

Plant Ethylene Detection Using Laser-Based Photo-Acoustic Spectroscopy

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Abstract

Analytical detection of the plant hormone ethylene is an important prerequisite in physiological studies. Real-time and super sensitive detection of trace amounts of ethylene gas is possible using laser-based photo-acoustic spectroscopy. This Chapter will provide some background on the technique, compare it with conventional gas chromatography, and provide a detailed user-friendly hand-out on how to operate the machine and the software. In addition, this Chapter provides some tips and tricks for designing and performing physiological experiments suited for ethylene detection with laser-based photo-acoustic spectroscopy.

Key words Ethylene, Laser-based photo-acoustic spectroscopy, Real-time measurements, ETD-300

1 Introduction

The plant hormone ethylene (C_2H_4) is a unique growth regulator due to its volatile nature and its pleiotropic effects on plant development and stress responses. Accurate detection of ethylene requires sensitive equipment that is suited to detect trace amounts of the gas. Ethylene (ethene according to IUPAC nomenclature) is the smallest unsaturated hydrocarbon with a double bond. Since the introduction of gas chromatography (GC) in plant science, ethylene became a detectable molecule opening new opportunities for research [1–3]. These first reports quantified ethylene production of apple fruit using GC. The versatility and (relative) affordability of GCs has made it the most used analytical technique for scientific and commercial detection of ethylene [4]. One of the major drawbacks of using GC for ethylene detection is that the analysis time can be quite long (2–10 min depending on the system used), eliminating the ability to monitor ethylene production in real time. Furthermore, the level of detection (around 1–0.1 ppm) is sometimes insufficient to detect trace amounts of ethylene, which can be physiologically relevant [5]. An alternative

technique that overcomes these last two limitations is laser-based photo-acoustic spectroscopy [4, 6]. This technique uses a CO₂ laser that excites ethylene molecules in the mid infrared region (absorption range 2–12 μm) resulting in heat production of the excited ethylene molecules. By continuously switching the light source on and off (by means of a chopper), the ethylene molecules are periodically excited, a process that generates heat pulses, that gives rise to periodic pressure changes (sound waves), which in turn can be detected by a sensitive acoustic microphone [4, 6]. The magnitude of the acoustic signal is proportional to the ethylene concentration in the sample. Unprecedented detection limits as low as 6 pL/L (6 ppt) have been reported [7]. Besides a low detection limit, a short response time of laser-based photo-acoustic spectroscopy facilitates real-time measurements of ethylene content without the necessity of long-term headspace accumulation, preventing any possible feedback effects of the accumulated ethylene [8].

2 Materials

1. A laser-based photo-acoustic spectrophotometer for ethylene detection. We use the ETD-300 (Sensor Sense, Nijmegen, NL) hereafter referred to as ETD, which is equipped with six channels (Fig. 1). This can also be a custom-built system (*see Note 1*).
2. Computer with ETD software (Valve controller 1.4.2, Sensor Sense, Nijmegen, NL), or similar.
3. Carrier gas tubing, connectors, and syringe needles (*see Note 2*).
4. Carrier gas supply and catalyzer (*see Notes 3 and 4*).
5. Control box (*see Note 5*).
6. Sample cuvettes, rubber septa, metal caps, and crimper (*see Note 6*).
7. Scrubbers (*see Note 7*).

3 Methods

The ETD can be used in three different modes of operation: the continuous flow mode, the stop-and-flow mode, and the samples mode. During operation in continuous flow mode, all channels attached to the control box (maximum six cuvettes) are flushed at the same time, while only one channel is analyzed by the ETD detector in real time. Thus, samples are analyzed one by one in a consecutive order, while constantly being flushed. When the amount of ethylene produced by the sample is too low to be

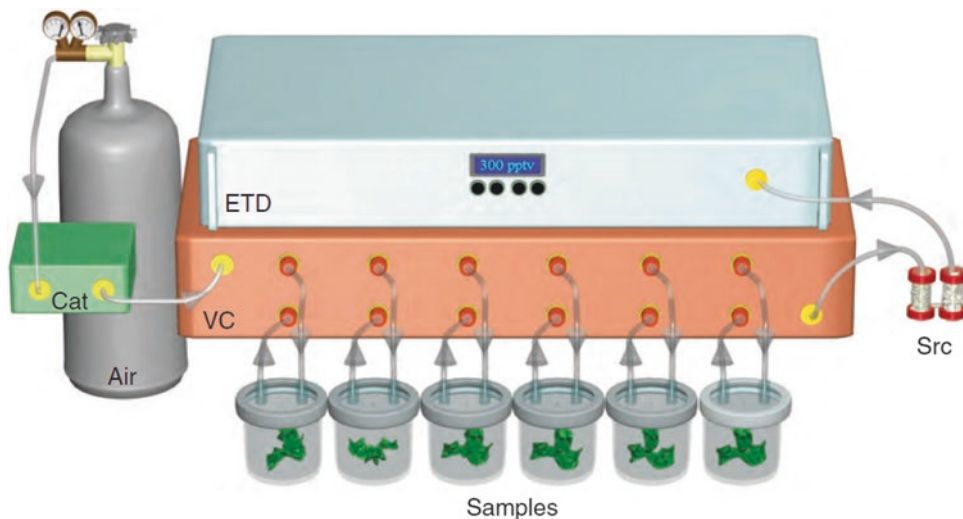


Fig. 1 Overview of the experimental setup of the laser-based photo-acoustic spectrophotometer (*ETD*). The flow-through system requires a carrier gas (air or a gas mixture of choice) originating from a gas bottle (or a compressor), which is passed through a catalyzer (*Cat*) to remove residual hydrocarbons. The hydrocarbon-free air is transferred to the control box (*VC*) containing a valve controller (to switch between channels) and flow controllers (to regulate the flow rate of each channel) which has six different channels, in order to consecutively measure ethylene in these six cuvettes. Built-in flow controllers regulate the flow rate (0–5 L/h) and the valve controller selects the channel that will be analyzed and/or flushed. Each channel is connected with a corresponding sample (cuvette) with an inlet and outlet tube. The VC selects the sample for which the headspace is flushed and redirected to the ETD detector. Before the air enters the ETD detector it passes over a scrubber (*Src*) to remove both CO₂ and water vapor by KOH and CaCl₂ respectively. After the gas sample has passed the ETD detector for analysis, it is exhausted into the room or can be redirected outside. Image courtesy of [4]

detected in the continuous flow mode, it is possible to use the stop-and-flow mode. In this mode, only one of the six cuvettes attached to the control box will be flushed with the carrier gas and simultaneously analyzed by the ETD detector. The other five cuvettes remain sealed during this measurement, allowing them to accumulate ethylene in their headspace. This will ensure an accrual of ethylene gas beyond the limit of detection. The stop-and-flow mode can be programmed so that up to six different samples are measured sequentially and repeatedly. The samples mode is used when a lot of samples need to be analyzed only once instead of over a certain time period, or when the sampling time is shorter than the analysis time of the ETD. When using the samples mode, it is important to make a snapshot sample by drawing a 1–2 mL gas specimen from the headspace of the vial that contains the plant sample, and injecting this specimen in a different empty airtight cuvette, which will be analyzed with the ETD at a later stage. The samples mode is particularly useful if many samples need to be analyzed shortly after each other.

3.1 Preparation of Measurements

1. Place your samples in an airtight cuvette. Three different types of samples can be analyzed: detached plant parts (e.g., a detached leaf or fruit), attached plant parts (e.g., an attached leaf), or whole plants (*see Note 8*).
2. Take into account the production of wound ethylene when using detached plant parts (*see Note 9*).
3. Connect your sample with one inlet tube and one outlet tube to the inlet and outlet connector, respectively, of the control box of the ETD (*see Note 10*).
4. Open the ETD software to start an experiment and select the desired settings (type of experiment, flow rate, measurement time, and schedule). Figure 2 shows an overview of the most important panels and configuration settings of the software (*see Note 11*). More details about the different settings are described below for each individual mode of operation.

3.2 Calibration of the ETD

1. The ETD is calibrated in the same way for the continuous flow mode and the stop-and-flow mode. For the samples mode, the ETD can be calibrated separately, taking into account the procedure how the snapshot sample was made (*see Note 12*).

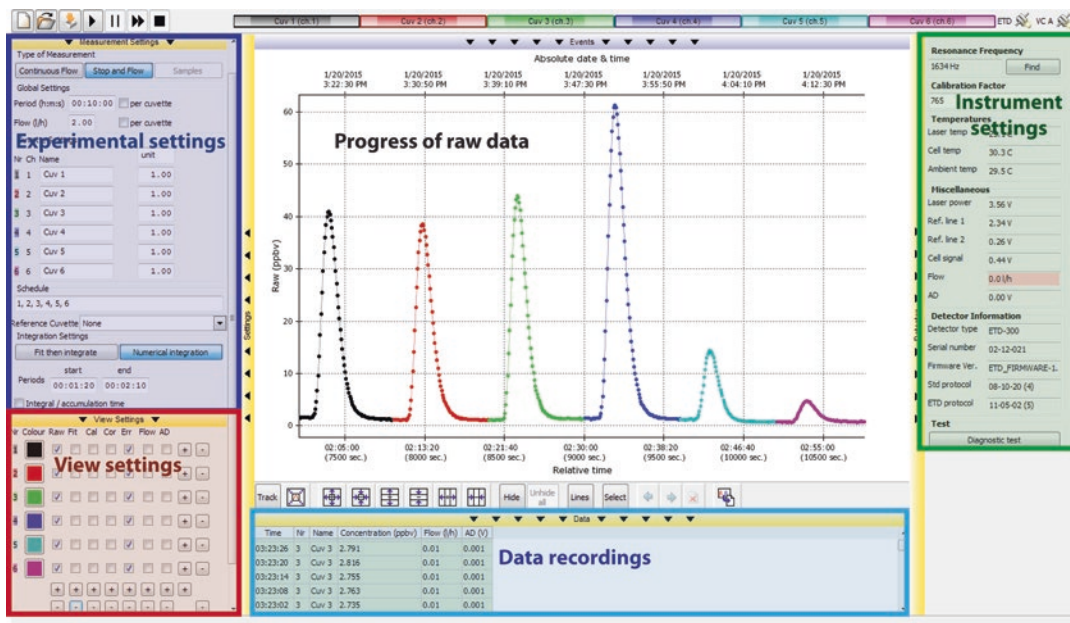


Fig. 2 Overview of the main panels of the ETD software (Valve controller 1.4.2, Sensor Sense, Nijmegen, NL). The *dark blue panel* (upper left, experimental settings) allows adjusting the settings of the experiment (mode of operation, flow rate, measurement time). The *red panel* (lower left, view settings) allows adjusting the view options presented in the raw data panel (center). The *light blue panel* (lower central panel, data recordings) lists the measured data points. The *green panel* (upper right, instrument settings) shows the actual instrument settings of the laser, the flow controller, and the detector

2. Attach a calibration bottle (e.g., 500 ppb ethylene) to the inlet of the valve control box to supply a constant flow of ethylene gas.
3. Do not connect the catalyzer in between the calibration bottle and the valve control box of the ETD.
4. Attach an empty cuvette to one channel (e.g., channel 1) which will be used for the calibration.
5. Analyze this channel (e.g., channel 1) in the continuous flow mode with a flow rate of 5 L/h for at least 30 min.
6. Wait until a stable recording of the online raw data points is reached (ethylene concentration in ppb).
7. Adjust the calibration factor (in the instrument settings panel) so that the online recordings of the raw data points match the concentration of the calibration gas. If the calibration factor is increased, the raw ethylene reads will also increase, while if the calibration factor is lowered, the raw ethylene reads will also lower.
8. Allow sufficient time for the online raw data points to equilibrate every time the calibration factor is adjusted.
9. Repeat **steps 7 and 8** until the raw online raw data recordings match the concentration of the calibration gas. The ETD is now calibrated.
10. Repeat the calibration procedure once every year, or more often when the ETD is used frequently.

3.3 Flushing the ETD

1. Before the start of a new set of measurements or a new experiment, it is important to flush the system to ensure that a stable baseline is reached, and any residual ethylene in the detector and/or tubing is removed.
2. Attach the tubing of all six channels to six different empty cuvettes.
3. Set the ETD software in the continuous flow mode.
4. Set the flow rate at 5 L/h.
5. Set the measuring time for each sample to 5 min.
6. Program the schedule of the samples so that each channel is flushed (set: 1–6).
7. Press start and wait until the online recordings of the raw data shows a stable baseline signal for each channel.
8. It is possible that the baseline is stable but not exactly zero.

3.4 Measurements in Continuous Flow Mode

1. Flush the ETD until a stable baseline is reached for each channel using empty vials (as described in Subheading 3.3).
2. Start a new continuous flow experiment in the ETD software.

3. Set the flow rate between 0.5 and 2 L/h (*see Note 13*).
4. Set the measuring time for each sample to 30 min (*see Note 14*).
5. Program the schedule for each sample that needs to be analyzed.
6. Incorporate one reference cuvette that does not contain a sample (empty cuvette or untreated control). This reference cuvette represents the background ethylene or the baseline signal.
7. Attach the tubing of each channel to its corresponding cuvette (as described in Subheading 3.1) after starting the measurement.
8. Press start.

3.5 Data Analysis in Continuous Flow Mode

1. Figure 3 shows an example of the raw ethylene recordings in the continuous flow mode. Each sample was measured for 20 min at a flow rate of 2 L/h, allowing sufficient time to reach and maintain an equilibrium state (*see Note 15*).
2. In the experimental settings panel, set the start and end point that corresponds to the time period during which the raw data recordings are stable for each sample, which corresponds to the equilibrium phase (*see Fig. 3*).
3. The software will automatically calculate the amount of ethylene measured (nL) during this averaging period.

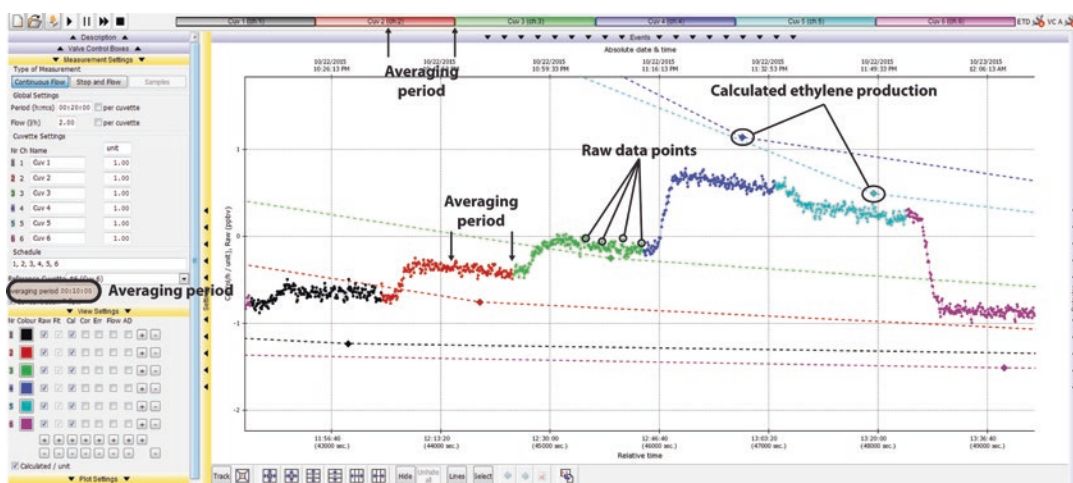


Fig. 3 Overview of a typical “continuous flow” output of the ETD software. Each sample is represented by a sigmoidal-shaped curve in a different color. Each sample reaches a plateau level, reflecting the steady-state situation of the measurement. This indicates that an equilibrium is reached between the amount of ethylene produced by the sample and the amount of ethylene that is flushed out of the headspace of the cuvette. The average amount of ethylene (ppb or nL/L) produced is calculated from the raw data points during which the equilibrium state is maintained (averaging period). The calculated rate of ethylene production can be adjusted for the flow rate (diamonds in nL/h). The x-axis represents the measurement time (s) and the double y-axis represents the amount of ethylene (raw data points in ppb) and the calculated ethylene production rate (diamonds in nL/h)

4. Check the tick box “concentration x flow” to calculate the exact ethylene production rate of each sample (nL/h).
5. Subtract the values of the reference cuvette by selecting the correct channel in the drop-box “Reference cuvette” in the experimental settings panel (channel number 6 in Fig. 3).
6. The calculated ethylene production values are saved in a separate Excel file, which can be viewed or analyzed at a later stage.

3.6 Measurements in Stop-and- Flow Mode

1. Flush the ETD until a stable baseline is reached for each channel using empty vials (as described in Subheading 3.3).
2. Start a new stop-and-flow experiment in the ETD software.
3. Set the flow rate between 2 and 3 L/h (see Note 16).
4. Set the measuring time for each sample to 10 min (see Note 16).
5. Program the schedule for each sample that needs to be analyzed (see Note 16).
6. Press start.
7. Attach the tubing of each channel to its corresponding cuvette (as described in Subheading 3.1) after starting the measurement. Make sure not to waste too much time in between the start of the measurement and the attachment of the first cuvette.

3.7 Data Analysis in Stop-and- Flow Mode

1. Figure 4 shows an example of the raw ethylene recordings in the stop-and-flow mode. Each sample was measured for 10 min at a flow rate of 2 L/h, allowing sufficient time to purge out all ethylene gas from the cuvette, resulting in a typical bell-shaped curve (peak) (see Note 17).
2. In the experimental settings panel, set the start and end point that corresponds to the time period that completely incorporates the peak (see Note 18).
3. The software will automatically calculate the amount of ethylene produced (nL).
4. Check the tick box “integral/accumulation time” to calculate the ethylene production rate per unit of time (nL/h) for each sample.
5. Subtract the values of the reference cuvette by selecting the correct channel in the drop-box “Reference cuvette” in the experimental settings panel. The reference cuvette is an empty cuvette, or an untreated control, representing background ethylene values.
6. The calculated ethylene production values are saved in a separate Excel file, which can be viewed or analyzed at a later stage.

3.8 Measurements in Samples Mode

1. Prepare a snapshot sample by taking 1–2 mL from the cuvettes containing the plant samples, and injecting this volume in

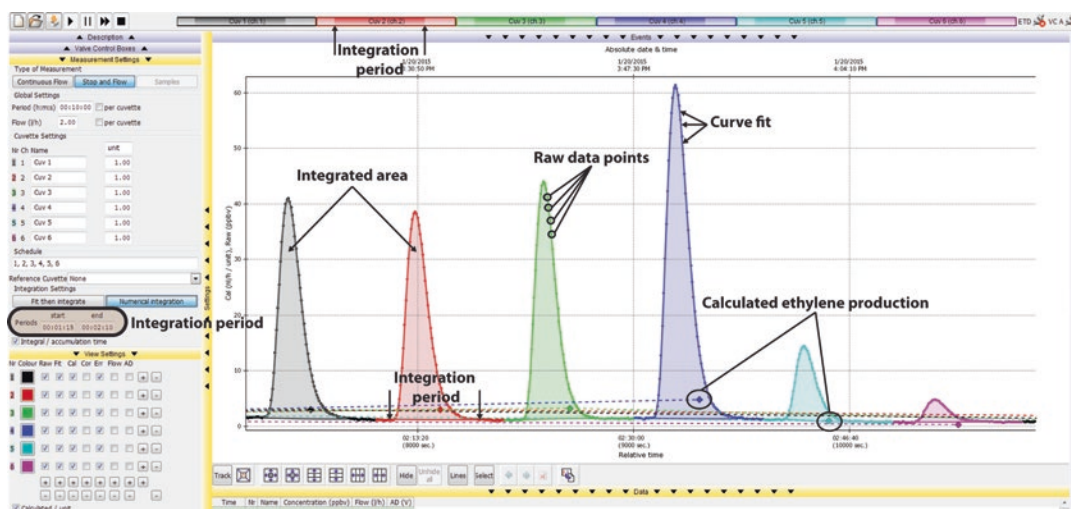


Fig. 4 Overview of a typical “stop-and-flow” output of the ETD software. Each sample is represented by a bell-shaped curve in a different color, which can be integrated (in this case a numerical integration was chosen) to quantify the amount of ethylene measured. Therefore, the start and end time point of the integration period should match the beginning and end of the peak. The area underneath the curve represents the total amount of ethylene (nL) multiplied by the flow rate. The total amount of ethylene can also be adjusted for the accumulation time to calculate the ethylene production rate (nL/h). The x-axis represents the measurement time (s) and the double y-axis represents the concentration of ethylene (raw data points in ppb) and the calculated ethylene production rate (diamonds in nL/h)

another empty airtight vial that will be analyzed by the ETD. Store the snapshot samples until analyzed by the ETD (*see Note 19*).

2. Flush the ETD until a stable baseline is reached for each channel using empty vials (as described in Subheading 3.3).
3. Start a “new samples experiment” in the ETD software (*see Fig. 5*).
4. Perform an experiment-specific calibration using the same sampling procedure as for the snapshot samples (*see Note 12*).
5. Set the flow rate between 2 and 3 L/h (*see Note 19*).
6. Set the measuring time for each sample to 10 min (*see Note 19*).
7. Program the schedule for each sample that needs to be analyzed and name each sample accordingly.
8. Select the “Pause after each cycle” tick box if you wish to pause the measurements after each loop as defined in the schedule.
9. Press start.
10. Attach the tubing of each channel to its corresponding cuvette (as described in Subheading 3.1) after starting the measurement. Make sure not to waste too much time in between the start of the measurement and the attachment of the first cuvette.

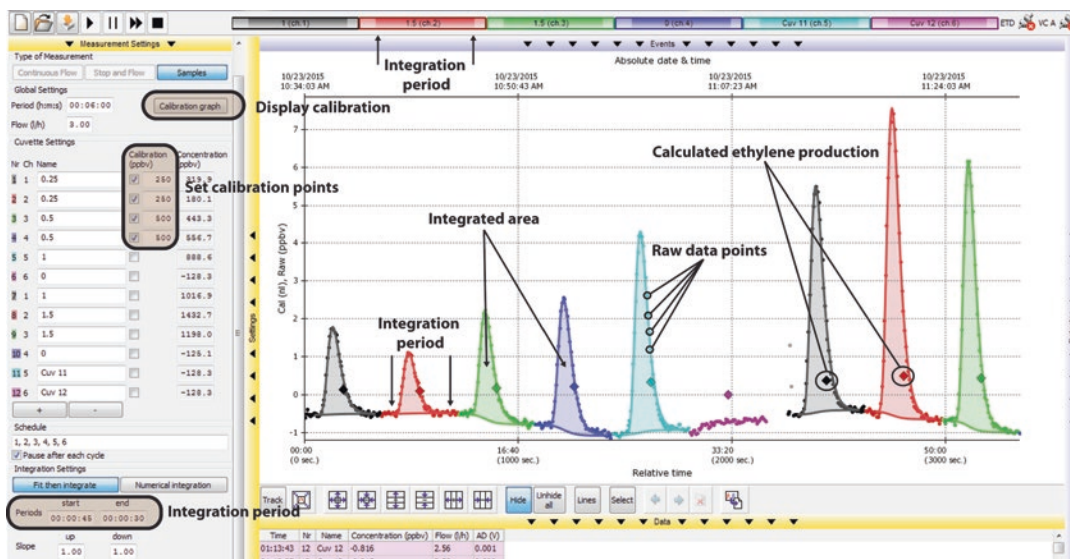


Fig. 5 Overview of a typical “samples mode” output of the ETD software. Many different samples can be programmed to be analyzed in series of six, with a pause in between each loop of six (by checking the tick box “Pause after each cycle.” Each sample is represented by a *bell-shaped curve* that can be integrated (in this case a “fit then integrate” integration was chosen using the Levenberg-Marquardt Algorithm for curve fitting with the slope parameters set to 1). The start and end time point of the integration period should match the beginning and end of the peak. The area underneath the curve represents the total amount of ethylene (nL) multiplied by the flow rate. The data cannot be corrected for the accumulation time in the samples mode. In this example, the first four samples are used for making a new calibration curve (the calibration tick boxes are marked and the concentration of ethylene is given). The calibration graph can be displayed by pressing the button “calibration graph.” The x-axis represents the measurement time (s) and the double y-axis the concentration of ethylene (raw data points in ppb) and the total amount of ethylene (diamonds in nL)

11. After each loop of six samples, a new loop of six different samples can be initiated by pressing the continue button.

3.9 Data Analysis in Samples Mode

1. The data analysis of the samples mode (Fig. 5) is similar to the stop-and-flow mode (*see* Subheading 3.7). The only difference is that it is not possible to correct the peak area (ethylene content in nL) with the accumulation time. This should be done manually by calculating the time each original sample was sealed up to the moment when the snapshot sample was made.

4 Notes

1. The ETD-300 (Sensor Sense, Nijmegen, NL) has a limit of detection of 300 ppt ethylene. This machine can be equipped with a control box (valve and flow controllers) and comes with an optional catalyzer that removes residual hydrocarbons from the carrier gas (in principle, air). The ETD is best operated in

a temperature-controlled environment between 10 and 26 °C, avoiding strong temperature fluctuations. Ideally, the equipment is placed in an air-conditioned room. For most plant science applications this temperature range is workable. In case different temperature conditions are required, tubes should be diverted from the machine into a different room/space where the samples are stored. It is important to switch on the ETD detector 60–90 min before the start of an experiment to ensure proper warm-up of the system.

2. All gas handling is best done with PFA (perfluoroalkoxy alkane) tubing with an outer diameter of 1/8". Connections can be made with Swagelok connectors, quick-lock connectors or flexible rubbers. Syringe needles attached to tubing ends using flexible rubbers can also be used if septa need to be punctured.
3. Carrier gas, mostly air, can be supplied by a compressor, or by bottles of compressed air. Bottles with compressed air will contain trace amounts of CO₂ (originating from ambient air), although the exact concentration is variable depending on time and method of fabrication (consult with the air supplier for more details). Compressed air is well suited for most experiments with plants, but sometimes it can be desired to treat plants with a known concentration of CO₂ or other gasses, and then a gas mixture with a predefined composition should be used.
4. The outlet of the gas bottle can be equipped with a two-stage pressure regulator that allows a precise control of the outlet pressure. Typically, a final output pressure of 1 atm is used for all experiments with the ETD. In order to ensure optimal valve and flow controller operation, the maximum outlet pressure cannot exceed 6 atm. The carrier gas from the bottle is directed to a catalyzer (Sensor Sense supplies the CAT1) to remove residual hydrocarbons and particulate matter. The catalyzer ensures that no external ethylene gets in the tubing of the experimental setup. The catalyzer only requires 5 min to warm up.
5. The control box contains a valve controller that directs the flow toward the six cuvettes that are connected to the control box. Each channel has a flow controller that precisely regulates the flow of the cuvette to which it is attached. The valve controller can be programmed to flush all six cuvettes at the same time (continuous flow mode) or only one cuvette at the time (stop-and-flow and samples mode). The flow rate of the supplied air can be adjusted between 0.25 and 5 L/h using channel-specific flow controllers.
6. Each cuvette (sample) has two tubes connected with the control box. One is the inlet tube that directs the carrier gas from the

control box to the cuvette, and the other one is the outlet tube that directs the carrier gas from the cuvette back to the control box. The control box is also connected with the ETD detector. This setup creates a loop that allows to purge out the headspace above the plants or plant tissues in the cuvette and directs it via the control box to the ETD detector for analysis. Different types of airtight cuvettes can be used, depending on the size and growth conditions of the samples. It is essential to try to use cuvettes that have similar dimensions as the plants/plant parts that are sampled, minimizing the free headspace. This avoids dilution of ethylene in the headspace and ensures that the ethylene concentration remains above the limit of detection. Sometimes cuvettes are vials that need to be sealed with a rubber septa, which can be punctured with a syringe needle to purge out the headspace. Make sure that these septa are sealed airtight, preferentially by capping them with a metal ring that is firmly attached using a crimper. Examples of different type of cuvettes are given by [9, 10] and are sold by several companies (e.g., Qubit Systems, Chromacol, Waters, Agilent, and others).

7. When the carrier gas, together with the headspace gas, is redirected from the cuvette via the control box to the ETD detector, it passes a scrubber to filter out water vapor and CO_2 to avoid interference with the ethylene signal. The scrubbers are placed in series, with first a CO_2 scrubber, containing KOH (or NaOH or soda lime) and second a water vapor scrubber, containing CaCl_2 (or CaSO_4 , also called Drierite). It is important to place the CO_2 scrubber before the water vapor scrubber because the CO_2 scrubber generates moisture when CO_2 is removed from the gas stream.
8. Whole plant samples can be germinated in the cuvette or transferred from a different growth medium into an airtight cuvette for analysis. When plants are germinated and grown in the cuvette it is best to use MS medium or an inert substrate in sterile conditions to prevent interference from unwanted ethylene production from microorganisms or decaying organic matter. When plants are transferred from a growth medium to an airtight cuvette, they can be placed on a sterile pre-wetted filter paper or miracloth tissue to avoid desiccation. Again, it is advised to work in sterile conditions to prevent unwanted ethylene production from microorganisms. It is also possible to measure ethylene levels in the headspace above liquid cultures (hydroponics, aquatic species, or algal cultures) in cuvettes, but it is important that a sufficiently large headspace is present to prevent water leakage into the control box. When working in liquid conditions one should take into account the solubility of ethylene in liquid (in principle water) using the law of Henry under atmospheric equilibrium conditions:

$$c_a = H^{cc} \times c_g$$

with c_a being the concentration (in molarity) of ethylene in the aqueous phase, c_g being the concentration (in molarity) of ethylene in the gas phase, and H^{cc} the Henry's law solubility constant [11]. More details on how to calculate Henry's law constant under different environmental conditions including different solutes, as well as examples of Henry's law constants for ethylene dissolved in water, are given in [11].

9. When using detached plant parts it is important to take into account the release of wound-induced ethylene [12]. Wound-ethylene is produced rapidly after wounding; hence, in principle, it is observed as a first peak. Plant parts can also be exposed to the surrounding air for 5–15 min before the start of the analysis to eliminate the first burst in wound ethylene.
10. Connections between two tubes can be made by using flexible rubbers or quick-lock connectors. This type of connections can also be made to attach tubing to the sample cuvette or the control box of the ETD. When samples are sealed in a cuvette using an airtight septum, the connection with the ETD can be made by puncturing through the septum with a syringe needle that is attached to the tubing by a flexible rubber.
11. The ETD software records the raw data separately from the analyzed data in different Excel files. This means that any software manipulations during data analysis do not affect the raw data recordings, but only the analyzed data.
12. The ETD can be calibrated separately in the samples mode, taking into account the procedure how the snapshot sample is prepared. This is done by first injecting a certain volume (e.g., 1–2 mL) of a calibration gas into an empty airtight vial (snapshot sample), by the same sampling procedure used for the analysis of unknown samples. Make sure to use at least two different concentrations for the calibration and prepare at least three technical replicates for each concentration. Subsequently, tick the “calibration (ppv)” box in the ETD software (in the experimental settings panel) for each sample that will be analyzed to generate a calibration curve. Define the concentration of each calibration sample in the ETD software (in the experimental settings panel) and measure each calibration sample. It is best to repeat the calibration procedure each time new experimental settings (flow rate and measuring time) or a new sampling procedure (volume of the snapshot sample) is used.
13. The flow rate in the continuous flow mode should be chosen wisely to ensure that an equilibrium is established between the amount of ethylene that is produced by the sample and the

amount of ethylene flushed out of the cuvette. A typical flow rate for these types of experiments is between 0.5 and 2 L/h, although this is best determined experimentally.

14. In the continuous flow mode, each sample is measured during a 10–30 min time period (depending on the programmed flow rate and the rate of ethylene emanation from the samples). The exact measuring time is best chosen experimentally to ensure sufficient time to reach the equilibrium state when switching from one channel to another. Especially if large differences in ethylene production are expected between different samples, it is important to increase the measuring time. Due to the very low level of detection and the fast response time (5 s) of the ETD, continuous real-time measurements of ethylene production of most plants or plant tissues are possible, in contrast to conventional GC setups that are not sensitive nor fast enough. In addition, the continuous flow mode prevents ethylene accumulation in the headspace of the cuvettes, eliminating possible unwanted effects of ethylene on the plant metabolism.
15. In the continuous flow mode, the raw data points have a sigmoidal shape for each sample, reaching a plateau level after a certain time (*see* Fig. 3). The initial lag phase of the curve corresponds to the rest air of the previous sample that needs to be flushed out from the tubing. Next, the signal increases (or decreases) because the first ethylene molecules of the current sample are detected. After a while the amount of ethylene produced by the sample is equal to the amount of ethylene that is flushed out the cuvette, resulting in a stable signal (equilibrium). It is important to maintain this equilibrium for several minutes (3–10 min) to have a good estimate of the average ethylene production rate.
16. In the stop-and-flow mode it is common to use a flow rate around 2–3 L/h, unless very high concentrations of ethylene are expected (then a higher flow rate should be used). The total time of ethylene accumulation in the headspace should be chosen so that all the ethylene present in the headspace of the cuvette is purged out, meaning that the raw data recordings are peak-shaped and return to the baseline at the end of the peak. If large differences in ethylene production are expected, it is advisable to program the time of analysis and the flow rate of each cuvette individually, allowing a longer accumulation time for samples with a lower ethylene production rate. When six samples are attached to the control box, it is common to analyze each sample for 10 min, so that each sample is analyzed once every hour. It is also possible that one of the six cuvettes is used as a blanc control, which can be analyzed for a longer time period, to create a longer accumulation period for the other five cuvettes. For example, cuvettes 1–5 contain samples

and are measured for 10 min, while cuvette 6 is empty and is measured for 2 h and 10 min. This way cuvettes 1–5 are only analyzed once every 3 h, ensuring a longer accumulation period, which can be convenient for samples that produce little ethylene.

17. During the stop-and-flow mode the ethylene concentration in the headspace of one cuvette is measured after a certain accumulation time, resulting in a bell-shaped curve (peak) that represents the total amount of ethylene flowing through the detector. The initial lag phase of the peak corresponds to the rest air of the previous sample that needs to be flushed out from the tubing. Next, the accumulated ethylene passes through the detector and will result in the bell-shaped output. At the end of the measurement, the signal drops again and reaches the equilibrium state (or the baseline). It is important that the analysis time is long enough so that the peak has reached the baseline or equilibrium state at the end of each measurement. This is achieved by setting an optimal flow rate and measurement time. A typical ethylene measurement of plant material would last 10 min when using a flow rate of 2 L/h. A rule of thumb is that the flow rate and analysis time should be set so that the volume of the headspace (including the volume of tubing) is flushed at least five times.
18. In the stop-and-flow mode, the area underneath a peak represents the total amount of ethylene of the corresponding sample, and is calculated by integrating this peak over a certain time period. The start and end point of the integration period should be chosen by the user. These time points are selected so that the peak is completely incorporated in the integration period. There are two integration methods available in the ETD software: the Levenberg-Marquardt Algorithm and a numerical integration. The Levenberg-Marquardt Algorithm will first calculate the best fit through the raw data points, facilitating the integration of the peak surface (=fit then integrate). It is important to adjust the shape of the “parabolic” fit to match the shape of the peak of the raw data points by adjusting the slope values (the higher the value, the steeper the slope). The numerical integration method is faster and does not require manual adjustment of the slopes. In practice, the results of the numerical integration method are not much different compared to the integration results of the Levenberg-Marquardt Algorithm curve (less than 1% based on an experimental comparison of 385 individual integration events). Therefore, the numerical integration method is more practical in use.
19. The samples mode is used when multiple (more than six) samples, originating from snapshot samples, need to be analyzed. These snapshot samples are made by taking a 1–2 mL gas specimen from

the sample vials that contain plants or plant parts, and injecting this gas specimen into another empty airtight vial. This way, a snapshot of the headspace of the sample is transferred to another vial. These secondary vials can be stored and subsequently analyzed with the ETD in the samples mode. It is thus possible to analyze more than six samples, although only six different samples can be attached to the control box in one loop of analysis. There is an option to pause the analysis after each loop, allowing the operator to change the cuvettes without having to worry that a new series of measurements has already started. The sample mode is typically programmed with a flow rate of 2 L/h for 10 min, but these settings can be adjusted according to the volume size of the cuvettes and the amount of ethylene present in the headspace. The data analysis procedure for the samples mode is the same as for the stop-and-flow mode and will result in the typical bell-shaped curves (*see* Fig. 5). There is no option available to automatically correct the amount of ethylene for its accumulation time. The calculated ethylene production values (nL) should be manually corrected for the accumulation time (time before the snapshot sample was made) by the operator using a separate software (e.g., Excel).

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