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GAS CHROMATOGRAPHY-MASS SPECTROMETRY METHOD FOR DETERMINATION OF MONOTERPENE AND SESQUITERPENE EMISSIONS FROM STRESSED PLANTS

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ABSTRACT. Emissions of biogenic volatile organic compounds (BVOCs) play important roles in plant biology and atmospheric chemistry at a wide range of spatial and temporal scales. This article describes a headspace method coupled with gas chromatography – mass spectrometry (GC-MS) for the detection of volatile terpenes (monoterpenes and sesquiterpenes) and other related compounds emitted from plants, especially under stress conditions. A protocol is developed for simultaneous detection and quantification of isoprene, and mono- and sesquiterpenes from plant emissions.

Keywords: BVOC emission, Mass spectrometry, Monoterpenes, Plant stress, Sesquiterpenes

INTRODUCTION

Plants emit more than 30000 chemically different volatile organic compounds (BVOC). The emissions of some of these compounds result from and serve as markers of activation of certain metabolic pathways in plants, end-products or intermediates of which are volatile [1]. Many of these volatiles also play important roles in plant communication with other plants and animals [2]. There are further constitutive emissions of several specific volatile compounds thought to be involved in plant non-specific defence to various abiotic and biotic stresses [1, 3]. Worldwide, it has been estimated that biogenic production of volatile organic compounds exceeds the anthropogenic production by an order of magnitude. Apart from the significance for plants, these emissions play important role in atmospheric chemistry and physics, in particular in formation of atmospheric pollutant ozone in the troposphere and formation of aerosols [4-7].

The volatile isoprenoids – isoprene (5 carbon atoms, C5) and volatile terpenes consisting of isoprene building blocks, monoterpenes (C10, MT) and sesquiterpenes (C15, SQT) – form a major part of plant-generated BVOC. Several widespread plant species are strong constitutive emitters of volatile isoprenoids, while volatile isoprenoid emissions can be induced by various environmental and biotic stimuli also in species not emitting these compounds constitutively [8, 9].

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Plant volatile isoprenoid emissions consist of a complex blend of chemically heterogeneous compounds. Often more than 20 different MT [10, 11] are emitted by a single plant species. These compounds have widely differing chemical reactivity and ozone-forming potentials in the atmosphere [12]. A number of SQT and some oxygenated sesquiterpene alcohols, aldehydes, and ketone derivatives ($C_{15}H_{22}O$, $C_{15}H_{24}O$, $C_{15}H_{26}O$) have further been identified in studies on BVOC emissions from both natural [13-17] and agricultural vegetation [18, 19]. All volatile isoprenoids, isoprene, MT and SQT, are anticipated to importantly participate in secondary aerosol-forming processes and in chemical reactions in the lower troposphere [6, 16, 21-23], but SQT are generally more reactive than isoprene and MT. The atmospheric lifetimes of reactive SQT such as β-caryophyllene as determined by their rapid gas-phase reactions with ozone, and OH and NO_3 radicals have been estimated to be on the order of only a few minutes[11, 15], while the life-time of isoprene and most monoterpenes varies between 36 min during daytime in summer and a few hours during nighttime in winter [24].

A vast array of volatile terpenes (MT and SQT) is involved in the communication between plants and between plants and herbivores [9, 25-29]. Under stress, the emissions of volatile isoprenoids may be amplified in constitutive emitters and be induced also in species not emitting these compounds constitutively [8, 9], modifying the plant communication with other organisms. For instance, emission of *de novo* synthesized compounds such as methylsalicylate, farnesenes, (*E*)-*β*-ocimene, linalool, *etc*, which serve as signals of the induced defence reactions in stressed plants, is often observed in plants not emitting isoprenoids in non-stressed conditions [30, 31]. Plant-insect relationships can also be altered by increased emissions of other stress compounds that occur simultaneously with enhanced isoprenoid emissions, e.g. emissions of green leaf volatiles (GLV, volatile aldehydes formed via the lipoxygenase pathway) occur in stressed plants as the result of the destruction of free fatty acids in lipoxygenase pathway [31, 32]. Apart from plant communication, volatile isoprenoid emissions are also thought to increase the plant tolerance to a variety of biotic and abiotic stresses [3 for a review].

Simultaneous measurement of the wide spectrum of plant volatiles with different physico-chemical characteristics constitutes an analytical challenge. Preconcentration of BVOCs on cartridges filled with solid adsorbents followed by thermal desorption and GC analysis has become a well-accepted analysis technique in a wide range of applications [14, 33, 34]. Additionally, other methods based on the sampling of headspace into stainless steel canisters followed by cryo-focusing and analysis by gas chromatography equipped with the flame ionization detector (GC-FID) analysis [35], or the technique for rapid collection of volatiles by solid-phase micro-extraction (SPME) coupled with GC-MS have been used [36-38]. The techniques described in the literature are generally for compounds in the range of volatility of C5–C10. In contrast, the

applicability of adsorbent sampling/thermal desorption for semi-volatile BVOC has received much less attention and application (see [39, 40] for review). The most commonly used adsorbents include diverse carbon adsorbents (active carbon, graphitized carbon, and carbon molecular sieves) and also synthetic polymeric adsorbents (Tenax, Chromosorb, etc.) [39, 41].

The goal of this study was to develop a quantitative method for simultaneous determination of mono- and sesquiterpenes in air samples from plants exposed to different biotic and abiotic stresses. The developed method is based on preconcentration of BVOCs on solid absorbents coupled with GC-MS analysis, and is optimized for quantitative determination of plant volatiles with widely varying physico-chemical properties.

RESULTS AND DISCUSSION

Combinations of adsorbents in a single adsorbent cartridge (volatile trap) have been previously used to trap and analyze compounds over a wider range of volatility than can be achieved with a single adsorbent [42]. In preliminary experiments of our study, the type and the amount of absorbent, and the cleanup method of adsorbent cartridges were optimized. We finally used the combination of Carbotrap C, Carbopack C and Carbotrap X for the multibed cartridges. These chemically neutral carbon-based adsorbents permit trapping of unsaturated compounds (*e.g.* monoterpenes) without intervening chemical reactions in the ambient air that can for instance happen with organic polymer adsorbents such as Tenax TA in oxidative atmosphere [43].

Several GC parameters and oven temperature program were optimized to achieve excellent separation of main mono- and sesquiterpenes emitted from the stressed plants in a single GC column. Different temperature regimes, flow rates of carrier gas and columns with differing polarity were tested. Ions based on the following criteria were selected for compound detection: (i) molecular ions for compound detection (together with the fragment mass spectrum); (ii) fragment ions with high abundance, such as base peaks; (iii) target ions with selectivity minimizing the cross-interferences between different BVOC's. Based on these criteria, scanning tests of the standard solutions trapped in cartridge of each monoterpene and sesquiterpene were carried out to describe the scanning mass spectrum and retention time of various isoprenoids. One target ion and two qualitative ions (qualifiers) for each compound were selected (Table 1). Operative conditions (oven program, time and temperature of desorption etc) were fixed on the basis of optimization results. Overall, the retention times of terpene standards were relatively concentrated in two parts of the chromatogram: one part featuring most of the monoterpene peaks and the other most sesquiterpene peaks. Among the tested columns (DB-1, DB-2, ZB-5) a fused silica ZB-624 column proved to provide the best separation of common monoterpenes and sesquiterpenes present in the emission of stressed plants (Table 1).

Table 1. Chromatographic and mass spectral data obtained by the GC-MS methods for all the tested terpene standards and methylsalicylate

	Molecular mass (g mol^{-1})	Retention time (min.)	Target ion (m/z)	Qualifiers* (m/z)
α-Pinene	136	18.74	93	92, 91
β-Pinene	136	21.44	93	41, 69
α-Phellandrene	136	22.56	93	91, 77
Δ^3-Carene	136	22.88	93	91, 77
α-Terpinene	136	23.33	93	121, 91
(*E*)-β-Ocimene	136	23.72	93	92, 91
Limonene	136	23.94	68	67, 93
β-Phellandrene	136	24.39	93	91, 77
1,8-Cineole (Eucalyptol)	154	24.78	43	81, 71
γ-Terpinene	136	25.60	93	91, 77
α-Terpinolene	136	27.46	93	121, 136
4,8-Dimethyl-1,3,7-nonatriene	150	28.80	69	41, 81
Linalool	154	29.87	71	43, 81
α-Thujone	152	31.74	81	110, 41
β-Thujone	152	32.47	110	81, 41
Methylsalicylate	204	37.73	120	92, 152
Bornyl acetate	204	42.67	95	43, 93
(*Z*)-β-Farnesene	204	49.51	69	41, 93
α-Cedrene	204	50.38	119	93, 105
(*E*)-β-Caryophyllene	204	51.08	41	69, 93
α-Humulene	204	53.25	93	80, 41, 121
(*E*,*E*)-α-Farnesene	204	53.83	93	41, 69
Nerolidol	204	56.33	69	41, 43

* The qualifier ion (or SRM transition) is often an isotope peak or a higher mass product ion as opposed to the second most abundant ion in the spectra.

Terpene quantification was based on the total ion current monitored by the GC-MS system calibrated with terpene standards.

The GC-MS response for all monoterpenes and sesquiterpenes was linear in the concentration range assayed ($3 \cdot 10^{-9}$-$1 \cdot 10^{-7}$ mol/L). Relevant data from the calibration plots are summarized in Table 2. It is apparent that the linear calibration ranges and the slopes (analytical sensitivity) of the calibration

graphs are similar for all the mono and sesquiterpenes investigated. The detection limits of the method were estimated as three times of standard deviation of the response (10 determinations) to a blank sample. The values found ranged between 0.1 and 4.7 nmol mL^{-1}, depending on the terpene. The average relative standard deviation was 0.2% for ten samples.

Table 2. Features of calibration plots for the determination of different terpenes

	Slope* (area mol^{-1} L^{-1})	Relative standard deviation of the slope (%)	Correlation coefficient	Detection limit (nmol mL^{-1})
α-Pinene	$6.76 \cdot 10^{16}$	1.0	0.995	1.6
β-Pinene	$5.87 \cdot 10^{16}$	0.9	0.992	1.8
α-Phellandrene	$3.90 \cdot 10^{16}$	5.1	0.994	2.7
Δ^3-Carene	$5.05 \cdot 10^{16}$	5.0	0.995	2.1
α-Terpinene	$4.71 \cdot 10^{16}$	3.1	0.995	2.3
(*E*)-β-Ocimene	$2.28 \cdot 10^{16}$	4.8	0.991	4.7
Limonene	$7.42 \cdot 10^{16}$	5.8	0.993	1.4
β-Phellandrene	$5.19 \cdot 10^{16}$	5.2	0.997	2.1
1,8-Cineole (Eucalyptol)	$7.52 \cdot 10^{16}$	6.1	0.994	1.4
γ-Terpinene	$4.71 \cdot 10^{16}$	6.0	0.995	2.3
α-Terpinolene	$7.72 \cdot 10^{16}$	4.9	0.998	1.4
4,8-Dimethyl-1,3,7-nonatriene	$3.29 \cdot 10^{16}$	4.9	0.992	3.3
Linalool	$7.51 \cdot 10^{16}$	3.5	0.991	1.4
α-Thujone	$3.24 \cdot 10^{16}$	2.2	0.990	3.3
β-Thujone	$2.06 \cdot 10^{16}$	6.1	0.991	5.2
Methylsalicylate	$5.94 \cdot 10^{19}$	1.2	0.993	0.1
Bornyl acetate	$9.93 \cdot 10^{17}$	1.1	0.994	0.1
(*Z*)-β-Farnesene	$2.90 \cdot 10^{16}$	5.2	0.992	2.9
α-Cedrene	$2.03 \cdot 10^{17}$	2.3	0.994	0.4
(*E*)-β-Caryophyllene	$1.39 \cdot 10^{17}$	3.2	0.996	0.6
α-Humulene	$1.23 \cdot 10^{17}$	4.5	0.997	0.7
(*E*,*E*)-α-Farnesene	$2.45 \cdot 10^{16}$	2.4	0.991	3.4
Nerolidol	$2.77 \cdot 10^{17}$	5.6	0.993	0.3

* Based on three calibration curves y = slope x, y: peak-area ratio, x: terpene concentration (mol/L).

In chemistry, the emission rate is defined as the amount of given substance released to the air per unit of time. In plants, the emission rate is normalized with leaf area or leaf dry mass. In the literature, area-based plant emissions are commonly expressed in nmol m^{-2} s^{-1}, while for mass-basis ng g dw^{-1} h^{-1} is often employed [44]. We recommend always to use SI units and express the compound emission in molar units.

Our GC-MS method was applied for quantification of a vast array of mono- and sesquiterpenes released from differently stressed plants. Figure 1 shows chromatograms of sunflower (*Helianthus annuus* L.) leaf emissions under controlled conditions (chamber temperature of 25 °C) (A) and after 5 min. temperature stress at 51 ^{O}C (B) measured 1 min. after application of the heat pulse. This pilot experiment demonstrates that cold stress significantly enhances the emissions of monoterpenes in *Helianthus annuus L.* (Figure 2).

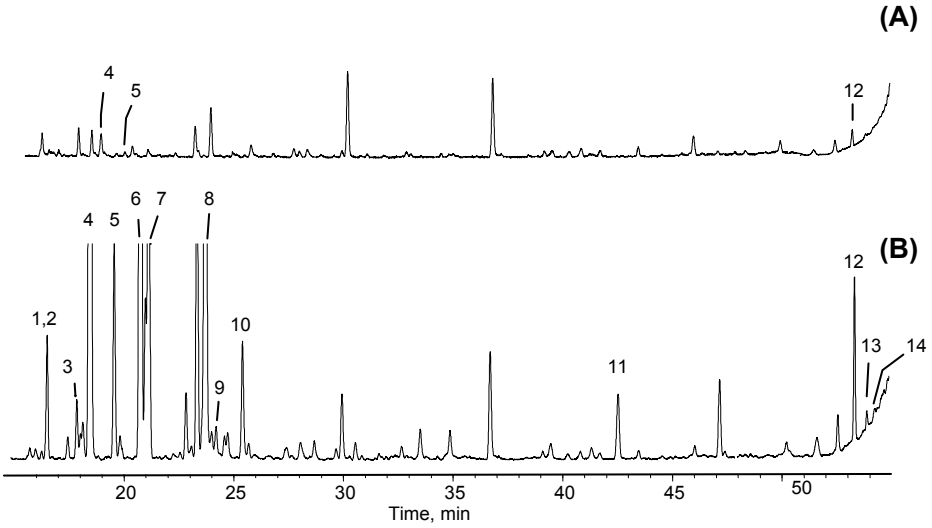


Figure 1. Chromatograms of sunflower (*Helianthus annuus* L.) leaf emissions. A: control plant; B: plant measured 1 min. after application of a 5 min. heat pulse of at 51 ^{o}C. Identified compounds – 1,2: (3*Z*)-hexenol co-eluted with (2*E*)-hexenal; 3: *α*-thujene; 4: *α*-pinene; 5: camphene; 6: sabinene; 7: *β*-pinene; 8: limonene; 9: *β* -phellandrene; 10: γ-terpinene; 11: bornyl acetate; 12: geranyl acetone; 13: (*E*,*E*)-*α*-farnesene; 14: germacrene D.

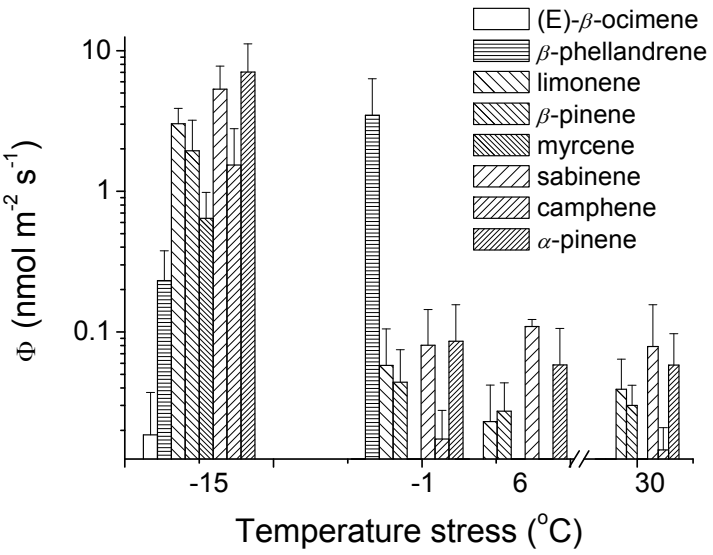
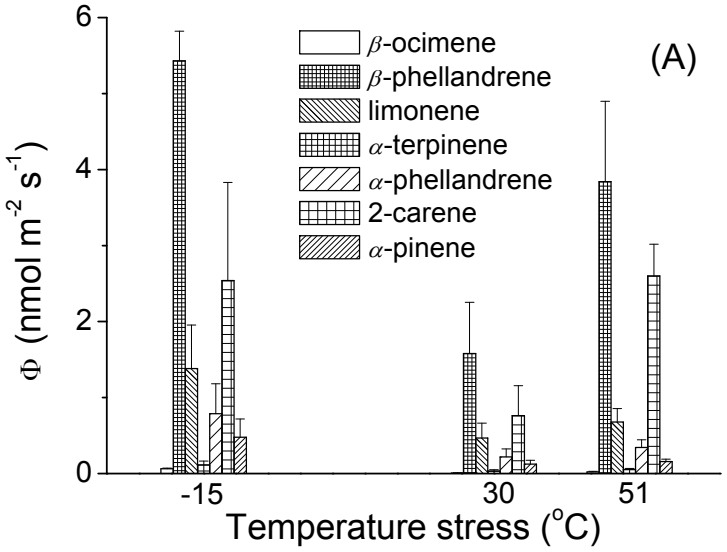


Figure 2. The emission rates (Φ) of monoterpenes from sunflower (*Helianthus annuus* L.) leaves measured after 1 min. exposure to 5 min. pulses of cold stress. *N* = 3 for all treatments.

Analogous results were obtained with tomato (*Lycopersicum esculentum* Mill.) plants under heat and cold stress (Figure 3).



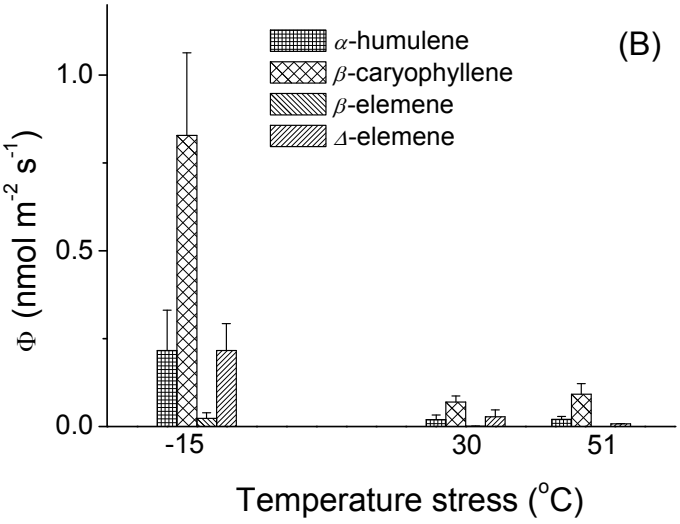


Figure 3. The emission rates (Φ) of monoterpenes (A) and sesquiterpenes (B) from tomato (*Lycopersicum esculentum* Mill.) leaves measured after 1 min. exposure to 5 min. pulses of heat and cold stress. *N* = 3 for all treatments.

CONCLUSIONS

The major drawback with current plant stress studies is that with very few exceptions [8] plant stress responses, including emissions, are not quantitatively assessed. Our method permits to obtain quantitative relationships between signal strength and plant response, and thus, to develop quantitative plant stress models. These models are needed to predict plant responses to environmental stress and simulate plant volatile compound emissions under different environmental conditions.

EXPERIMENTAL SECTION

All chemicals were purchased from Sigma-Aldrich, (St. Louis, MO, USA, GC purity) with exception of 4,8-dimethyl-1,3,7-nonatriene (DMNT) which was synthesized from commercial citral using methyltriphenylphosphonium bromide and butyl lithium at Kuopio University, Kuopio, Finland [45].

All plants used in measurements were grown from seeds. Plants were grown in 1 L clay pots filled with commercially-available potting soil and were watered daily. The gas exchange measurements were performed using a custom-built gas-exchange system described in detail in Rasulov et. al. [46]. Shortly, the flow-through plant chamber of 1.2 L was made of two glass layers. Water with set temperature was circulated between the outer and

inner glass layers to control the chamber temperature. All tubing in the system was made of stainless steel or Teflon. Flow rate through the system was maintained at 1.4 L min^{-1}. Light was provided by four perpendicularly positioned 50 W halogen lamps, and light intensity could be regulated between 0-1000 μmol m^{-2} s^{-1} by changing the lamp voltage. In these experiments the light intensity was kept at 650 μmol m^{-2} s^{-1}.

Volatiles from the chamber exhaust air were adsorbed at a flow rate of 200 ml min^{-1} for 20 min. (altogether 4L air) onto multibed stainless steel cartridges (10.5 cm length, 3 cm inner diameter, Supelco, Bellefonte, USA) filled with Carbotrap C 20/40 mesh (0.2 g), Carbopack C 40/60 mesh (0.1 g) and Carbotrap X 20/40 mesh (0.1 g) adsorbents (Supelco, Bellefonte, USA). Before the collection of volatiles, the traps were cleaned by the passage of a stream of ultrapure helium at a flow rate of 200 ml min^{-1} and at temperature of 250 °C for 2 hours. Sampling of plant volatiles was done at room temperature of 25 °C. Background air samples were collected from the empty chamber before and after the measurements. Adsorbent cartridges were analyzed with a combined Shimadzu TD20 automated cartridge desorber and Shimadzu QP2010 plus GC MS instrument (Shimadzu Corporation, Kyoto, Japan). The following TD20-parameters were used: He purge flow 40 ml min^{-1}, primary desorption temperature 250 °C, primary desorption time 6 min, second stage trap temperature during primary desorption: −20°C, second stage trap desorption temperature 280°C, hold time 6 min. Adsorbent cartridges were backflushed with high purity He during thermal desorption. A Zebron ZB-624 fused silica capillary column (0.32 mm i.d.×60 m, 1.8 µm film thickness, Phenomenex, USA) was employed for the volatile separation using the following GC oven program: 40 °C for 1 min, 9 °C min^{-1} to 120 °C, 2 °C min^{-1} to 190 °C, 20 °C min^{-1} to 250 °C, 250 °C for 5 min. The GC carrier gas was He (99.9999%, Elmer Messer Gaas AS, Tallinn, Estonia) with 1.48 ml min^{-1}. Shimadzu QP2010 Plus mass spectrometer was operated in the electron impact mode. The transfer line temperature was set at 240 °C and ion-source temperature at 150 °C. The absolute amounts of terpenes were calculated based on an external standard consisting of known amounts of mono and sesquiterpenes. The compounds were identified by comparing the mass spectrum of an individual compound to the spectra of compounds in external standard and in the NIST Library.

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