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Metabolomic Profiling of Natural Volatiles

Headspace Trapping: GC–MS

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Summary

Plants are a fabulously rich source of naturally volatile metabolites, which are derived from a range of contrasting biochemical pathways (e.g., mono-, di-, and sesquiterpenoids, benzoates, alcohols, esters). Such volatiles may immediately be released from the plant or they may be stored, e.g., in glycosylated form for release later "on demand." Certain roles for these molecules have already been determined in that they can function as attractants (e.g., to pollinators, seed dispersers, and others) or as protectants (repellants, pathogen inhibitors, and so on). The flavor and fragrance of plant materials to humans and other animals are also, to a great extent, determined by natural volatiles. Other more sophisticated roles have also been elucidated where plant volatiles have been shown to be involved either as signal molecules to attract the predators of damaging herbivorous insects or potentially even as signal molecules warning other plants of imminent danger. As such, detailed knowledge of these components can be valuable in relation to breeding crop varieties for enhanced product quality or for achieving improved resistance to pathogens and insects. Furthermore, knowledge of the metabolites can result in a corresponding knowledge of the genes responsible for their synthesis and this can lead to dedicated strategies for their in vitro production through, e.g., reverse genetics in heterologous microbial expression systems in fermentors for the production of high-value fine chemicals. Various analytical techniques based on gas chromatography–mass spectrometry have been devised for the analysis of this complex group of metabolites. Two of these key methods are detailed in this chapter.

Key Words: Headspace trapping; GC–MS; natural volatiles.

1. Introduction

A significant part of the plant cell metabolome—comprising the volatile metabolites—is of particular interest not only in fundamental research into, e.g.,

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signaling mechanisms and interorganism interactions *(1)*, but also are equally important in applied research as these metabolites play an important role in plant product quality in terms of, e.g., flavor and fragrance *(2)*. Headspace trapping techniques combined with gas chromatography–mass spectrometry (GC–MS) are analytical approaches that are suitable for metabolomics studies of natural volatiles. Specially designed porous polymers, used as the adsorbant to fill flow-through cartridges (e.g., Tenax), or to coat solid-phase microextraction (SPME) fibers, are used to collect and concentrate the volatiles released into the air space above the plant material. These technologies have high sensitivity, reproducibility, and robustness *(3–10)*. Such approaches can be used to collect and concentrate naturally released volatiles from either parts of, or whole plants, over a period of time—so-called headspace analysis. In addition, similar approaches can be applied after, e.g., plant material has been pretreated (frozen, ground to a powder, and so on) where certain procedures can be applied in order to drive off (purge) the major volatile fraction from the plant matrix for subsequent collection and detection. Headspace trapping includes several basic steps: (1) sample preparation, which is aimed at stabilizing the biological material (for example plant tissue) and inducing the release (purging) of volatiles from the material into the air above (the headspace); (2) the volatiles are then extracted (trapped) from the headspace using a solid-phase adsorbant in the form of a fiber (in the case of SPME; **Fig. 1**) or of a flowthrough cartridge filled with a porous polymer (e.g., Tenax). Different adsorbants are available that have different affinities to different classes of volatile metabolites. As such, polymer choice can be used to give preference to those groups of metabolites of greatest interest; (3) the trapped volatiles are subsequently released from the solid phase for analysis. In the case of SPME this only happens after the fiber has been inserted into the injection port of the gas chromatograph at which time it is exposed to a high temperature. When using cartridges, the volatiles can either be chemically extracted using organic solvents and then be concentrated further prior to injection into the GC or alternatively, they may, using direct thermal desorption, be released directly into the GC using dedicated equipment supplied with the machine; and (4) chromatographic separation then occurs using a long capillary column, usually in combination with a suitable temperature gradient. During the analysis the individual molecules are first separated as they pass through the chromatography column at different rates, and, on entry into the mass spectrometer, they are fragmented into characteristic fragments that are then detected as charged molecular ions. These ions are qualified on the basis of their mass-to-charge ratios (*m/z* value) and are quantified based on relative abundance, which results in a distinct spectrum for each compound. These spectra can then be used to interrogate existing databases (e.g., National Institute of Standards and Technology [NIST] and Wiley compound libraries) to assist in compound identification.

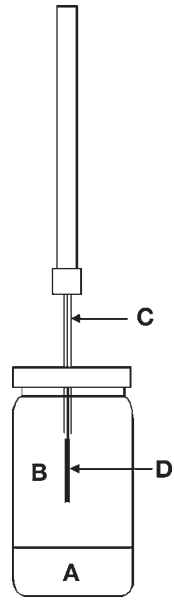


Fig. 1. Schematic representation of a solid-phase microextraction fiber exposed in the headspace of a glass vial containing biological material. The fiber **(D)** has been removed from the protective sheath **(C)** into the headspace **(B)** of the pulped plant material and $CaCl_2$/EDTA **(A)**.

Modern investigations in the field of metabolomics require (1) reliable profiling of the metabolic composition of large numbers of biological samples and (2) an unbiased comparative (statistical) analysis of the datasets obtained. In some cases (e.g., SPME), the samples are often not subjected to any pretreatment procedures using organic solvents and, thus, the components remain as native as possible and the majority of natural volatile components that the living plant tissue consists of, are represented in the analysis as a sort of metabolic snapshot. However, in certain cases it may be desirable to choose a modified approach in order to maximize volatile release while avoiding potential artifacts caused by, e.g., many biological processes moving into senescence or complicating matrix effects. Undesirable effects may also be initialized owing to cell disruption altering the original metabolic composition, thus failing to provide a true picture of the living material. In this respect, the most important step in the procedure is making the correct decision concerning sample preparation. This should be made with the aim of stabilizing the biological matrix during the period of volatile collection and/or to drive off all volatiles into to headspace so that they can be extracted.

In this chapter, we describe reliable protocols for automated, sequential metabolomic profiling of volatile metabolites released from plant material. The first example chosen, by way of illustration, uses readily available tomato fruits in combination with a headspace SPME-GC–MS approach involving a pre-treatment strategy to maximize volatile release. In a second example, a method is presented where natural *Arabidopsis* flower volatiles, which are inevitably released in small amounts, are collected and concentrated over an extended period for subsequent further concentration and analysis. Information on how to proceed with an unbiased comparative analysis of metabolic data obtained from such an analysis is also presented.

2. Materials

2.1. Natural Volatiles

2.1.1. SPME Adsorption

1. Red ripe tomato fruits.
2. A supply of liquid N_2.
3. Freezer at –70°C.
4. MilliQ water or the double distilled equivalent.
5. NaOH/EDTA water solution: 100 m*M* EDTA adjusted to pH 7.5 using 1 *M* NaOH.
6. $CaCl_2$ powder.
7. 50-mL Plastic centrifuge/storage tubes (e.g., Corning, Corning, NY).
8. 4-mL Glass screw-cap vials, 15–45 mm (e.g., Bester, Amstelveen, The Netherlands).
9. 10-mL Polypropylene bimetal crimp-cap vials (e.g., Bester), with silicon/Teflon septa, 20 mm (Interscience, Breda, The Netherlands).

2.1.2. Headspace Trapping/Tenax Adsorption

1. Flowering plants of *Arabidopsis thaliana.*
2. 2-mL Glass vials containing 1 mL tap water.
3. Teflon tape.
4. Pentane, redistilled.
5. Diethyl ether, redistilled.
6. Tenax, TA, 20/35 mesh (Alltech, Breda, The Netherlands).
7. Glass cartridges, 140 × 4 mm with reduced diameter at one end to assist loading.
8. Flexible Teflon tubing.
9. 1-mL Pipet tips with the points removed to create a wider opening.
10. Glass jar (ca. 500 mL) sealed with a Teflon-lined lid equipped with inlet and outlet ports.
11. Flow meters and a vacuum pump.
12. 4-mL Screw-cap vials (Bester).
13. Polypropylene screw caps with polytetrafluoroethylene (PTFE)-lined rubber septa.
14. 2-mL Wide-mouth crimp-cap vials with crimp caps equipped with polytetrafluoroethylene (PTFE)-lined septa (Bester).
15. 250-µL Vial inserts (Bester).
16. Source of N_2 gas.

2.2. Equipment

2.2.1. Natural Volatiles

2.2.1.1. SPME

1. Metal electric grinder, basic analytical mill A11 (IKA, Staufen Germany).
2. Shaking water bath at 37°C.
3. Ultrasonic bath.
4. SPME fiber assembly (65-µm polydimethylsiloxane-divinylbenzene [PDMS-DVB]) (Supelco, Zwijndrecht, The Netherlands) (*see* **Note 1**).
5. Gas chromatograph (e.g., Fisons 8060) coupled to a MD 800 mass spectrometer (Fisons, Beverly, MA) and fitted with a CombiPAL auto-sampler (CTC Analytics, Zwingen, Switzerland) (*see* **Note 2**).
6. Capillary column: HP-5 (50 m × 0.32 mm, 1.05-µm film thickness; Hewlett Packard, Amstelveen, The Netherlands) with a supply of helium as carrier gas (*see* **Note 3**).

2.2.1.2. Headspace Trapping/Tenax Adsorption

1. Gas chromatograph (e.g., Hewlett Packard 5890 series II) coupled to a mass spectrometer (e.g., Hewlett-Packard 5972A).
2. Capillary column: HP-5MS, 30 m × 0.25 mm, 0.25-µm film thickness (Boom, Meppel, The Netherlands); retention gap 5 m × 0.32 mm, deactivated (Alltech).

3. Methods

3.1. SPME: GC–MS of Tomato Fruit Volatiles

3.1.1. Tomato Fruit Sampling (see **Note 4**)

1. Preferably, use freshly harvested undamaged tomato fruits of similar age and developmental stage. Use at least five fruits per sample. Cut each fruit into quarters with a sharp knife and immediately freeze one one-fourth part of each fruit in liquid N_2 (*see* **Note 5**).
2. Grind the five-pooled frozen tomato parts in liquid N_2 in a metal electric grinder. Keep the powder deep-frozen at all times and transfer it to 50-mL storage tubes, again using a precooled spatula (*see* **Note 6**).
3. Store the pooled powder sample obtained in the freezer at approx –70°C until all samples are ready to be analyzed together.

3.1.2. Preparation of Samples for SPME-GC–MS

1. Samples removed from the freezer should be placed conveniently available for use but nevertheless must remain deep-frozen (in liquid N_2 or in a –70°C freezer) until being used one at a time for the following steps.
2. Quickly but carefully weigh out 0.5 g of frozen powder using a metal spatula with the end prefrozen in liquid N_2 into a prefrozen 4-mL screw-cap vial.
3. Immediately close the vial with its screw cap and incubate in a preheated water bath at 37°C with gentle agitation for 10 min (*see* **Note 7**).

4. Open the vial and quickly add 0.5 mL of NaOH/EDTA (100 m*M* EDTA, pH 7.5) and then immediately after 1.1 g $CaCl_2$ powder (*see* **Note 8**). Immediately close the vial and shake thoroughly. Sonicate in an ultrasonic bath for 5 min.
5. Transfer the pulp obtained using a 1-mL pipet tip into a 10-mL crimp-cap vial and immediately close it (*see* **Note 9**). The samples are now ready to be analyzed by SPME-GC–MS.

3.1.3. SPME-GC–MS Analysis (see **Note 10**)

1. Set the GC–MS parameters to the following values:
   a. Helium pressure is maintained at 37 kPa.
   b. The GC interface and MS source temperatures are 260 and 250°C, respectively.
   c. Set up the following GC temperature gradient program: start at 45°C (2 min), raise to 250°C at a rate of 5°C/min and finally, hold at 250°C for 5 min.
   d. After each run the column is automatically cooled from 250°C back to the starting temperature.
   e. The total run time including the final oven cooling back to the starting temperature is 60 min.
2. Prior to volatile adsorption, incubate the vials containing the plant material at 50°C for 10 min with continuous agitation (*see* **Note 11**).
3. Insert the SPME fiber (**Fig. 1D**) through the septum and into the headspace of the vial, and expose the coating to the air above the sample (**Fig. 1B**). Incubate for 20 min while maintaining the temperature at 50°C with continuous agitation (*see* **Note 12**).
4. Insert the fiber into the GC injection port and desorb the volatiles for 1 min at 250°C.
5. Run the temperature gradient and record the mass spectra in the 35–400 *m/z* range using the MS set at a scanning speed of 2.8 scans/s with an ionization energy of 70 eV (*see* **Fig. 2**).
6. Use the commercial software supplied with the mass spectrometer for subsequent data analysis (*see* **Note 13**).

3.2. Headspace Trapping Analysis of Arabidopsis *Flower Volatiles*

3.2.1. Sampling of Plant Material

1. For each glass jar to be used, fill two glass cartridges each with 150 mg Tenax (*see* **Note 14**).
2. Cut the inflorescence and part of the stem of a healthy *Arabidopsis* plant with a scapel blade and place in a 2-mL vial containing 1 mL tap water. Seal the vial neck with Teflon tape (*see* **Fig. 3**) (*see* **Note 15**).
3. Place the sample in the glass jar and replace the lid bearing the two outlet ports.
4. Connect up the two Tenax-filled cartridges using Teflon tubing to the inlet and outlet ports (*see* **Note 16**).
5. Connect the other end of the outlet port cartridge to the vacuum pump and establish an air-flow rate through the system of 100 mL/min and keep running for 24 h (*see* **Note 17**).

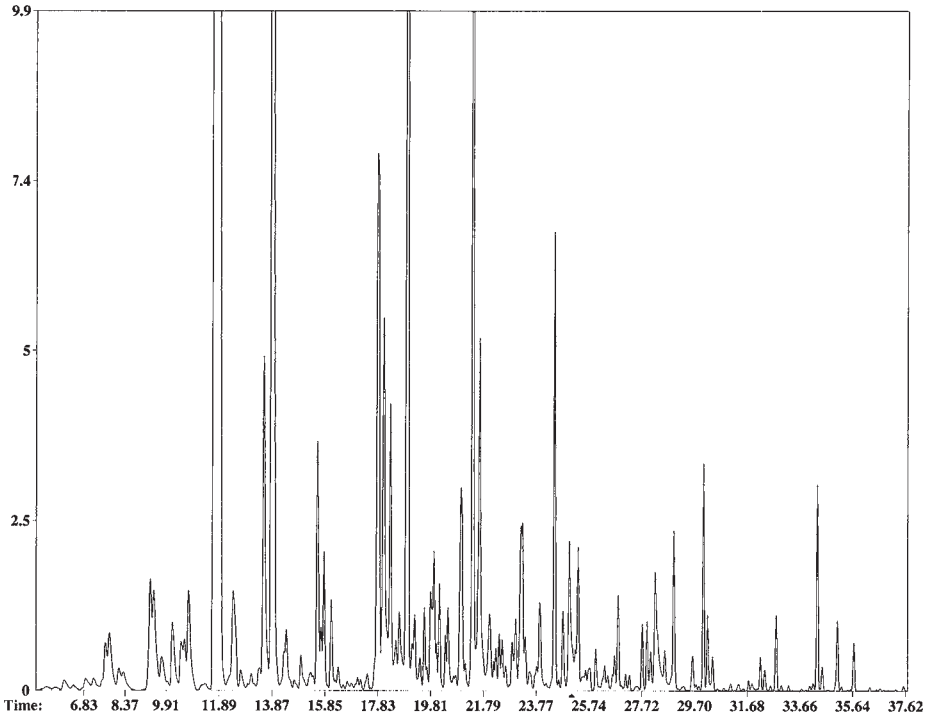


Fig. 2. A typical gas chromatography–mass spectrometry profile of tomato volatiles.

6. Repeat for the other samples and include one empty jar as negative control (*see* **Note 18**).
7. After 24 h make a mixture of pentane:diethylether (80:20) and elute the Tenax from the outlet cartridge with 1 mL of the mixture. Collect the run-through in a 4-mL screw-cap vial and repeat twice to give in total a 3-mL of eluant (*see* **Notes 19** and **20**).
8. Concentrate the eluant down to 50 µL using a gentle flow of N_2 in a fume hood.
9. Transfer the eluant into a vial insert and place into a 2-mL crimp-cap vial and seal with a crimp cap for GC analysis (*see* **Note 21**).

3.2.2. GC–MS Analysis of Purge and Trap Eluates

1. Use the following gradient for the GC run: 45°C for 1 min, increase the temperature to 280°C at a rate of 10°C/min, maintain the temperature at 280°C for 5 min. After each run the column temperature will automatically be returned to the starting temperature. Set the injection port to 250°C, the interface to 290°C, and the MS source to 180°C.

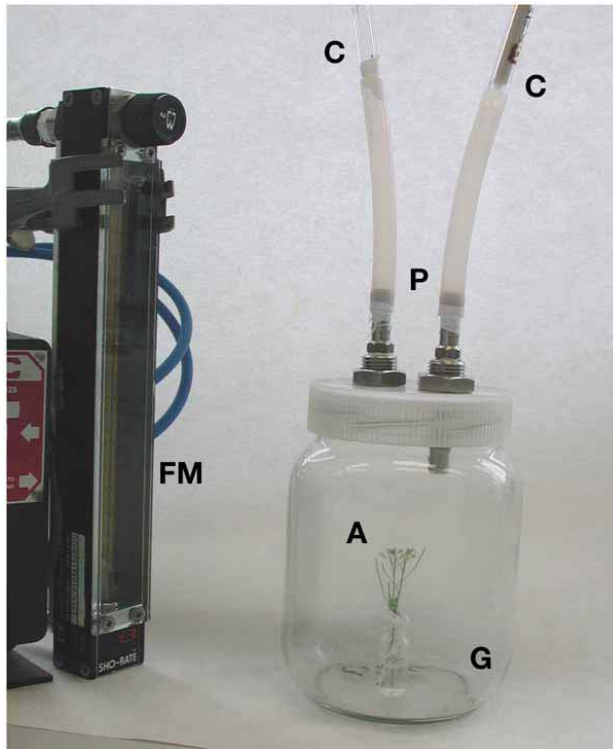
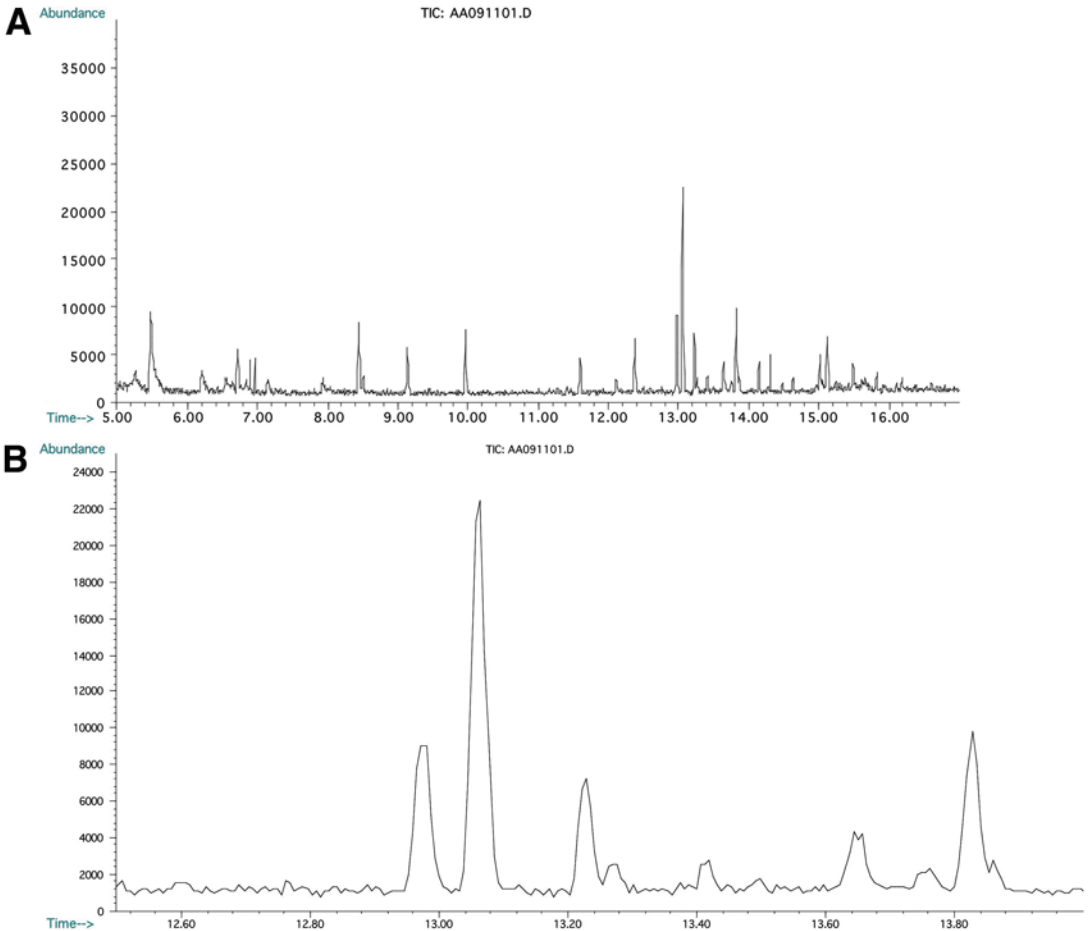


Fig. 3. Glass jar (G) containing a piece of *Arabidopsis* inflorescence (A). The inlet and outlet ports (P) are both connected by Teflon tubing to glass cartridges (C) containing Tenax. Air is then sucked through the system by a vacuum pump connected to a flowmeter to control the flow rate (FM).

2. Set the helium inlet pressure with the electronic pressure control to give a constant flow rate of 1 mL/min. Set the ionization potential to 70 eV.
3. When the equipment is stabilized, inject a 2-µL aliquot of the Tenax eluate in splitless mode into the GC.
4. Repeat for all the samples and the control.
5. Analyze the data further using the commercial software supplied with the mass spectrometer (*see* **Note 22**; **Fig. 4**).

Fig. 4. *(opposite page)* **(A)** Typical headspace volatile fingerprint of natural volatiles released from *Arabidopsis* flowers. **(B)** Enlargement of a section of the upper trace showing characteristic peaks present in the sesquiterpene region of the chromatogram.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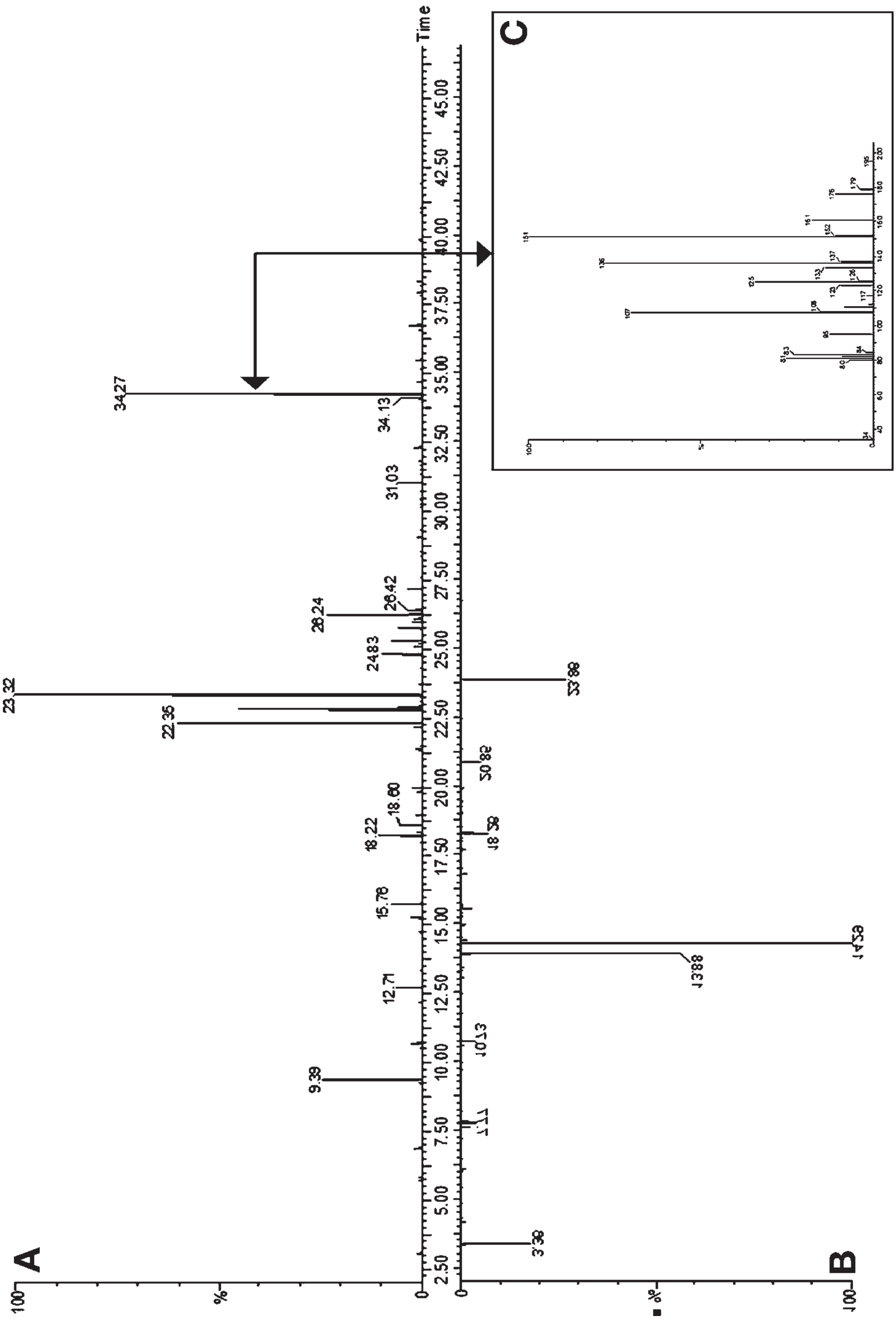
3.3. Data Analysis

When unfamiliar with GC–MS data it is recommended to begin data analysis using the packages supplied with the equipment used. You can then proceed to export the differential mass peaks into programs such as AMDIS, and databases such as the NIST mass spectral library (http://www.nist.gov) to help identify interesting compounds. For large-scale metabolomic comparisons of many datasets, premanipulation of the data is likely to be required. Packages such as metAlign™ (www.metalign.nl) can be used for this data preprocessing. This involves, e.g., baseline correction and noise elimination, full spectral alignment of datasets, that is, alignment of every mass peak (*m/z*) situated in a particular retention time throughout all datasets analyzed, and searching for statistically significant metabolic differences between datasets. An example of the results of one such comparative analysis of the volatile composition of two tomato lines is presented in **Fig. 5**. The difference chromatograms represent mass peaks (molecular fragments), which are either more, or less, abundant in one line compared with the other. Using AMDIS and NIST, known volatile metabolites corresponding to these peaks can then be determined.

4. Notes

1. Different SPME fiber types are available. For nontargeted metabolomics, fiber assemblies that have a more universal adsorption capacity should be chosen. The one listed has been chosen for its broad affinity to volatiles with different chemical origins. When a more targeted approach is desired, more specific fiber types can be selected to give maximal preference to specific compound classes (*see*, e.g., http://www.sigmaaldrich.com/Brands/Supelco_Home).
2. There are many different producers and models of GC–MS available. Much manufacturer information is available to help you chose which machine is most appropriate to meet your needs and budget.
3. A range of different types and lengths of columns are available from several different manufacturers and the information these supply can be used to make choices. The HP-5 column used here is often a good starting point and its use is frequently reported in the literature. There is a lot of information on KI values available for this type of column, which helps to facilitate compound identification.

Fig. 5. *(opposite page)* Difference chromatograms of fruit volatiles from two contrasting tomato cultivars **(A**,**B)**. **Figure 3A** contains those peaks of volatiles that are more abundant in fruit of the cultivar A compared with the cultivar B. **Figure 3B** contains the peaks of volatiles that are more abundant in fruit of the cultivar B compared to the cultivar A. **Figure 3C** represents the mass spectrum of one of the differential peaks. When compared with the National Institute of Standards and Technology mass spectral library the mass spectrum 3-C represents *trans*-geranylacetone.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4. A significant level of biological variation will always be present between different replicate plants of the same genotype, or even different samples from the same plant (fruits for example). Consequently, to reduce the intersample variation it is strongly recommended to work with pooled materials taken from several comparable sources. In addition, a number of biological replicates of each sample to be analyzed (e.g., genotype, developmental stage, tissue type—depending on what is to be compared) must be taken to get statistically reliable information. Actual numbers are dependant on the type of material used and the uniformity of its prehistory. However, a good rule of thumb is to use five replicates each comprising of five-pooled tissue samples. Experience will then tell if more or less is required. In the case of truly high-throughput screening where perhaps hundreds of samples are to be screened, each genotype can initially be represented by a single sample obtained by pooling of a number of biological replicates. Nevertheless, analysis of such pooled samples should again be repeated two to three times randomly throughout the entire period of analysis to assess and compensate for any analytical (instrumental) variation.
5. It is essential to obtain the most reliable and reproducible overview of the metabolic fingerprint of the living material. Unfresh or damaged material is, therefore, to be avoided as some volatile compounds are only produced as a consequence of damage or senescence. Very sharp knives should be used for cutting and the cut segments should be plunged carefully into the liquid nitrogen within seconds. Subsequent uniformity in handling is also essential and when working with multiple samples the whole process needs to be streamlined to ensure uniform treatment.
6. We use this type of analytical mill as it is capable of withstanding the extreme temperatures of liquid nitrogen. If such a grinder is not available then a large pestle and mortar can readily be used. Both should be prefrozen by pouring in liquid nitrogen and allowing everything to cool down before use. Grinding should also take place in the presence of liquid nitrogen to ensure the sample remains frozen at all times. As liquid nitrogen is being used, appropriate safety precautions should be taken at all times (eye and hand protection, protective clothing, and others). Follow the exact recommendations of your organization.
7. Normally, the chemical composition of biological material should be preserved from any undesirable changes occurring before and during experiments. However, some volatile compounds are only released after cell disruption through enzymatic and nonenzymatic processes. Such compounds can be of considerable relevance specifically in flavor and fragrance studies. Consequently, a strictly controlled incubation of fixed duration and temperature is used prior to analysis to enable these compounds to be included in the analysis. After the incubation period all reactions must be immediately stopped (*see* **Note 8**).
8. When analyzing a series of biological materials in a sequential automated mode, their chemical composition must be preserved during the entire analytical procedure. This means that any enzymatic and nonenzymatic processes occurring in a biological matrix and, thus, altering its chemical composition must be controlled in a uniform manner and terminated on demand. Several ways have been described

to achieve a relative stability of a biological material in time, e.g., boiling, short microwave treatment, and saturation with salts ***(11)***. Here, we use probably the most popular way—saturation of the material with $CaCl_2$, which not only inactivates the enzymes but also facilitates driving off the volatiles into the headspace above. $CaCl_2$ can be added either as a powder—in the case of material like fruit, which normally contains greater than 90% water, or in an aqueous solution when using completely, or relatively dry, material. In both cases, the final concentration of $CaCl_2$ must be not less than 5 *M*. To prevent a nonenzymatic oxidation of the volatiles an aqueous solution of EDTA and NaOH is used. Both have a chelating effect while at the same time increasing the pH from 4.5 (a typical pH of tomato fruit) up to 6.2–6.3. Such a procedure has been found to preserve the volatile composition of tomato fruit pulp for at least 12 h.

9. The easiest way to transfer the pulp is to use a pipet fitted with a 1-mL tip that has had the end cut off to create a wider opening. Avoid getting any material to be analyzed onto the vial wall—such material will slowly dry out and subsequently change its polarity, which can then alter the composition of volatiles in the headspace.
10. When analyzing a series of samples, the steps of the headspace extraction and the injection of extracted compounds into GC should be performed by a CombiPAL auto-sampler or its equivalent. In this way, all steps can be made uniform. This is essential to obtain reliable and reproducible results.
11. Agitation and heating to drive off the volatiles is appropriate for the material used here but when using living plant material (e.g., intact leaves, whole small fruits, or unground fruit parts) agitation and heating are usually to be avoided.
12. Make sure no plant material gets deposited on the inside of the vial septum and never allow the fiber (**Fig. 2D**) to touch the plant material at the bottom of the vial (**Fig. 2A**) during headspace extraction.
13. In the case of the SPME analysis, the method described for tomato samples has been shown to give stable mixtures for 12 h after sample preparation. With, e.g., run times of 60 min only ≤12 samples should, therefore, be prepared at one time. For other plant materials, sample stability should first be thoroughly checked before planning large-scale analyses of multiple samples. As analytical conditions can vary slightly during longer runs, and often unexpected changes in data acquisition can occur, it is strongly recommended to have a monitoring system in place to check for this. To monitor for such divergences and to be able to use this information to estimate the degree of reliability of the data, a constant amount of a standard compound (one not present in your sample and which has a retention time different to the sample components) should be added to your samples prior to analysis. In addition, a reference mixture (a collection of standard compounds related to the chemical composition of your sample) should be routinely analyzed at regular intervals (e.g., every tenth run). Information from these runs can both be used to test for abnormalities in data acquisition and also can be useful for subsequent statistical analysis of the whole dataset. SPME is known as a semi-quantitative analysis method. It is useful for the reliable detection of qualitative differences between samples and for larger quantitative differences. This

is because the dynamic range for individual components is limited (linearity in response is usually limited to a fourfold difference) and the adsorption properties can be dependent on chemical composition of the sample. This complicates quantification when using this method but relative amounts can be used reliably for comparative analysis of the biological composition of related biological samples.

14. Depending on the chemical properties of the compounds of greatest interest it is possible to chose different adsorbants with differing affinities. *See*, e.g., http://www.sigmaaldrich.com/Brands/Supelco_Home or http://www.markes.com for details of the range of possible materials that are available.
15. Cut plant material may give off extra volatiles owing to the wounding effect. Avoid, therefore, any unnecessary damage to the explant and seal in the stem into the vial fully with Teflon tape. Chose plants with a number of open flowers and few dying/dead ones.
16. It is imperative to only use inert materials such as glass, stainless steel, and Teflon. All connections should be made using Teflon tubing (although the final connection between the outlet cartridge and the vacuum pump may be made of, e.g., silicone tubing. Plastics must be avoided. Noninert materials may release contaminating volatiles which interfere with the analysis. Their contact with organic solvents will also result in irreversible contamination of your spectra through, e.g., the dissolution of the softners often used in plastics (e.g., phtalates).
17. The chosen flow rate should be in relation to the size of the glass jar used—a total sample chamber purge time of 5 min is recommended. The inlet port cartridge is essential to clean the air entering the system of all environmental volatiles adsorbed by the polymer used. In this way all the volatiles trapped in the outlet cartridge must have come specifically from the plant material in the sample chamber.
18. These are best set up in parallel to ensure maximum uniformity in experimentation.
19. Trapping is essentially a chromatographic process, which is influenced by the amount of Tenax, the trapping time, temperature, and gas-flow rate. If you are worried that saturation might be achieved early or that leaching through the system may be occurring you can test for this in an initial trial run by connecting two Tenax inserts in series. Under the right conditions the second insert should still be empty of volatiles at the end of the sampling period chosen.
20. Release of volatiles, especially from flowers, is often regulated or at least influenced by circadian or light/dark cycles. This must be taken into account when wishing to measure volatile release for shorter periods than 24 h. In these cases, a proper time series must be performed prior to experimentation in order to characterize volatile release. Only then can the right choice of adsorbance time be made to ensure uniformity of sampling.
21. Again, at all stages avoid the use of plastics for any of the handling steps and always take the required precautions when dealing with these highly volatile solvents.
22. Depending on the scientific goal there are different ways to analyze the data. For compound identification, peak comparison with spectra in commercial databases (NIST, Wiley) is a useful starting point. For definitive confirmation of identity,

running authentic standard compounds is necessary. If you wish to proceed to fully quantify individual interesting components it is also necessary to run a dilution series of each authentic reference compound to make the necessary calibration curve. *See also* **Note 13** for comments on spiking samples and reproducibility tests.

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