Practical approaches to plant volatile analysis

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Summary

Plants emit volatile organic compounds (VOCs) that play important roles in their interaction with the environment and have a major impact on atmospheric chemistry. The development of static and dynamic techniques for headspace collection of volatiles in combination with gas chromatography–mass spectrometry analysis has significantly improved our understanding of the biosynthesis and ecology of plant VOCs. Advances in automated analysis of VOCs have allowed the monitoring of fast changes in VOC emissions and facilitated in vivo studies of VOC biosynthesis. This review presents an overview of methods for the analysis of plant VOCs, including their advantages and disadvantages, with a focus on the latest technical developments. It provides guidance on how to select appropriate instrumentation and protocols for biochemical, physiological and ecologically relevant applications. These include headspace analyses of plant VOCs emitted by the whole organism, organs or enzymes as well as advanced on-line analysis methods for simultaneous measurements of VOC emissions with other physiological parameters.

Keywords: VOC, SPME, headspace sampling, GC-MS, real time analysis.

Introduction

While all plants exchange non-organic volatiles (CO₂, O₂) during photosynthesis or respiration, most of them also have the ability to emit volatile organic compounds (VOCs) from different organs such as flowers, fruits and leaves. The physicochemical constraints on volatility limit plant VOCs to low-molecular and largely lipophilic products that belong to the classes of terpenes and non-terpene aliphatics (including nitrogen- and sulfur-containing compounds), phenylpropanoids and benzenoids.

Because many VOCs produced by plants, such as constituents of essential oils, are widely used commercially as flavorings and fragrances, their analysis in the food and perfume industry has a long tradition (Bicchi, 2004). However, the significance of VOCs in plant physiology and ecology has been extensively investigated only in the past 10–15 years and has revealed roles for VOCs in the mutualistic interaction of plants with other organisms and biotic and abiotic stress responses (Dudareva et al., 2004). Probably the most well known are volatiles emitted from floral tissues attracting pollinators (Pichersky and Gershenzon, 2002). Several plant species store mixtures of VOCs in specialized secretory structures such as glandular trichomes or resin ducts (Gershenzon et al., 2000; McGarvey and Croteau, 1995) which release their contents in response to tissue damage and may deter herbivores or inhibit microbial growth (Langenheim, 1994). Moreover, research in the past decade has revealed that herbivory on aerial or below-ground plant tissues commonly induces de novo biosynthesis and
emission of VOCs including C₆ green-leaf volatiles (e.g. (Z)-3-hexenal and (Z)-3-hexenyl acetate), methyl salicylate, methyl jasmonate, indole, terpenes and others. These volatiles can act as direct defense compounds (Andersen et al., 1994; De Moraes et al., 2001) or play a role in indirect defense by attracting natural enemies preying upon or parasitizing herbivores (Dicke et al., 2003; Kessler and Baldwin, 2001; Rasmann et al., 2005; Turlings et al., 1995). Finally, chemical volatile signals released from injured plants not only affect herbivores or pathogens but may also signal alarm to neighboring plants by triggering defense responses (Arumuganathan et al., 2000; Engelberth et al., 2004).

Independent of tissue damage by other organisms, numerous plants emit VOCs in response to light and temperature changes or other abiotic stresses like flooding or drought (Ebel et al., 1995; Holzinger et al., 2000; Kreuzwieser et al., 2000). Low-molecular-weight terpenes such as isoprene (C₅), monoterpenes (C₁₀) and sesquiterpenes (C₁₅) are released in substantial amounts from woody plants. They have significant impact on atmospheric chemistry since they contribute to the formation of ozone and secondary organic aerosols in the presence of anthropogenic pollutants (Atkinson, 2000; Hoffmann et al., 1997; Kesselmeier and Staudt, 1999; Sharkey and Yeh, 2001). Furthermore, isoprenoid emissions influence levels of atmospheric hydroxyl radicals (·OH) and the atmospheric residence time of methane, an important greenhouse gas (Thompson, 1992). The physiological role of such terpene emissions is still not completely understood. Volatile terpenes are believed to improve the thermotolerance of photosynthetic tissues since they are likely to intercalate into thylakoid membranes and may stabilize them at high temperatures (Loreto et al., 1998; Sharkey et al., 2001). There is increasing evidence to show that terpene volatiles exhibit antioxidative activities in planta by quenching reactive oxygen species (Loreto and Velikova, 2001; Loreto et al., 2001a). Moreover, terpene volatile emissions are thought to act as a metabolic safety valve to avoid the undue sequestration of phosphates (Rosenstiel et al., 2004).

The increasing scientific interest in the biochemistry, physiology, ecology and atmospheric chemistry of plant VOCs has led to the development of a variety of systems for the collection and analysis of volatiles (Linskens and Jackson, 1997; Millar and Sims, 1998). In the past decade in particular, volatile analysis has improved by the design of relatively inexpensive but sensitive bench-top instruments for gas chromatography–mass spectrometry (GC-MS). The developed headspace analysis techniques provide a more representative volatile profile of living plants than traditional methods of solvent extraction or steam distillation. Besides manually operated headspace sampling methods, automated VOC analysis systems with high time resolution and on-line capability have become indispensable for monitoring fast changes of volatile profiles during plant development or under stress conditions. The need for real-time measurements has led to considerable interest in non-chromatographic methods, most often involving chemiluminescence or infrared photoacoustic (PA) spectroscopy, or mass spectrometry (e.g. proton transfer reaction (PTR)-MS). Such systems can be combined with additional equipment for parallel measurements of other stress-sensitive physiological parameters such as photosynthetic activity. VOC analysis systems that are sensitive, fast and fully automated are also of increasing importance to elucidate the biosynthesis of plant VOCs. In addition, functional genomics approaches for dissecting the metabolic pathways of plant VOCs demand time-efficient techniques for volatile product analysis of recombinant enzymes or high-throughput profiling of volatile metabolites of mutant and transgenic plant lines.

In this paper we will describe the principal methods and latest technologies used in the analysis of plant VOCs in different areas of research. The advantages and disadvantages associated with each method will be pointed out to facilitate the choice of the most appropriate method and equipment required for a particular research problem.

**Sampling and analyzing plant volatiles – which method to choose?**

All methods for the analysis of plant volatiles attempt to identify the authentic profile of volatile blends emitted by a plant. However, the choice of which system to use in a particular experiment for collection and analysis of plant volatiles usually depends on the biological problem and plant material being investigated (Figure 1). First, it needs to be decided whether volatiles should be analyzed from plants grown under laboratory conditions or in their natural habitat. To address ecologically or physiologically relevant functions of volatiles in natural populations, field collections of VOCs require portable, robust and often simplified equipment. In comparison, system set-ups in the laboratory can include computer-assisted sample collection and additional devices to reduce contamination and to precisely control temperature, light intensity, photoperiod and relative humidity. Standardized conditions allow the monitoring of VOC emissions in response to changes of a single variable such as herbivore damage.

Volatile emissions are most conveniently collected in situ from whole plants. However, it is often required to sample VOCs from plant parts or organs, for example to distinguish the volatiles of reproductive and vegetative tissues, to determine stress-induced VOC emissions as local or systemic responses or to correlate VOC emissions with tissue-specific enzyme activities. In this case, VOCs are sampled either from detached plant parts or preferably in situ from enclosed plant organs to avoid additional emission of VOCs due to wounding effects.
Dependent on the plant species investigated, VOC emission rates may vary significantly and hence determine which instrumentation is required for adequate collection efficiency and analysis sensitivity. While trace amounts of trapped volatiles may be sufficient for analytical purposes, larger quantities are necessary for NMR analysis or bioassays. In addition, the researcher has to decide whether to take a more qualitative ‘snap-shot’ of volatiles released at a particular time point or to measure quantitative, developmental or stress-related changes in VOC emissions with appropriate time resolution. Finally, specialized equipment allowing on-line or high-throughput analysis has to be considered for studies of plant volatile metabolism or mutant screenings.

To guide the inexperienced researcher in the choice of appropriate VOC analysis protocols, the most important methods used to investigate the biological roles of plant volatiles and their metabolism are presented in more detail in the following sections.

**Sampling volatiles in the headspace of whole plants and plant organs – practical aspects and applications**

Volatiles have been investigated most extensively in the airspace (headspace) surrounding above-ground plant parts. Major fields of study include analyses of floral volatiles in relation to pollination biology, measurements of volatiles such as isoprene released from photosynthetic tissues in response to changes in light and temperature, and volatile emissions induced by herbivore damage. In most cases, the emitted volatiles have to be sampled and concentrated prior to subsequent analysis. Headspace sampling is a non-destructive method for collecting volatiles. Compared with solvent extractions of volatiles from plant tissues, headspace analysis gives a more realistic picture of the volatile profile emitted by plants and detected by insects that respond to plant volatiles, making this method most suitable for many ecologically relevant applications. In general, devices used for headspace collections should be free of materials that retain volatiles or cause bleeding of compounds that may contaminate the system and interfere with volatile analysis. Commonly used materials that do not show bleeding include glass, metal and special plastics such as Teflon, although even this material may not be completely inert. Details of materials suitable for the construction of headspace collection chambers can be found in Millar and Sims (1998).

**Sampling volatile organic compounds in static headspace**

For static headspace analysis, the plant or its parts are enclosed in a container and the emitted volatiles are trapped onto an adsorbent. The air surrounding the plant remains ‘static’, which means it is not circulated in the chamber. Volatiles are enriched on the adsorbing matrix without sampling impurities of a continuous air stream that may obscure the detection of low-abundant VOCs. Thus, this
method is advantageous for sampling VOCs from low-emitting plants.

An important advance in static headspace analysis was the development of solid phase microextraction (SPME) which is a fast and simple method for collecting volatiles at detection limits in the ppbv (parts per billion by volume) range. Solid phase microextraction is based on adsorption and desorption of volatiles from an inert fiber coated with different types of adsorbents. The fiber is attached within the needle of a modified syringe and volatiles can be sampled by inserting the needle through a septum of a headspace collection container and pushing the plunger to expose the fiber (Figure 2). Following equilibration between the fiber and the volatile sample (a few minutes to half an hour), the fiber is retracted into the needle and can be transferred to a gas chromatograph for direct thermal desorption. Solid phase microextraction fibers can be reused approximately 100 times. Thermal desorption of VOCs from the fiber eliminates the need for solvents that may contain impurities which will interfere with sample analysis. However, by desorbing the entire sample into the injector, no repeated injections of the sample are possible.

By carefully selecting the polarity and thickness of the fiber coating, compounds of different polarity and volatility ranging from high-boiling or semivolatile to volatile compounds can be sampled (Table S1). While thin coatings ensure a fast diffusion and release of semivolatile compounds, thicker coatings may better retain highly volatile compounds until thermal desorption. The amount of compound adsorbed by the SPME fiber depends not only on the thickness of the fiber coating but also on the distribution constant of the analyte, which generally increases with its molecular weight and boiling point. Solid phase microextraction will not allow trapping of sufficient amounts of volatiles for structure elucidation of unknown compounds. Larger amounts of VOCs can be sampled on iron stir bars, which are coated with the same sorbents as SPME fibers and were originally developed to extract organic compounds from aqueous samples (Bicchi et al., 2000). The bar is placed in the headspace of the plant sample and trapped volatiles are released by thermal desorption after transfer of the bar into the GC injector liner.

Quantification of volatiles by SPME is generally possible by the application of internal or external calibration. To obtain reproducible quantitative results, the fiber and sample should reach equilibrium, at which the amount of analyte removed from the fiber is proportional to the concentration of the compound in the sample. Equilibration time is dependent on the volatility and polarity of the analyte and the properties of the adsorbent. Careful control of sampling parameters (sample volume, temperature, time) and the use of appropriate standard mixtures for calibration are crucial for quantitative analysis. However, quantification by SPME may still be difficult or impractical when dealing with a wide range of compounds with different distribution constants.

The SPME device and further detailed information on the theory, optimization and different types of fiber adsorbents are available from Supelco (Belleville, PA, USA). Portable field samplers are also available that have a sealing mechanism to allow storage of samples for later analysis in the laboratory. Several companies offer GC autosamplers with a SPME option for rapid processing of multiple vials. However, the size and shape of vials in these samplers are fixed and often impractical for sampling from plants. For rapid screening of multiple samples in variable types of containers, an automated adjustable robotic arm has been developed (Pham-Tuan et al., 2001). Automated SPME-GC allows high-throughput analysis of volatile profiles emitted from plant parts such as flowers or leaves of natural variants, mutant or transgenic lines as shown by Aharoni et al. (2003) for Arabidopsis thaliana.

Numerous studies have used SPME extraction for the analysis of a broad range of volatile compounds in food, air, soil and water samples. Biological applications include SPME analyses of VOCs from whole plants or flower and bark tissues (e.g. Flamini et al., 2002; Rohloff and Bones, 2005; Sha et al., 2004) and essential oils (e.g. Tomova et al., 2005).

As an alternative to trapping VOCs on adsorbents, direct headspace sampling is possible by removing an aliquot of the headspace with a gas-tight syringe and injecting it directly into the gas chromatograph (see also section on

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**Figure 2.** Static headspace sampling with a solid phase microextraction (SPME) device.

For volatile extraction by SPME, the plant sample is enclosed in a glass container with a broad opening for easy removal of the plant. The adsorbent-coated fiber is mounted on a SPME fiber holder, similar to a modified syringe that is injected through the septum of the sample container. By pushing the plunger of the SPME fiber holder, the fiber is extended from the needle and exposed to volatiles. After collection, the fiber is retracted into the needle and the SPME device can be removed from the container for GC analysis.
VOC analysis techniques for studying the biosynthesis of plant volatiles and their role in stress physiology. The process can be automated with commercial headspace autosamplers. However, direct headspace sampling requires a sufficiently high concentration of VOCs in the headspace to provide at least nanogram quantities in the sample taken for GC analysis. Thus, the method is limited by the need for satisfactory sensitivity.

Despite the latest advances in SPME technology, static headspace collection has some clear disadvantages. The static airspace accumulates humidity as well as heat, especially when samples are collected under illumination, and these conditions may interfere with normal physiological processes and affect the emission of volatiles. Because not all of the emitted volatiles are removed during one sampling time, changes in emission over time will be difficult to determine. In conclusion, static headspace sampling is suited for qualitative analyses of VOCs and surveys of VOC profiles of different plant species or cultivars at a single time point rather than for quantitative measurements of changing VOC emissions.

Dynamic headspace sampling techniques

Dynamic headspace sampling represents the most frequently used technique in all areas of plant volatile analysis. In this sampling method, a continuous air stream flows through the sample container as a carrier gas, which increases the headspace sample size. While the analytes are trapped on adsorbents, the carrier gas is circulated through or purged out of the container, allowing for the collection of amounts of volatiles sufficient for detection and even structure elucidation. In open dynamic headspace systems in particular, some of the problems related to a static headspace such as increases in temperature and humidity or an accumulation of deleterious volatiles in the headspace are eliminated by a constant air stream. However, care needs to be taken to provide clean incoming air that is filtered, for example through activated charcoal, to avoid interference of impurities with headspace volatile compounds in the ensuing analytical steps.

In most cases of dynamic headspace analysis, volatiles are trapped and enriched on an adsorbing matrix prior to their analysis by GC. A large number of different adsorbent materials are available and several reviews have provided excellent information regarding their choice and applications (Dettmer and Engewald, 2002; Linskens and Jackson, 1997; Millar and Sims, 1998; Raguso and Pellmyr, 1998). Table S1 gives an overview of the most common matrices including carbon-based adsorbents and organic polymers, with information on their thermal stability, binding affinities and artifacts caused by reactions of the adsorbing material itself or the adsorbed VOCs on the matrix surface.

The adsorbent material is usually packed inside narrow glass or metal tubes in beds of approximately 2–50 mm between glass wool plugs or metal grids. The air containing the VOCs is passed through the adsorbent bed at a particular flow rate during the sampling process. Trapped volatiles can be eluted from the adsorbing matrix into glass vials with pure solvents or mixtures of low-boiling-point organic solvents. The solvent should contain a defined amount of one or two standard compounds (e.g. 1-bromodecane, n-octane) for semiquantitative analysis. Adsorbent materials with high thermal stability such as Tenax, carbon molecular sieves and graphitized carbon blacks can be employed in thermal desorption of VOCs as an alternative to solvent extraction. In thermal desorption, VOCs are directly released from the adsorbent under high temperatures and are usually concentrated by cryofocusing prior to separation by GC (see below). Compared with solvent extraction, thermal desorption provides increased desorption efficiencies and prevents sample dilution, leading to increased analytical sensitivity. Other advantages are reduced manual sample preparation times and the avoidance of impurities in the organic solvent interfering with GC analysis. However, thermal desorption is not free of limitations, which include the lack of repeated sample injections and the occurrence of artifacts due to the degradation of thermally unstable compounds or reactions of the trapping media (Table S1).

A major problem with all trapping materials is their incomplete adsorption of VOCs. For example carbon-based matrices have very specific affinities for VOCs. Those materials that effectively trap VOCs of low polarity and low molecular weight are inefficient for adsorbing VOCs with opposite characteristics. Therefore, when collecting complex mixtures of VOCs care must be taken, and eventually more than one adsorbent may be required to trap a blend that is qualitatively and quantitatively representative. This is especially critical when the mixture is used for subsequent bioassays such as insect attraction experiments. This problem can be solved by ‘multiple layer adsorption’ where carbon matrices with different retention characteristics are placed in series with air flowing first into the matrix adsorbing very volatile VOCs (e.g. Carbograph and Carbotrap C). The theory and practice of VOC sampling with multi-bed tubes go beyond the scope of this report and has been extensively detailed elsewhere (Ciccioli et al., 2002). Multi-bed tubes are commercially available (e.g. Carbotrap/Carbosieve SII beds, Markes International, Pontyclun, UK) or can be self-made (Schnitzler et al., 2004b).

Closed-loop stripping. Closed-loop stripping systems have broad utility for the collection of herbivore-induced volatiles, as shown for induced VOC emissions from lima bean, as well as for trapping volatiles from detached flowers (Dudareva et al., 2005; Koch et al., 1999; Tholl et al., 2005). In these
systems, volatiles are collected during continuous circulation of headspace air inside closed chambers. A simple closed-loop stripping system developed by Boland et al. (1984) and Donath and Boland (1995) consists of small 1- or 3-l glass desiccators to which air circulation pumps are attached (Figure 3). Plants or plant parts are placed inside the glass chambers and headspace air is continuously circulated through a volatile trap placed in a stainless steel housing, allowing quantitative trapping of the emitted VOCs (Donath and Boland, 1995). Since the circulation of air in a closed system minimizes trapping of air contaminants as compared with the air flow in an open system (see below), closed-loop stripping is applicable to VOC analyses of plants with low volatile emissions. For example, the small flowers of the model plant A. thaliana show a significantly reduced emission rate of volatiles compared with flowers of highly scented plants (Chen et al., 2003) making it virtually impossible to collect volatiles from a single flower. For a detailed analysis of Arabidopsis floral terpene volatile profiles, 70–120 detached inflorescences were placed in small, water-containing, glass beakers inside a sealed 1-l desiccator, and the closed-loop stripping procedure according to Donath and Boland (1995) was applied. Following the collection of volatiles on 1.5 mg charcoal or 25 mg Super Q traps for 8 h, compounds were extracted from the traps with 40–100 µl dichloromethane and analyzed by GC-MS. For comparative analysis of volatiles from undetached flowers, floral volatiles were sampled by semi-open dynamic headspace volatile trapping (see below) for a similar length of time from whole flowering plants with an equivalent number of inflorescences. Profiles and compositions of floral volatiles obtained by both methods were highly comparable. However, volatile profiles obtained with the closed-loop stripping method showed a significantly higher signal to noise ratio due to the trapping of fewer air contaminants. In this way, minor compounds of the complex floral terpene volatile mixture could be analyzed (Chen et al., 2003; Tholl et al., 2005). Signal to noise ratios may be improved even further by sampling VOCs from a larger number of inflorescences.

Other advantages of closed-loop stripping systems are that they are easy to set up in controlled climate chambers and it is possible to collect VOCs from several individual plants at the same time, which makes this method suitable for screening purposes. However, results from closed-loop stripping sampling should always be compared with those obtained by open headspace trapping to exclude artifacts due to effects on the enclosed plant caused by changes in the atmosphere of the chamber in the absence of air exchange with the outside of the chamber. In addition, volatiles such as ethylene that are not trapped on the adsorbing material may accumulate in the chamber, and relative humidity might increase without occasional venting of the system between trapping cycles.

`Pull' and 'push–pull' systems. In contrast to closed-loop stripping devices, pull or push–pull systems operate with a constant flow of air which is taken up from the outside and leaves the system with the volatiles emitted by the plant. In a pull system, air is pulled over the plant sample through an adsorbent trap that is connected to a vacuum pump. A simple form of a pull system is an adsorbent trap that is directly positioned next to a plant or a plant organ without any further enclosure of the plant (Burger et al., 1988). In open-top chambers, the plant or its parts are placed in a container that allows the flow of ambient air without prior cleaning (Figure 4a,b). Halitschke et al. (2000) have used such system to investigate herbivore-induced volatile emissions of hydroponically grown Nicotiana attenuata plants or leaves of soil-grown plants, and Kaiser (1991) has applied similar devices for sampling of floral volatiles. The set-up is relatively simple, inexpensive and highly portable making it suitable for simultaneous collections from many chambers and for sampling in the field as shown by Kessler and Baldwin (2001). This system works well for plants that emit high amounts of volatiles; however, the risk is high of trapping impurities from the ambient air unrelated to the investigated VOC blend that will obscure the detection of minor sample compounds during GC analysis. By enclosing plants, leaves or flowers entirely in a glass container or a polyacetate cooking bag that releases very few volatiles (Dobson, 1991), the amount of impurities from ambient air may be reduced. Air enters the collection chamber through a purifying filter and is drawn from the chamber by pulling a

**Figure 3.** Volatile collection by the closed-loop stripping procedure. The photograph shows collection of volatiles from detached snapdragon flowers during feeding with isotope-labeled precursors (Dudareva et al., 2005). Air is continuously circulated through the glass container at flow rates of 2–3 l min⁻¹. P, circulation pump; T, steel housing containing the volatile trap; S, Teflon stopper with holes for pump adaptors; D, 1-l desiccator; F, detached snapdragon flowers in a glass beaker with the feeding solution.
defined volume through the adsorbent trap that is extracted for further analysis. Such devices have been described by Dudareva et al. (2003) and Raguso and Pellmyr (1998) for headspace analysis of volatiles from snapdragon and Clarkia breweri flowers. Care must be taken to achieve homogeneous air flow over the plant and a sufficient flow rate must be set to prevent increases in temperature and humidity when the system is exposed to direct sunlight. If a flexible cooking bag is used, it may collapse and damage the plant, which may alter volatile emission.

In push–pull systems (Figure 4c), air is pushed into the headspace sampling container at a rate regulated by a flow meter. Prior to entering the chamber, the air is cleaned by flowing through a purifying filter material such as charcoal that adsorbs impurities and may be humidified at a desired rate by mixing it with a second air stream with saturating humidity. Inside the container, a uniform air flow over the plant should be created. A portion of the air is pulled out of the chamber through an adsorbent trap connected to a vacuum pump. The flow rate of the outgoing air stream is regulated by a second flow meter, allowing the collection of a defined percentage of the volatiles emitted by the plant. The remaining air flow escapes the collection chamber through a vent preventing overpressure. Compared with pull collection devices, this system offers more flexibility in regulating in- and outgoing air flow dependent on the emission rates of VOCs in the investigated plant. We have employed this system to collect volatiles from aphid-infested plants over several days (Kunert et al., 2005) and trap VOCs from flowering Arabidopsis plants over 8 h (Chen et al., 2003).

In some applications it is desirable to collect volatiles from only certain parts of the plant, such as those released from undamaged leaves after localized herbivore damage (Figure 4d). While leaving the herbivore-damaged leaves outside the collection system, headspace from upper undamaged leaves can be collected for several days as demonstrated for VOC emissions from Gossypium hirsutum (Röse et al., 1996). Such measurements were made possible by using open-bottomed glass collection chambers set on top of a multiport guillotine base. The guillotine base contains concentric gas-sampling ports and two Teflon-coated removable blades that close the bottom of the chamber around the stem of the plant, leaving an opening for the stem where the blades fit together (Figure 4d). The positive-pressure venting around the stem prevents ambient air from entering the collection chamber. This system allows for the collection of volatiles from parts of intact growing plants while completely isolating the lower section of the plant including soil and pots (Heath and Manukian, 1994; Röse et al., 1996). The technique was described in detail by Heath and Manukian (1992) and has been automated to
monitor time-dependent changes in VOC emissions by switching traps for drawing samples at defined time intervals over several days (Heath and Manukian, 1994; Manukian and Heath, 1993). For example, rhythmic volatile emissions from flowering Mirabilis jalapa were measured with this system over a time course of 48 h (Effmert et al., 2005), and it was also successfully applied for time-course analyses of herbivore-induced VOC emissions from maize (Degenhardt and Gershenzon, 2000). The entire collection chamber can be installed in a greenhouse or in a climate chamber to control environmental parameters.

Besides push–pull systems, other laboratories have designed collection chambers in which pressurized air entering the container is pushed through the adsorbent trap without applying additional vacuum (‘push’ systems, Dicke et al., 1999; Kunert et al., 2002). In these systems containers must be sealed well to prevent leakage of the air containing VOCs, and new unclogged volatile traps should be used to prevent overpressure and ensure efficient air flow.

In most of the systems presented above environmental parameters are simply regulated by the conditions of the climate chamber and manual adjustment of the rate of air flow and air humidification inside the collection device. Systems with improved automated parameter controls have been described by Jakobsen (1997), for example, which contain electronic humidity sensors inside the headspace chamber and temperature control by continuous circulation of water between double walls of the glass container. Smaller systems using cuvettes with comparable parameter control have been developed (for a detailed description see the section on VOC analysis techniques for studying the biosynthesis of plant volatiles and their role in stress physiology) for measurements of VOCs from leaves or branches.

Gas chromatographic separation and detection of plant VOCs

Plant VOCs trapped on adsorbing matrices are routinely analyzed by the standard technique of GC. A wealth of literature is available describing GC analysis protocols and recent advances in GC analysis technology, of which only a small portion will be covered in this paper (e.g. Dewulf and Van Langenhove, 2002; Handley and Adlard, 2005; Lockwood, 2001; Marriott et al., 2001; Merfort, 2002; Ragunathan et al., 1999).

For GC analysis of VOCs, samples are either injected as solvent extracts into the heated injector in a split or splitless mode or desorbed from the adsorbent by placing it directly in a thermal desorption tube, heated to 250–300°C. In a two-stage thermal desorber, the thermally released volatiles are concentrated by a cold trap (or cryotrap) prior to their injection into the GC column. Recent technology has led to the development of on-line systems which combine volatile sampling with automated thermal desorption (see below). Furthermore, an interesting method has been described for direct thermal desorption of volatiles from floral organs by placing them in quartz microvials that are inserted into a modified GC injector (Jürgens and Dötterl, 2004). Care must be taken to prevent decomposition of compounds at high temperatures.

For analytical purposes, volatiles are commonly separated on fused silica capillary columns with different stationary phases, such as the non-polar dimethyl polysiloxanes (e.g. DB-1, DB-5, CPSil 5), and the more polar polyethylene glycol polymers, including Carbowax™ 20M, DB-Wax, and HP-20M. More in-depth guidance on column selection can be obtained from column vendors.

Following separation on a GC column, volatile compounds can be analyzed by a variety of different detectors. Flame ionization detectors (FID) are commonly used for quantitative analysis because of their wide linear dynamic range, their very stable response and their high sensitivity with detection limits of the order of picograms to nanograms per compound. Another detector, preferred for the analysis of volatile terpenes, is the photoionization detector (PID) which is considerably more sensitive than the FID in the presence of reactive double bonds but requires careful calibration for quantitative analysis.

Mass spectrometry (MS) detectors are the most popular type of detector for routine plant volatile GC analysis. In the mass spectrometers of most standard GC-MS benchtop instruments, compounds exiting the GC column are ionized by electron impact (EI) and the resulting positively charged molecules and molecule fragments are selected according to their mass-to-charge (m/z) ratio by entering a quadrupole ion trap or a quadrupole mass filter. Total ion chromatograms are obtained, which provide information on the retention time of each compound and its mass spectrum consisting of a characteristic ion fragmentation pattern. Detection limits of highly sensitive mass spectrometers are in the picogram range for the full scan mode (scanning ions over a wide molecular range) and may be as low as in the femtogram range (in quadrupole mass filters) in the selected ion monitoring (SIM) mode scanning selected ions that are representative of a compound. Quantification is possible in full scan or SIM mode, but requires thorough calibration for each individual compound.

For identification of compounds in GC-MS analysis, suggestions can be obtained from popular mass spectral libraries such as Wiley and NIST MS databases and databases providing retention index data such as the Kovats index system, related to n-alkanes (see Table S2). However, identification based solely on retention indices or library mass spectral data is not reliable and often leads to false assignments. A correct identification requires at least the determination of Kovats indices on two columns with
different polarities and a match of the mass spectrum of the compound of interest with that of an authentic standard. A simple co-injection of sample and standard on only a single column is not sufficient. Ion-trap-derived mass data may be less suitable for identifying unknown compounds due to poor matches with available MS libraries. Mass spectra obtained by softer chemical ionization (Cl) instead of El show limited molecular fragmentation but are helpful for obtaining stronger molecular ions, especially for aliphatic hydrocarbons.

In addition to the identification of the basic structure of plant VOCs, it is often desired to determine their chirality since the enantiomeric composition of volatiles can be crucial in their role as chemical signals. Several enantioselective capillary columns are available (Schurig, 2001) with chiral phases such as different hydrophobic cyclodextrin derivatives (Bičchi et al., 1999; König and Hochmuth, 2004). Generally, acetylated cyclodextrin derivatives are preferred for separating polar analytes, while non-polar compounds are better resolved on pre-alkylated cyclodextrin derivatives. The application of chiral separation in flavor and fragrance analysis and enantiomeric identification of sesquiterpene hydrocarbons has been well documented by Maas et al. (1994a,b), Schreier et al. (1995) and König and co-workers (e.g. König and Hochmuth, 2004; König et al., 1992).

Because blends of plant volatiles can consist of many chemically diverse compounds as well as isomers, a simple GC-MS analysis run might not be sufficient for the identification of all products. Tandem mass spectrometry (MS-MS) has been established allowing separate analyses of single compounds of complex GC peaks (Granero et al., 2004; Raganathan et al., 1999). In MS-MS, ions of interest (parent ions) are mass-selected and typically fragmented by collision with a neutral gas followed by mass analysis of the resulting product ions. Furthermore, GC-MS analysis can be complemented by capillary GC–Fourier transform infrared spectroscopy (FT-IR). This spectroscopic method has been employed to differentiate closely related isomers with very similar EI mass spectra (Raganathan et al., 1999). Fourier transform infrared spectroscopy provides information on the intact molecular structure and allows unique spectra to be obtained even for similar isomers. The utility of GC-FT-IR is limited by difficulties in quantification and time-consuming data interpretation, although a growing collection of high-quality data is provided by the Sadler database (Sadler Division of Bio-Rad, Philadelphia, PA, USA).

If it is impossible to separate complex volatile mixtures sufficiently on a single column, two-dimensional capillary gas chromatography can be used. Here, compounds are separated on a first column from which selected fractions (heart-cuts) are directed to another column as the second dimension. This approach has been used, for example, to determine the enantiomeric composition of monoterpene hydrocarbons in tissues of *Picea abies* by combining a conventional GC column with a chiral column (Borg-Karlson et al., 1993). More recently, so-called ‘comprehensive GC × GC systems’ have been developed that consist of two columns with differing polarity that are directly coupled by a cryogenic modulator compressing regions of the effluent from the first column and injecting them in the second column. The simultaneous two-column separation leads to a significant increase in total separation space. The method is of particular interest in the field of analysis of essential oils to increase peak resolution and improve quantification or identification of volatile components (Di et al., 2004; Marriott et al., 2003). The identification of chemical fingerprints via comprehensive GC × GC may be of interest in the study of plant species with very complex patterns of natural variation in volatile blends.

Another recent trend in GC analysis of VOCs particularly favored by researchers involved in essential oil analysis is the development of fast gas chromatography. Fast GC analysis is carried out in 3–5 min with a comparable efficiency to conventional high-resolution GC on the same matrices (Matisova and Domotorova, 2003). The application of fast GC could be considered for high-throughput analyses in VOC metabolite profiling. However, fast GC requires additional non-standard instrumentation to support, for example, fast temperature changes and high inlet pressures. For efficient detection of compounds in combination with fast GC analysis and comprehensive GC × GC, the use of time-of-flight (TOF)-MS detectors has been discussed (Matisova and Domotorova, 2003). The technique of TOF-MS, generating instantaneous mass spectra, in combination with peak deconvolution algorithms, results in increased rates of acquisition of mass spectral data (up to 5000 mass spectra sec−1) compared with those of quadrupole MS and allows the identification of overlapping peaks.

Whether the researcher needs high-speed GC analysis or improved sample resolution capacity, as described above, has to be decided on a case by case basis. The identification of all compounds of a volatile blend is not always required and relatively simple VOC profiles may still be analyzed by standard GC-MS. However, ongoing advances in GC technology will certainly further improve separation efficiency and analysis time even in routine analysis.

Finally, the elucidation of the structure of unknown volatile compounds merits some general remarks, although details are not within the scope of this paper. Multiple analytical steps usually need to be considered for unequivocal determination. Most often amounts of a single compound sufficient for one- and two-dimensional nuclear magnetic resonance (NMR) analysis techniques need to be isolated on a preparative scale using preparative packed GC columns and thick-film capillary columns with highly selective cyclodextrin matrices (König and Hochmuth, 2004). A recent publication by Nojima et al. (2004) describes a simple...
and efficient technique for preparation of NMR samples of volatile chemicals using a micropreparative GC system.

To determine the absolute configuration of a novel compound, it can be compared with a synthetic reference compound using enantioselective GC. If no synthetic reference compound is available, chemical correlation is applied, meaning that the new structure is chemically modified to a known compound or a product that can be compared with a product derived from a structure with known absolute configuration (König and Hochmuth, 2004).

Monitoring environmentally dependent changes of VOC emissions

Many investigations have documented that emissions of plant volatiles show time-dependent changes. For example, emission of floral volatiles often follows rhythms controlled by diurnal or circadian cycles. Many plants emit low-molecular terpene volatiles such as isoprene and monoterpenes from photosynthetic tissues in a light- and temperature-dependent manner. Furthermore, the release of volatiles as direct or indirect defense signals can be induced by herbivore damage. In order to monitor changes in volatile emissions in response to these environmental factors and correlate them with alterations in physiological parameters, gene expression and enzyme activities, volatile profiles and emission rates need to be determined at multiple time points. Automated dynamic headspace collection systems as described earlier allow sampling of VOCs at predefined time intervals. However, samples trapped in these systems need to be desorbed and subsequently analyzed by GC. This standard off-line GC analysis excludes the possibility of recording fast-changing emissions of volatiles, which occur on timescales of seconds and minutes. This deficiency has led to the development of several on-line techniques combining a fast time response with low detection limits.

Dynamic headspace sampling with on-line thermal desorption

A VOC analysis system with moderate time resolution was developed by attaching an automated dynamic headspace sampling system to an on-line thermal desorption device (Gerstel Online-TDS G, Gerstel, Germany). The Gerstel Online-TDS consists of a thermodesorption unit connected to a cryofocusing device for refocusing of the analyte prior to GC injection. By regulating the mass flow, the system can automatically draw volatile samples and switch between sampling and thermodesorption. Vercammen et al. (2001) connected the TDS-G system to two sample chambers, the headspaces of which were analyzed consecutively using a rotary valve. A time resolution of 5–60 min can be achieved depending on the time necessary to collect sufficient amounts of VOCs from the emitting plant and the time for GC separation. On-line thermal desorption was also employed by Aharoni et al. (2003) for collections of VOCs from Arabidopsis.

Fast and transportable GC (zNose®)

A recently developed miniaturized GC instrument, the zNose® (Electronic Sensor Technology, Newbury Park, CA, USA; Figure 5), combines fast trapping of VOCs with fast on-line GC analysis. The instrument operates with a highly sensitive surface acoustic wave (SAW) quartz microbalance detector, in which VOC analytes are condensed on the surface of an oscillating crystal. This leads to an increase in the oscillator mass and lowers the vibrational frequency proportional to the amount of condensate. Due to the high sensitivity of the SAW detector (in the ppbv range) the time for volatile sampling on a Tenax trap can be reduced to 20–40 sec. Following rapid thermal desorption, compounds are separated on a capillary column (DB-5) of 1 or 5 m length and quantitatively analyzed by the SAW detector. Because of the short operation time and fully automated sampling and data acquisition, air samples can be collected in time intervals as low as 3 min over longer periods without supervision.

The zNose® has been employed for monitoring rhythmic VOC emissions from flowers and induced VOC emissions from herbivore-damaged plants (Kunert et al., 2002).
limitation of this GC is the short column which reduces the resolution of VOCs with similar retention times such as monoterpenes or sesquiterpenes. However, the instrument has been a useful tool for fast quantitative estimations of known volatile profiles, which makes it applicable for measuring fast changes in VOC emissions or screening VOCs on a high-throughput basis. Even more important, the portability of the instrument allows such measurements not only in the laboratory but also in field experiments.

Real-time analysis of volatile terpenes and other VOCs in plant headspace and the atmosphere

By far the most advanced technology employed for the analysis of plant volatile emissions in real time has been developed in recent years in the field of atmospheric chemistry. The particular interest in fast monitoring of VOCs such as isoprene and other low-molecular-weight isoprenoids that have significant impact on ozone and aerosol formation has stimulated the development of different online detection systems. High-frequency sampling is desired for measurements of volatile flux in tree canopies where terpene volatiles show high and rapid reactivity with active chemical species in the atmosphere.

Hills and Zimmerman (1990), from NCAR in Boulder, Colorado, described the FIS (fast isoprene sensor) chemiluminescence detector for on-line detection of isoprene in air. This sensor, in combination with a cuvette system, allowed the first real-time observation of fast changes in isoprene emissions from leaves (Hills and Zimmerman, 1990; Monson et al., 1991). In the chemiluminescence detector, isoprene reacts with ozone, producing formaldehyde in an excited state. This reaction product subsequently relaxes to the ground state emitting light at 450–550 nm, which is detected by a blue-sensitive photomultiplier tube with a detection limit of 400 parts per trillion by volume (pptv) for isoprene and a response time of 0.1 sec. The detection is specific for isoprene since other naturally abundant volatiles, for example monoterpenes, are weak emitters of ozone-induced chemiluminescence (Hills and Zimmerman, 1990). This instrument has brought about significant progress in the understanding of isoprene emission by plants (e.g. Brüggemann and Schnitzler, 2002a; Rosenstiel et al., 2003; Singsaas and Sharkey, 1998) and the determination of isoprene fluxes from forest canopies (e.g. Fuentes et al., 1996) and will remain a significant instrument for the study of isoprene fluxes. However, because of its specificity, its use is limited to isoprene research.

Over the last decade, laser-based infrared photoacoustic (PA) spectroscopy has been developed as an additional versatile tool for sensitive real-time detection of plant volatile compounds. For example, CO₂-based PA spectrometers were used for the detection of the phytohormone ethylene (Arimura et al., 2002; Wächter et al., 1999). The technique is based on the strong absorption of the infrared waveband (3–10 μm) by volatile hydrocarbons, aldehydes and alcohols. The measuring principle is the detection of sound waves: absorption of distinct pulsed laser light by volatiles leads to heating and consequent expansion of the gas sample, resulting in the appearance of sound waves which can be detected photoacoustically by very sensitive microphones (Harren and Reuss, 1997).

Recent technical developments showed that CO (carbon monoxide) overtone lasers can be used for on-line detection of isoprene (Dahnke et al., 2000; Wolfertz et al., 2003, 2004), and oxygenated compounds such as acetaldehyde (Kreuzwieser et al., 2000; Zuckerman et al., 1997) enlarging the field of application of PA spectroscopy in plant physiology. For example, the technique allows the detection of isoprene down to a few ppbv with a time resolution of 1 min in a continuous gas stream. However, PA spectroscopy is still a very sophisticated on-line technology, which needs some further developments to find its way into the repertoire of techniques commonly applied in analysis of biogenic volatiles. In particular, future efforts are necessary to reduce the size of the instruments used, for greater mobility, and to demonstrate a broader application of this spectroscopic technique to the analysis of other volatile terpenes.

Hansel et al. (1995) and Lindinger et al. (1998a,b) reported on the development of proton transfer reaction–mass spectrometry (PTR-MS), a combination of a proton transfer reaction drift tube and a quadrupole mass spectrometer. The instrument, which is commercially available from Ionicon Analytik GmbH, Austria, principally allows fast detection of most VOCs in combination with low detection limits (10–100 pptv). Proton transfer reaction–mass spectrometry can replace GC-MS in many applications of VOC analysis and achieve a response time measured in seconds per compound. The fundamental difference from conventional MS is that PTR-MS uses ‘soft’ chemical ionization of VOC molecules by reaction with hydroxonium ions (H₃O⁺) produced by an external ion source.

The principle of operation of the PTR-MS instrument is shown schematically in Figure 6. A hollow cathode discharge acts as an ion source, which produces H₃O⁺ ions from pure water vapor. Primary H₂O⁺ ions enter the drift tube that is flushed continuously with ambient air and undergo non-reactive collisions with any of the common components in air (N₂, O₂, Ar, CO₂). A small fraction (typically 1%) of the primary H₂O⁺ ions transfers its protons to VOCs which are present as trace gases in air and which have proton affinities higher than that of water. Volatile organic compounds become protonated in the following reaction:

\[ \text{H}_3\text{O}^+ + \text{VOC} \rightarrow \text{VOCH}^+ + \text{H}_2\text{O} \]
(with the reaction constant $k_t$). Unlike conventional electron impact ionization, protonation does not generally cause a fragmentation of the ionized molecule. The ions are transported through the drift tube by a homogeneous electric field. This provides a constant reaction time and energy, so that the proton transfer reactions are well defined. The factor determining the time response is the time the air spends in the drift tube (<0.2 sec). Primary and product ions enter a small intermediate chamber where air is pumped out and the ions are extracted into the detection chamber where their masses are analyzed using a quadrupole mass spectrometer. The detection limit at a signal to noise level of 2 is typically in the order of a few 10 pptv for a 1 sec integration time.

While PTR-MS has been used primarily in field campaigns for trace atmospheric measurements of a variety of compounds including aromatics, isoprene, ketones, leaf wound aldehydes and alcohols (Fall et al., 1999; Hansel and Wisthaler, 2000; Sprung et al., 2001; Wisthaler et al., 2002), this technology has also become an alternative and useful addition to GC analysis of plant enclosure measurements. The following VOCs, which are relevant to plant physiology, have been measured by PTR-MS: isoprenoids (isoprene, monoterpenes, sesquiterpenes), aldehydes, alcohols, ketones and others (e.g. Beauchamp et al., 2005; Fall et al., 1999; Graus et al., 2004; Holzinger et al., 2000; Steeghs et al., 2004). While PTR-MS detects most volatiles as their molecular mass plus one (e.g. methanol m33, acetaldehyde m45, acetone m59, isoprene m69), some VOCs including C$_6$ wound alcohols or monoterpenes undergo some degree of fragmentation within the instrument.

Although the PTR-MS method has found numerous applications and has greatly expanded the capability for relatively fast measurements of VOCs there remain significant weaknesses such as difficulties in the detection of very fast-reacting VOC species (e.g. monoterpenes and sesquiterpenes). Furthermore, interference in the detection of VOCs is possible due to the limited selectivity obtained from monitoring only the protonated parent mass. For example, PTR-MS measures only the total concentration of monoterpenes or sesquiterpene isomers and gives no information on the terpene composition of an air sample (Tani et al., 2003). To enable distinction between isobaric compounds like, for example, alcohols and organic acids, a combination of a proton transfer reaction drift tube with a TOF-MS is required which will dramatically improve the duty cycle (all masses can be monitored simultaneously) and add a high mass-resolving power. To distinguish even between isomers, MS-MS capability needs to be added, which seems possible by using ion trap or triple quadrupole MS technology. At present, combined experiments using slow GC technology (providing high selectivity) together with the fast PTR-MS method (having poor selectivity) are most suitable to monitor and identify fast-changing concentrations of VOCs at trace levels.

**VOC analysis techniques for studying the biosynthesis of plant volatiles and their role in stress physiology**

*The use of gas exchange cuvette systems*

Cuvette systems have become increasingly popular in the field of on-line analysis of plant volatiles, and especially for VOCs emitted from trees. In association with suitable detection systems, cuvette measurements allow precise and rapid monitoring of the air composition and the release or uptake by plants of gaseous compounds such as CO$_2$, H$_2$O and VOCs enabling accurate *in vivo* studies of the relationships between primary metabolism (photosynthesis) and secondary metabolism (VOC synthesis). Cuvette systems also allow the investigation of physical constraints to VOC emissions such as those caused by stomatal opening (Niinemets et al., 2004).
Two types of cuvettes are in general use, leaf and branch cuvettes. Leaf cuvettes are made of a frame in which a leaf, or a portion of it, is sandwiched. They generally have a very small and fixed volume, rarely exceeding 0.5 l. Cuvettes used for measuring VOC emissions from tree branches, have a larger volume (>2 l) and are often inflatable (without solid frames) and of variable volume. A collage of different cuvettes is shown in Figure 7. Cuvettes have transparent windows of glass or plastic to allow illumination of the enclosed plant material on the adaxial and, occasionally, also on the abaxial side of the leaf. Light is provided by external, generally artificial sources such as light-emitting diodes (LEDs) with wavelengths needed for physiological light reactions to occur (Tennessen et al., 1994). As a cold and easy modular light source, LEDs can generate very high and uniform light intensities without overheating the leaf cuvette. However, LEDs are currently difficult to use in branch cuvettes due to insufficient and inhomogeneous lighting.

Figure 7. Examples of cuvette systems for measurements of plant VOCs and physiological parameters.
(a) A simplified sketch of a typical cuvette allowing measurements of the concentration of CO₂ and H₂O (by infrared analysis) and VOC emissions by on-line analysis via PTR-MS or GC, or off-line analysis via cartridge trapping and subsequent analysis by GC-MS.
(b) Special cuvette for simultaneous measurements of gas exchange and fluorescence properties of the leaf.
(c) Cuvette for analyzing ozone exchange by leaves without interference of cuvette material (this cuvette is entirely made out of Teflon).
(d) Cuvette system used to monitor gas exchanges from entire branches under field conditions (courtesy of R. Baraldi, CNR-IBIMET, Bologna, Italy).
(e) Cuvette system for gas exchange analysis from whole plants of Arabidopsis thaliana.
All cuvettes (except (d)) allow full control of the principal environmental parameters (temperature, light intensity and quality, wind speed and relative humidity) and can therefore be used to parameterize the dependence of VOC emissions from key environmental and physiological factors. IRGA, infrared gas analyzer.
illuminated over large and uneven surfaces. Leaf cuvettes are generally thermoregulated by Peltier resistances or, more simply but less effectively, by water circulating in their body walls at the desired temperature. In both cases, the actual leaf temperature is constantly measured by thermocouples appressed to the side of the leaf which is not directly exposed to incident light. Large-volume branch cuvettes are often not thermostated and temperature is simply monitored by one or more thermocouples appressed to leaves in strategic positions.

In all cuvette systems, air circulates inside the cuvette entering from one or more inlet holes and generally exiting through only one outlet. The geometry of the cuvette must favor rapid and turbulent air motion to avoid air stagnation, which may in turn cause condensation of water on cold spots of the cuvette and the formation of a boundary layer resistance restraining the exchange of gases between the leaf and the atmosphere. Therefore, built-in fans are often used to stir the air inside the cuvettes. In general, the volume of air must turn-over at least twice per minute to avoid the above-mentioned problems. The humidity of the air in the cuvette can be easily controlled when working with laboratory equipment by bubbling air into water and setting the desired relative humidity by abating the excess into a condenser (a simple container) maintained at a lower temperature. This system is difficult to use in field experiments, but excess humidity can be controlled with the use of desiccants such as magnesium perchlorite and silica gel.

Laboratory equipment also allows excellent control of the composition of the air by mixing air components from gas cylinders. Although measurements under contaminant-free conditions may not reflect ‘real life’ conditions, they are desired to determine species-specific emission rates of VOCs, and for their parameterization on the basis of single environmental or physiological factors. To eliminate rapid reactions of VOCs with ozone present in the inflowing air, ozone-free synthetic air from cylinders can be used or ozone may be scavenged from ambient air by appropriate chemical traps or physical devices such as a simple piece of metal. In addition, special care should be taken in the choice of ozone-free fans. To simulate the presence of contaminants or pollutants such as ozone or anthropogenic hydrocarbons, these gases can be simply added to the air flow. This also allows the fumigation with enriched isotopes (typically $^{13}$CO$_2$), to study their incorporation in metabolites (see below).

Like other devices used in the collection of VOCs, cuvettes should be entirely constructed with materials which are inert to VOCs, including glass or special plastic and transparent materials such as Tedlar or Teflon film (Figure 7b). If metal is to be used, the metal parts (generally the cuvette frame) can be wrapped in Teflon film or sprayed with a liquid layer of Teflon. In addition, the use of soft Teflon O-rings is recommended to avoid wounding the sandwiched leaf and eliminate wound-induced VOC emissions. In case of fumigating leaves with very reactive compounds such as ozone, fittings and pipelines external to the cuvette should consist of inert materials (Figure 7c).

Cuvette measurements are a favorite in vivo method for studying VOC biosynthesis. They have been employed for the simultaneous detection of isoprenoid emissions and CO$_2$ uptake by photosynthesis in response to changes in light and CO$_2$ and the application of photosynthesis inhibitors. These studies led to the early prediction that isoprenoid VOCs can be formed from photosynthesis intermediates (Loreto and Sharkey, 1990). Concurrent measurements of photosynthesis and VOC emission also allowed calculations of the fraction of photosynthetically fixed carbon that is directly metabolized in VOC biosynthesis (Kreuzwieser et al., 2002; Sharkey and Loreto, 1993). Simultaneous measurements are best performed when air leaving the cuvette is split to reach an infrared gas analyzer for CO$_2$ detection and a fast sensor for VOC emissions such as PTR-MS, allowing, in addition, the examination of labeled compounds.

Special leaf cuvettes allow integrated measurements of gas exchange and photochemical characteristics of the leaf by chlorophyll fluorescence detection (Figure 7b). The rationale for these measurements is that (i) part of the electron transport rate driving photosynthesis and photosynthesis may also drive VOC biosynthesis, (ii) the emission of some VOCs may be related to the dissipation of excess light and (iii) VOC-induced photoprotection may be monitored with a rapid, non-invasive and remote technique. Simultaneous measurements of gas exchange and chlorophyll fluorescence suggested that the physiological role of VOCs (mainly isoprene) is not related to energy dissipation mechanisms (Loreto and Sharkey, 1990), as supported by the early work of Jones and Rasmussen (1975). Measurements of gas exchange and chlorophyll fluorescence, on the other hand, enabled calculation of the fraction of photosynthetic electron transport needed for biosynthesis of terpene volatiles and allowed modeling of monoterpenes and isoprene synthesis on the basis of this physiological parameter rather than by the indirect relationship with environmental (empirical) variables (Niinemets et al., 2002). Fluorescence was successfully used to monitor VOC-induced protection of photosynthesis in response to stress, when gas exchange measurements are prevented by fumigation with toxic compounds (e.g. ozone, Loreto and Velikova, 2001; Loreto et al., 2001b) or when remote screening of photochemical efficiency was sufficient to detect protection from heat damage by isoprenes (Sharkey and Singsaas, 1995).

Finally, special cuvettes allow rapid freeze-clamping of the enclosed leaves for biochemical analysis of enzyme activities and metabolites with very rapid turnover. In a system developed by Sharkey (Loreto and Sharkey, 1993) the leaf sandwiched in the cuvette is frozen extremely quickly (<0.1 sec) by smashing it between the plastic
windows of the cuvette under high pressure (>100 bar) with metallic drums previously frozen in liquid nitrogen. This system was effectively used, with slight modification, to determine pools of dimethylallyl diphosphate (DMADP), the last intermediate in isoprene biosynthesis (Loreto et al., 2004; Wolfertz et al., 2003), and to measure the activities of isoprene and monoterpene synthases (Loreto et al., 2001a).

The use of PTR-MS in analyzing the origin of carbon and metabolic intermediates in VOC biosynthesis

One of the most powerful and valuable abilities of PTR-MS as well as PA spectroscopy – the full potential of which has not yet been tapped – is the detection of stable isotopes allowing study of the metabolic origin and flux of intermediates in the biosynthesis of plant volatiles. On-line analysis using either stable $^{13}$C- (e.g. Karl et al., 2002; Schnitzler et al., 2004a) or deuterium-labeled precursors (e.g. Wolfertz et al., 2003, 2004) have demonstrated the versatility of PTR-MS and PA spectroscopy for elucidating the origin of carbon and metabolic intermediates in plastidic isoprene biosynthesis and providing new insights into the dynamics of metabolic turnover. Figure 8 shows an example of $^{13}$C-labeling of isoprene in the presence of $^{12}$CO$_2$ followed by a loss of the $^{12}$C-labeled isotope by subsequent exposure to $^{13}$CO$_2$ in poplar (Populus × canescens) leaves measured by PTR-MS. Depending on the scientific question and the availability of stable isotope precursors, this approach can be applied to study the biosynthesis of many other plant volatiles such as monoterpens, alcohols and aldehydes.

Determining the biosynthetic origin of VOCs by isotope ratio

In some VOC analyses, $^{12}$C/$^{13}$C isotope ratios have been determined by coupling GC with isotope-ratio mass spectrometry after conversion of the eluting compounds into CO$_2$ and H$_2$O (Gleixner et al., 1998). The isotopic carbon ratios of plant VOCs depend on the differential incorporation of $^{12}$C and $^{13}$C isotopes in the course of different enzymatic reactions. Therefore, determination of the isotope ratios of plant volatiles can give valuable hints about their biosynthetic and subcellular origin. Early use of this method indicated that volatile isoprene and carotenoids share the same biosynthetic pathway and that environmental stresses could increase the supply of carbon to isoprene from pathways other than photosynthetic carbon fixation (Sharkey et al., 1991). Recent $^{12}$C/$^{13}$C isotope ratio studies of volatile terpenes confirmed the different biosynthetic origin of monoterpenes and sesquiterpenes from the plastidic methylenylalcohol phosphate (MEP) pathway and the cytosolic mevalonate pathway respectively, but also indicated the metabolic crosstalk between both pathways (Jux et al., 2001).

Analysis of volatile plant enzyme products

Techniques used for analyzing VOCs can also be applied to determine the activity of enzymes catalyzing the formation of plant VOCs. Since the solubility of many VOCs such as terpene hydrocarbons is extremely low in the hydrophilic phase, these compounds rapidly evaporate from an aqueous enzyme solution and can be analyzed by headspace collection techniques. The sensitive method of SPME (see

Figure 8. Proton transfer reaction–mass spectrometry measurements of $^{13}$C-labeled isoprene isotopes. Kinetics of $^{13}$C labeling of isoprene following exposure to $^{13}$CO$_2$ (a) and wash-out of labeling after return to normal $^{12}$CO$_2$ (b) in poplar leaves. Top panel: Enclosure concentrations of $^{12}$CO$_2$ and $^{13}$CO$_2$ as response to switching from one CO$_2$ source to the other at $t = 0$. Bottom panel: Leaf emission rates of isoprene isotope species ($m69$, unlabelled; $m70$, single labeled; $m71$, double labeled; $m72$, triple labeled; $m73$, fourfold labeled; $m74$, fully labeled) and the sum of all isoprene isotopes. Data are redrawn from Graus (2005). VMR, volume mixing ratio.

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above) was successfully applied to qualitative analysis of terpene volatiles produced in vitro by recombinant plant terpene synthases (Chen et al., 2003, 2004; Köllner et al., 2004; Tholl et al., 2005). A relevant protocol is provided as Protocol S1. Automated SPME extraction may be applied for the rapid analysis of a large number of assays, for example to evaluate different product profiles of recombinant enzymes obtained in a site-directed mutagenesis approach. Quantitative analysis with SPME may be possible but requires extremely careful calibration.

Direct headspace analysis has been used as an alternative method for measuring the activity of terpene synthases. This technique was successfully used for the analysis of the activity of isoprene synthase (Schnitzler et al., 1996; Silver and Fall, 1991) and has become the standard approach for determining the activity of this enzyme from many plant species. The principle of the enzyme assay is rather simple: Protein extracts containing appropriate concentrations of substrate (DMADP) and co-factors are incubated in gas-tight vials under optimized conditions. To terminate the enzyme reaction, the liquid phase containing the enzyme, substrate and co-factors is removed by a gas-tight syringe while the evaporated enzyme product remains trapped in the headspace of the gas-tight vial (Figure 9). An aliquot of the headspace is then injected by a gas-tight syringe for GC analysis. Analytical pre-requisites for the application of automated headspace analysis are a pre-column, filled with absorbent material such as Tenax TA for trapping the volatile compounds upstream of the GC column, and a temperature-programmed injection system for focused compound injection. In a similar approach, the activity of monoterpene synthases has been qualitatively and quantitatively determined in cell-free protein extracts of other plant species (Picea abies, Quercus ilex; Fischbach et al., 2000; Loreto et al., 2001a) with a highly linear detection range of monoterpene volatiles in the assay headspace and a detection limit of 1.5 ppmv (Fischbach et al., 2000). The method has also been exploited for the analysis of cellular DMADP levels via quantitative conversion of DMADP to isoprene under acidic and high-temperature conditions (Brüggemann and Schnitzler, 2002b; Fisher et al., 2001; Loreto et al., 2004; Rosenstiel et al., 2002, 2003). The same technique can be used to measure the activity of isopentenyl diphosphate: dimethylallyl diphosphate (IDP) isomerases (Figure 9, Brüggemann and Schnitzler, 2002a).

**Evaluation of the methods used for VOC analysis**

Although the large array of available techniques for volatile analysis allows selection of the method most suitable for studying specific research problems, it also leads to the difficulty of comparing results biased by each individual method. A floral or leaf volatile profile may vary depending on the technique used. Unfortunately, almost no comparative studies of different methods have been performed. Some of the most critical factors influencing volatile data acquisition are the environmental conditions in the collection chamber or cuvette, including light intensity, temperature and relative humidity, since they directly influence volatile metabolism and the physiology of the plant including photosynthesis and transpiration. The effects of light intensity and temperature have been intensively investigated for isoprene as well as other volatiles emitted from leaves and flowers. Control of the air flow is critical for regulating temperature and relative humidity and providing

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**Figure 9.** Scheme for headspace analysis of terpene synthase and isopentenyl diphosphate (IDP) isomerase activities. Enzymes are incubated with their substrates and co-factors in sealed GC vials. After termination of the reaction, the water phase is withdrawn from the vial and VOC products are analyzed via GC by automated headspace injection.

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optimal gas exchange between the plant tissue and the surrounding air. Another cause of qualitative and quantitative variation of volatile results obtained by different analysis protocols is the use of different volatile adsorbents and desorption methods. Raguso and Pellmyr (1998) observed significant variation in volatile blends from flowers of C. breweri due to the use of different sorbents and organic solvents for compound elution. In addition, they reported on the influence of higher flow rates in trapping compounds with lower volatility, but simultaneously causing increased contaminant background levels in GC analysis.

Another variable in volatile analysis is the time at which volatiles are collected in headspace sampling techniques. The time for volatile collection should always be optimized, since emissions are affected by diurnal and circadian rhythms. Changes in volatile profiles in response to herbivory or mimicked herbivory (Röse and Turlington, 2005; Turlings et al., 1990) can often take several hours to several days, depending on the plant species and the part of the plant chosen for volatile measurements (Röse and Tumlinson, 2004). Initial collection of volatiles may be performed over several days with 24-h collection intervals, and subsequently compared with results from several shorter sampling intervals. For low-emitting plants in particular, longer collection times are applied to trap enough volatiles for analysis. To further enhance the detectability of volatile compounds, one can increase the amount of plant material, decrease the volume of surrounding air space and increase the air flow, although limitations such as increased transpiration (in the case of too much plant tissue) or air contamination (in the case of higher flow rates) have to be considered. A risk will remain that some volatiles may be emitted in quantities below technical detectability, which may be functionally relevant, for example, for insect attraction.

Results obtained from volatile collections under field conditions will always vary from those acquired in the laboratory due to fluctuating environmental parameters and the differential reactivity of plant VOCs with reactive molecules in the atmosphere, such as ozone, when ambient air is applied.

Intersample variability of a particular method occurs due to the plant material being investigated. Even if clonal lines are used, care must be taken to standardize plant age, development, light, nutritional and soil-moisture conditions as well as the time of day for volatile sampling. Furthermore, it has to be considered that the emission of VOCs is strongly affected by insect and microbial damage or even pesticide treatment, and plants have to be selected or monitored accordingly prior to volatile analysis.

Overall, researchers are advised to perform their volatile analyses with more than five replicates and to test several different parameters using, if possible, more than one method of analysis. Experimental conditions should be reported as carefully as possible to facilitate comparison of results.

Conclusions

Within the past decade we have seen enormous progress in the analysis of plant volatiles that has been stimulated by very different areas of research such as plant biochemistry, ecology and atmospheric chemistry. Particular achievements have been made in real-time VOC analysis in monitoring rapid and subtle changes of volatile emissions in response to abiotic or biotic factors. These advances have created opportunities for detailed views on the time courses of VOC emissions, whether they are involved in the attraction of pollinators or the deterrence of herbivores. Furthermore, these new analytical capabilities can match or exceed the timescale and sensitivity of contemporary genomic and proteomic techniques to provide more precise correlations with the biochemistry underlying the biosynthesis and regulation of VOCs. The recent developments in gas chromatography and mass spectrometry described here have reduced detection limits and improved separation efficiencies and time response factors, and these will facilitate high-throughput screenings of plants to detect transgenics, mutants or natural variants with altered VOC emission profiles. Despite this progress in VOC analysis, traditional VOC collection techniques and off-line GC analyses will remain essential for many laboratories and especially for remote field studies. Therefore, this review has described some important guidelines and parameters regarding VOC collection devices and GC separation techniques. In summary, the methods presented here should give the VOC researcher the flexibility to investigate multiple aspects and still unexplored areas of the biosynthesis, emission and function of plant VOCs. For example, little is known so far about the biosynthesis of VOCs in roots and their role in the rhizosphere and soil ecology. Furthermore, integrative studies of primary metabolism and VOC biosynthesis have mostly been conducted with isoprene and should be a model for comparative investigations of the biosynthesis of floral and wound-induced volatiles.

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Supplementary Material
The following supplementary material is available for this article online:
Table S1 Common adsorbents for trapping of plant VOCs
Table S2 GC-mass spectral and/or retention index databases for plant VOC identification
Appendix S1 Analysis of volatile products of terpene synthases by SPME or automated headspace sampling.
This material is available as part of the online article from http://www.blackwell-synergy.com

References


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