Extracting and trapping biogenic volatile organic compounds stored in plant species

Elena Ormeño, Allen Goldstein, Ülo Niinemets

Biogenic volatile organic compounds (BVOCs), released by practically all plants, have important atmospheric and ecological consequences. Because BVOC-emission measurements are especially tedious, complex and extremely variable between species, two approaches have been used in scientific studies to try to estimate BVOC-emission types and rates from plant species. The first, which has known little success, involves grouping species according to plant-taxonomy criteria (typically, genus and family). The second involves studying the correlation between BVOC content and emission (i.e. how leaf content could be used to estimate emissions). The latter strategy has provided controversial results, partly because BVOCs are amazingly chemically diverse, and, as a result, techniques used to study plant BVOC content, which we review, cannot be equally adequate for all analytes.

In order to choose an adequate technique, two patterns must be distinguished. Specifically stored compounds – mainly monoterpenes and sesquiterpenes that dominate the essential oil obtained from a plant – are permanently and massively present in specific storage structures (e.g., secretory cavities, trichomes) of the order of µg/g–mg/g and usually allow emissions to occur during stress periods when terpenes are weakly synthesized. These BVOCs can be studied directly through traditional extraction techniques (e.g., hydrodistillation) and novel techniques (e.g., application of microwaves and ultrasound), and indirectly by trapping techniques involving the collection, within adsorbent material, of BVOCs present in the headspace of a plant.

Non-specifically stored compounds (e.g., isoprene, 2-methyl-3-buten-2-ol, and, in species without storage structures, monoterpenes and sesquiterpenes) can only be temporarily accumulated in leaf aqueous and lipid phases in small concentrations of the order of ng/g. As a result, studying their concentration in leaves requires the use of trapping techniques, more sensitive to trace amounts. Unlike for specifically stored BVOCs, knowledge of the concentration of non-specifically stored BVOCs cannot provide any information regarding the emission potential of a species but, instead, provides crucial information to understand why BVOC emissions may be uncoupled from the physiological processes that drive their synthesis.

We describe both extracting and trapping techniques and discuss them in terms of the technical choices that may cause losses of thermolabile constituents, chemical transformations, different volatile recoveries and suitability to represent plant content of BVOCs faithfully. The second part of this review addresses technical shortcomings and biological and environmental factors that may alter the correlations between BVOC content and emission from plants.

Keywords: Biogenic volatile organic compound; BVOC content; BVOC emission; Hydrodistillation; Leaf BVOC; Microwave-assisted extraction; Solid-phase microextraction; Storage structure; Terpene

Abbreviations: DHT, Dynamic headspace trapping; HD, Hydrodistillation; MAHD, Microwave-assisted hydrodistillation; MASE, Microwave-assisted solvent extraction; PSE, Pressurized solvent extraction; SDE, Simultaneous distillation solvent extraction; SFE, Supercritical fluid extraction; SFME, Solvent-free microwave extraction; SWE, Subcritical water extraction; UAE, Ultrasound-assisted extraction

Elena Ormeño*
Institut Méditerranéen d'Ecologie et Paléoécologie, Institut Ecologie et Environnement, Centre National de la Recherche Scientifique (INNE-CNRS), Aix-Marseille University, 13331 Marseille Cedex 03, France
Allen Goldstein
Division of Ecosystem Sciences, Department of Environmental Science, Policy, and Management, University of California, Berkeley, CA 94720, USA
Ülo Niinemet
Department of Plant Physiology, Estonian University of Life Sciences, Kreutzwaldi 1, 51014 Tartu, Estonia

*Corresponding author. Tel.: +33 (0) 4 13 55 12 26; Fax: +33 (0) 4 13 55 11 51;
E-mail: elena.ormeno@univ-provence.fr

1. Introduction

Plants release to the atmosphere important amounts of biogenic volatile organic compounds (BVOCs) that account for up to 30% of the photosynthetically fixed carbon under stress conditions [1]. These metabolites may act as plant defenses as they repel herbivores and facilitate the foraging behavior of natural enemies of herbivores, and protect leaf cells from a variety of abiotic stresses [2]. Likewise,

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BVOC storage in leaves is a key defense trait, not only altering the success of a given plant species in the environment, but also influencing ecosystem functioning due to the toxicity of most BVOCs for omnivorous herbivores, forcing them to change their dietary habits. In the atmosphere, BVOC emissions affect atmospheric chemistry, since their oxidation in the atmosphere leads to ozone and secondary organic aerosol formation, and thereby affect air quality and climate [3].

Regarding the way that BVOCs are stored within leaves, two patterns are distinguished. The first refers to specifically stored compounds (i.e. metabolites whose storage is permanent, reaching important concentrations within the leaf of the order of μg/g–mg/g and occurring either in leaf internal structures (e.g., secretory cavities and secretory canals or ducts), or in structures located on the surface of the leaf (e.g., trichomes). These specific structures mainly contain terpenes, the largest and most diverse class of BVOCs, whose abundance is correlated with the density of storage structures [4]. Non-terpenoid compounds, mainly benzenoids, may also be present therein [4].

The second pattern refers to non-specifically stored compounds, which account for those volatiles that are temporary stored in very small concentrations, of the order of ng/g in leaf aqueous and lipid phases. This pattern occurs always (i.e. independently of the presence of storage structures) for water-soluble volatiles [e.g., 2-methyl-3-buten-2-ol, green leaf volatiles (C_5, C_6 and C_9) emanated from mechanically damaged leaves, acetone, acetaldehyde, methanol and linalool that can be stored in the leaf liquid phase]. When species lack these structures, this second pattern also occurs for most hydrophobic monoterpenes and sesquiterpenes that can accumulate in the leaf lipid phase. Under non-stress conditions, and in species that do not possess these structures, the major fraction of these compounds is directly emitted to the atmosphere after being synthesized without being accumulated, while environmental stress results in stomatal closure that may lead to the build up of these compounds inside the leaf.

Unlike in ecology, little attention has been given to terpene storage within foliage in atmosphere-related studies. Linking potential emissions to content is nevertheless of special interest since terpene emission and concentration of specifically stored terpenes from a given species have been found to be strongly linked in some studies [5–7], although numerous factors often impede the good correlation between the emission and content. Ormeño et al. [5] recently demonstrated that plants featuring high and low terpene concentrations also possess correspondingly high and low emission rates. This result provides a basis for estimating the magnitude of plant emissions for a wide diversity of species. Moreover, BVOC content allows emissions to occur during periods when terpenes are weakly synthesized (e.g., during water-stress conditions). It also allows the evaluation of plant capacity to produce highly reactive compounds (e.g., sesquiterpenes), which can potentially be released to the atmosphere but are barely detected by the current analytical systems due to their high reactivity and stickiness. Also, a significant concentration of terpenes in plant material of the order of μg/g and more increases plant flammability with the consequent effects on fire risk, a phenomenon that is highly related to air-pollution episodes.

In the first part of this review, we focus on techniques that allow study of plant BVOC content. Both, extraction techniques, which rely on the plant matrix as substrate, and trapping techniques, based on BVOC collection from the plant headspace, can be used directly or indirectly, respectively, to study specifically stored BVOCs. Only trapping techniques, typically used to estimate plant blend and highly sensitive to very low BVOC concentrations, are useful to study non-specifically stored BVOCs [8]. The second part of the review analyzes the factors that can impede finding a significant correlation between content and emission from leaves. We do not focus on the analytical techniques utilized to identify and to quantify BVOCs [typically by gas chromatography (GC) coupled to mass spectrometry (MS) and flame ionization detectors (FIDs), respectively], although we refer to them in due course.

2. Techniques to study leaf reservoirs of BVOCs

2.1. Extraction of specifically stored BVOCs

2.1.1. Traditional methods. BVOCs can be extracted from harvested leaves, providing quantitative and qualitative information on the spectrum of compounds produced and their amount. Distillation and extraction with organic solvent(s) are the two main traditional ways to extract the stored BVOCs from harvested foliage.

Distillation, most often carried out as hydrodistillation (HD), is used to liberate the volatiles from plant material into a gaseous form. As the yield of HD is typically low, a substantial amount of foliage of fresh and sometimes dried leaves is placed in the plant chamber of the still in contact with water. The volume of water – selected according to the amount of foliage and the essential oil yield desired – is heated to boiling. The steam temperature is thereby high enough to break down the leaf structures that hold the volatiles, but is much lower than the boiling point of BVOCs. This avoids decomposition of most of the compounds in the essential oil. Since heating is performed in the presence of water, water-vapor pressure increases and so does the vapor pressure of BVOCs. Volatiles are consequently carried with the steam through a tube into the still condensation chamber where both water and volatiles condense. The hydrophobic essential-oil components form a film on the

surface of the water. The film is decanted or skimmed off the top to obtain the final essential oil. HD is a time-consuming technique requiring several hours (Table 1). To reduce the length of the process, to limit the alteration of the natural constituents by possible oxidation, to reduce losses of the most polar compounds and to save energy, analysts can apply steam distillation (SD), which involves forcing the steam through the plant material [9]. Hydrolysis of extracted compounds has nonetheless been observed through SD [10]. The overall disadvantage of distillation methods is that it is hard to determine quantitatively the essential oil of small amounts of foliage as the yields are typically low (Table 2).

BVOCs from leaf material can also be extracted by organic solvents. The extraction efficiency depends on the correct choice of solvents (e.g., pentane, hexane), the use of agitation and choice of temperature to increase the solubility of BVOCs and improve the mass transfer. Extraction under 25–30°C and agitation on small amounts of ground foliage (~1 g), for a short period of time (20–30 min) gives optimal recoveries [6,11]. This simple solvent extraction permits recovery of many monoterpene compounds that are lost during HD due to the high temperatures (Table 1). Solvent extraction can also be achieved by Soxhlet apparatus, whereby the foliage is constantly eluted with fresh solvent [11]. A solvent reservoir is gently heated, allowing the solvent to vaporize. By means of a condenser, the solvent turns back into liquid and then drips back onto the foliage, performing the BVOC extraction. The foliage is contained in a porous cup allowing the solvent to flow back to its reservoir. For both techniques, the resulting plant extract, so-called concrete, can be evaporated by vacuum pressure without the use of heat. The resulting concentrated solution is called the absolute – a highly concentrated plant extract without natural waxes [12].

An extraction combined with distillation [i.e. simultaneous distillation solvent extraction (SDE)] can be achieved by a Likens-Nickerson instrument (Fig. 1a) [13]. A flask with heating bath contains the plant sample, while another flask with heating bath contains the solvent [typically low boiling solvents (e.g., pentane)]. A cooler and a condenser separator permit efficient condensation trapping of the volatiles. Despite the long extraction time required, especially when the matrix features important lipid content, this is a very common method [14].

There is no clear consensus on the volatile-extraction efficiencies of the different techniques. For example, on the one hand, the Soxhlet extraction has better monoterpene-extraction efficiencies than simple solvent extraction but provides poorer recoveries than SD [15] (Table 3) for walnut-tree leaves, a species that features glandular trichomes. On the other hand, Soxhlet extraction appears to be a more convenient technique than SD for extraction of monoterpenes of thyme, which also features glandular trichomes [16]. The method set-up and morphological differences within the same type of storage structure are likely to influence such differences.

2.1.2. Recent methods. Important progress has been made in the development of novel separation techniques with shortened extraction times, reduced solvent consumption, and enhanced prevention of oxygenation and isomerization, especially for thermolabile and chemically highly active constituents.

In a variety of combinations, microwaves are increasingly being used as the heat source to assist the extraction of essential oils, as recently reviewed [17]. They are, for example, applied to assist during solvent extraction (microwave-assisted solvent extraction, MASE). The microwaves are used directly to heat up a solvent (e.g., methanol) [17]. The solvent chosen must be able to absorb microwave energy and pass it on as heat to the plant matrix. Microwaves have also been used to assist during HD (microwave-assisted HD, MAHD, Fig. 1b), resulting in slightly or strongly higher extraction yields [18,19]. The usefulness of this advanced HD technique partly relies on the sudden eruption of lipophilic compounds from storage structures of leaf exposed to the microwaves [18]. There is evidence that some isomerization occurs when using high microwave power [20] (Table 1).

Unlike microwave extraction, which utilizes polar and non-polar solvents, solvent-free microwave extraction (SFME) has been developed [21,22]. Fresh leaves – without addition of water or any other solvent – are placed into a reactor of a microwave apparatus that ensures homogeneous microwave distribution. Under atmospheric pressure, the vapor generated by the water contained in the fresh leaves is enough to extract the BVOCs from plant material. Constant temperature and vapor conditions are guaranteed by the return flow of condensed water, which is achieved by a circulating cooling system. However, heating the sample enclosure is needed during at least most of the microwave-radiation stage to compensate for the temperature drop resulting from water evaporation from the biological material.

In improved SFME, dried material can also be used. In this case, a solid medium with a higher microwave absorption capacity than water (e.g., carbonyl iron powder) is mixed with the material, resulting in a shorter extraction time [21]. A higher recovery of oxygenated monoterpenes occurs with improved SFME, compared with HD and MAHD [21] (Table 3), but this high recovery has been attributed to analyte oxidation with oxygen in air since the sample is not submerged in water.

Supercritical fluid extraction (SFE) is a solvent-free extraction method, usually carried out using CO_2 due

Table 1. Advantages and disadvantages of techniques used to study terpene content in vegetation

Technique	Advantages	Limitations[1]
Hydrodistillation [23,44]	⊕ No solvent residues ⊕ Low yield of essential oil/plant amount (of concern if a replicate is needed for young plants or if the plant volatiles are studied over time)	⊖ Potential losses of most polar terpenes (oxygenated ones) and chemically most active compounds ⊖ Loss of volatile compounds ⊖ Low efficiency (in terms of volume of essential oil per 1 g of plant). Foliage mass required ~100 g ⊖ Long extraction time
Stirring or simple solvent extraction [11,12]	⊕ No heat ⊕ High yield of essential oil/plant amount (an aliquot of 0.5 g may suffice for some species	⊖ Co-extraction of non-volatile matter (mainly cuticle waxes) if the ''concrete'' is not processed ⊖ If non-volatile analytes are removed, the clean-up step that may cause loss of volatile analytes
Soxhlet solvent extraction [16,58]	⊕ Enables the extraction of the desired volatile, where the lipid has only a limited solubility in a solvent ⊕ Solvent recycling	⊖ Poor recovery of high-volatile or heat-labile compounds ⊖ High extraction times required (3–24 h) ⊖ Possible thermal decomposition of the stored compounds cannot be ignored as the extraction usually occurs at the boiling point of the solvent for a long time ⊖ Co-extraction of non-volatile compounds ⊖ Losses of volatile compounds if concentration steps are required due to the use of large volumes of organic solvent
SDE: simultaneous distillation solvent extraction [14,24,59]	⊕ One-step extraction technique ⊕ Fast ⊕ Allows great reduction of solvent volumes due to the continuous recycling ⊕ Extracts are free from non-volatile materials (e.g., cuticular waxes or chlorophylls) ⊕ Micro versions of SDE allow use of small amounts of extraction solvents without requiring subsequent concentration of the extract, thereby reducing losses of volatile compounds	⊖ Time consuming ⊖ Some compounds in the foliage extracts arise from pyrolysis or hydrolysis during the process
PSE [2]: pressurized solvent extraction [15,16,28]	⊕ Faster extraction time and lower solvent consumption than Soxhlet, sonication, ⊕ The final extracts are clean enough for direct analysis by GC/MS without need of any pretreatment. This is the great benefit of the method, because, for volatile analytes, every additional handling of samples increases the danger of losses	⊖ Very high temperatures and thus very low monoterpene yield: 5-fold less (limonene) recoveries than UAE and Soxhlet extractions ⊖ Co-extraction of non-volatile species
SFE: Supercritical fluid extraction (with CO_2) [16,23,25,60]	⊕ Low temperature avoiding modifications from heat ⊕ No solvent residue ⊕ High efficiency (in terms of volume of essential oil per plant mass) ⊕ CO_2 is inexpensive and abundant in comparison with organic solvents ⊕ Allows continuous modification of selectivity by changing the solvent density ⊕ It has the density of a liquid and solubilizes solids like a liquid solvent, but has a diffusion power similar to a gas and permeates through solid materials very easily	⊖ Organic solvents, so-called modifiers, may be needed for the CO_2 extracting fluid to alleviate the polarity limitations ⊖ Co-extraction of waxes is unavoidable, ⊖ although this point may be seen as advantageous since some waxes (wax esters) stabilize the essential oil in and delay the evaporation of the fragrances
Microwave-assisted extraction techniques [17,20,21,61]	⊕ No addition of solvent or water ⊕ Short extraction time (30 min) ⊕ Suitable for thermolabile species, since it uses low temperature ⊕ Full reproducible extractions completed in seconds or minutes with high reproducibility	⊖ Use of high microwave energies may lead to isomerization or to compound destruction

(*continued on next page*)

Table 1. (*continued*)

Technique	Advantages	Limitations[1]
SWE [3]: subcritical water extraction [26]	⊕ Rapid (15 min) ⊕ Efficient ⊕ Inexpensive method ⊕ High efficiency (only ~1 g of plant mass is required to obtain the plant extract)	⊖ High temperatures (150°C) result in destruction of chemically-active compounds ⊖ Highly selective: Useful to extract oxygenated terpenes whereas non-oxygenated terpenes are barely detected
UAE [4]: ultrasonic-assisted extraction [20,30]	⊕ Fast ⊕ High yield (i.e. low amount of material required)	⊖ Formation of free radicals and ⊖ consequently potential changes in the constitutive molecules
Combination of the previous techniques with SPME [33,35]	⊕ Collection of the volatile compounds, without interferences from the matrix ⊕ Solvent-free method	⊖ Only qualitative and semi-quantification of extracted volatiles can be achieved ⊖ Semi-quantification will be very sensitive to humidity

[1] Disadvantages of most methods depend on the temperature applied.
[2] Also known as pressurized liquid extraction (PLE), pressurized fluid extraction (PFE) or accelerated solvent extraction (ASE).
[3] Also known as continuous subcritical water extraction (CSWE).
[4] Also known as ultrasound-assisted extraction and sonication.

to its advantages as a solvent for BVOCs (Table 1). Under high pressure, CO_2 turns into a liquid and acts as a solvent that can be used to extract the essential oil from plant material [10,23]. CO_2 is forced into a stainless-steel tank containing plant material, then the pressure is released. As pressure decreases, CO_2 returns to a gaseous state and only the plant extract remains. Compared to HD, SFE results in extraction yields that may be about 6 times higher [23]. Sesquiterpene yield and the number of compounds extracted via SFE are also higher than using SDE, HD and MAHD [24]. However, SFE does not seem suitable to extract monoterpenes, unlike SDE, which has been highlighted to be more efficient than SFE, HD and MAHD [24] (Table 3). One of the main drawbacks of SFE is its limitation to non-polar and medium-polar substances, since it is mostly applied with CO_2 [16].

Continuous subcritical water extraction (SWE) – which allows for the extraction of more polar terpenes, such as oxygenated terpenes – has been proposed as an alternative to SFE. Continuous SWE is based on the use of water as the solvent for extraction (Table 2). Plant material in an extraction chamber releases the volatiles in response to heating (e.g., the chamber is placed in an oven). Pressure is regulated to keep water in the liquid phase [25]. Temperatures are in the range 125–150°C, although, in oregano samples, temperatures over 125°C already degrade the extract [26]. Afterwards, a liquid-liquid extraction is required with an organic solvent to concentrate the volatiles contained in the aqueous solution [27]. The method is fast, with 10–20 min needed to process a sample, and obtains higher yields than traditional HD [26,27]. As summarized in Table 3, SWE is particularly useful for extracting oxygenated terpenes, as their affinity for water is greater than that of non-oxygenated species. However, losses of oxygenated monoterpenes would occur if temperatures above 175°C are used [27].

Pressurized solvent extraction (PSE) relies on the use of heated and pressurized organic solvent (Table 2). The solvent is pumped into an extraction vessel where the sample is contained in a porous bag (thimble) [16,28,29]. High pressure keeps the solvent from boiling, while high temperature accelerates the extraction process by increasing the penetration of the solvent into plant matrix. These features together with the solubility of the analyte in the solvent (increased partition coefficient for non-polar solvents) enhance the rate of desorption of the analyte from the sample matrix. PSE reduces solvent consumption and sample-preparation time from hours, in the case of traditional methods, to minutes. Compared to HD, SFE and Soxhlet extraction, PSE is the most suitable method to obtain the essential oil of thyme herb [16]. Compared with the other methods, its efficiency is exceeded only by that of Soxhlet extraction, but PSE is less time-consuming.

Ultrasound-assisted extraction (UAE) has been combined with different techniques (e.g., SFE and conventional stirring solvent extraction). The ultrasonic equipment can be used for the extraction of BVOCs localized in both surface glands, where a mild ultrasonic treatment is enough, and inside the cells, where stronger treatment is needed. For cellular BVOCs, pre-treatment by size reduction is necessary to maximize surface area [30]. UAE increases the performance of solvents and is performed at lower temperatures [31], which are less likely to result in losses of thermally unstable compounds, but isomerization and decomposition may occur for chemically unstable compounds [20]. UAE provides smaller extraction yields than most classical (HD, SD, Soxhlet) and some recent extraction methods (e.g., PSE) [15,31,32] (Table 3).

Table 2. Parameter set-up to extract and trap plant BVOCs from plant material

Method	Ref.	Plant material (g)	Extraction time (min)	Extracting solvent (mL)	Extraction temperature (°C)	Extraction pressure (MPa)	Specific instrumentation or parameters	Yield (%)
Distillation (hydro – and steam – distillation)	[12,15,16, 18,22,25,29]	10–1000 (mostly fresh leaves)	180–360	500–4000 water	50–100	Atmospheric	Dering apparatus	0.03–3.44
(Stirring) simple extraction with organic solvent	[11,12,15, 27,31]	1–500 (fresh or dried ground leaves)	20–1440	5–1500 cyclohexane, pentane, hexane, dichloromethane	Room–69	Atmospheric		0.001–2.6 0.09–9(1)
Soxhlet extraction	[15,16,31]	2–5 (fresh or dried ground leaves)	60–300	5–100 hexane	Room–160	Atmospheric		0.013–3.14
SDE : simultaneous distillation extraction	[14,39,48]	1–100 (fresh leaves)	60–360	60–400 water Followed by liquid–liquid extraction with pentane, ethyl ether or dichloromethane	Unspecified–120	Atmospheric	Modified Likens-Nickerson Microscale simultaneous distillation-extraction apparatus	2.22–2.9
PSE[2]: pressurized solvent extraction	[15,16,29]	0.5–3 (fresh or dried ground or non-destroyed leaves)	15–180	Hexane, dichloromethane, ethyl acetate, distilled water	20–175	6–15	Dionex ASE200 instrument	0.02–2.8 1.3–26.7(1)
SFE: supercritical fluid extraction (CO_2)	[16,60,62]	0.5–800 (fresh or air dried ground or non-destroyed leaves)	20–35	CO_2	20–80	2–50	One-PSE instrument; Suprex MPS/225 system	0.48–2.7
SFME: solvent-free microwave extraction	[21,22]	100–250 (fresh or dried spices and leaves)	30–50	None	Not controlled–100	Atmospheric	Milestone DryDIST (2004) apparatus; Household system	Unspecified–0.4
Microwave-assisted extraction techniques (e.g., MAHD: microwave-assisted hydrodistillation)	[18,21]	50–60 (spice, leaves)	60–90	0.3–1.2 water	Unspecified	Atmospheric	Irradiation frequency: 20 GHz; Power: 990 W	Unspecified–3.66
SWE: subcritical water extraction	[25–27]	0.4–4 (fresh or dried ground leaves)	15	Water Followed by liquid–liquid extraction with 4–5 mL hexane	125–150	2–5		3–20(1)
UAE: ultrasonic-assisted extraction	[15,31,32]	2–50 (fresh ground leaves)	15–60	5–100 ethanol, hexane	Room–69	Atmospheric	Ultrasound power: 150 W; Sonication frequency: 20 kHz	0.006–2.87
HS-SPME (headspace solid-phase microextraction)	[12,14,16,33]	Unspecified–2 (fresh or dried ground or living)	2–90	None	Unspecified–60	Atmospheric	SPME coatings: Carboxen/PDMS; CAR/PDMS; PDMS PA	

(1) Times the yield obtained by distillation.
(2) Time the SPME is exposed to the HD.

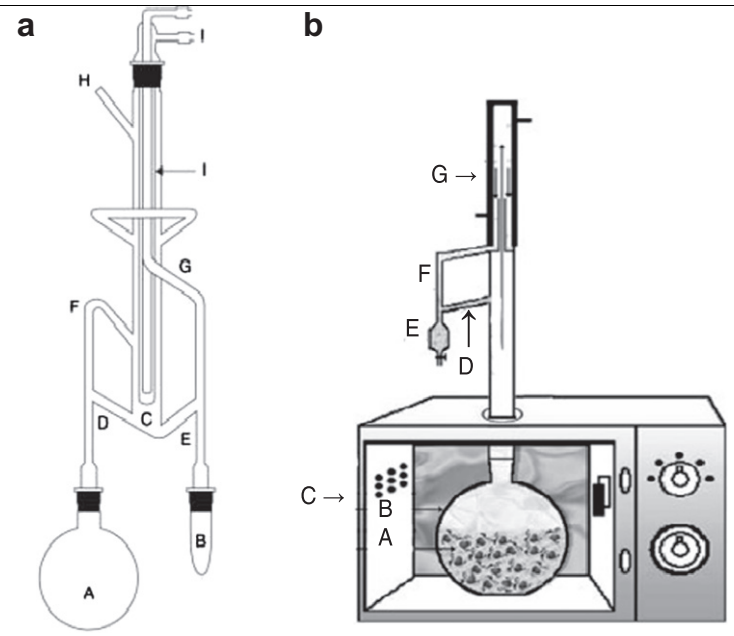

Figure 1. (a) *Simultaneous distillation extraction (SDE)* (from [12], reprinted with permission): sample A and solvent B flasks are heated to their boiling points. Their vapors are mixed in the separation chamber (C) and condensed on the cold finger (I). The organic and water liquid phases return to their original flasks through the return tube for water (D) and the return tube for solvent (E), while volatiles are gradually transferred from the water (F) to the organic phase (G). The water and the organic solvents, which are never in contact during the whole process, are constantly reutilized for the same sample matrix, reducing the liquid consumption. An inlet/vent (H) allows work under atmospheric pressure. (b) *Microwave-assisted hydrodistillation (MAHD)* (from [14], reprinted with permission). Plant material (A) is placed in a sample flask containing water (B), which is introduced in an oven (C). Water (E) flows through a water-reflux tubing (D) and vapor condenses in a condenser (G). The collected essential oil (F) is finally decanted from the condensate.

According to a few studies that have compared the efficiencies of different extraction techniques, SDE is the most appropriate for extracting non-oxygenated monoterpenes, while MAHD is particularly suitable for oxygenated monoterpenes, and SFE for sesquiterpenes [24]. PSE also seems to be a promising technique, since it shows an extraction efficiency for walnut-tree leaves for all volatile groups superior to that of SD, Soxhlet extraction, UAE and simple solvent extraction with agitation [15] (Table 3). However, to be able to establish a clear ranking of extraction efficiencies across different extraction techniques, we suggest that future research studies should develop more exhaustive comparisons by considering classical and modern techniques and comparing various techniques across species featuring different specific storage structures.

2.2. Trapping BVOCs released by harvested foliage

Many of these techniques can be combined with solid-phase microextraction (SPME) (Fig. 2a,b), which results in collection of a fraction of stored compounds, previously volatilized from the matrix to the headspace, instead of the absolute extraction of the stored compounds. SPME-related techniques, unlike extraction techniques, cannot be compared in terms of BVOC yield but just in terms of relative composition [12]. One of these techniques is MASE followed by HS-SPME (MASE-HS-SPME), a fast, efficient technique to study terpene composition in plants [33] (Fig. 2a). Volatiles released from foliage after application of microwaves are adsorbed onto the fiber coatings of the SPME.

SPME is considered a quick, cheap and useful technique for trapping and characterizing the fractional composition of the BVOCs stored in plants. SPME comprises a fiber coated with a solid (sorbent), a liquid (polymer), or a combination of both [34]. Solid SPME fibers provide semi-quantitative information for a fraction of the stored BVOCs, since the adsorbed amount depends on the fiber-coating affinity for the compound and the coating-free sites where compounds are adsorbed [35], in addition to their concentration in the headspace or the leaf. As a result, the BVOC composition may misrepresent some volatiles and over-represent others. If liquid fibers are used (e.g., PDMS), the affinity limitation is eliminated, since compounds are absorbed by the fiber resulting in more accurate quantitative results, but a lower number of compounds is recovered. For any coating, the exposure time of the SPME to the headspace and the sampling temperature must at least be tested and rigorously reproduced in order to obtain reliable results. After BVOC trapping, the SPME fiber is transferred into the analytical instrument, typically GC/MS, for desorption and analysis of the target metabolites. Desorption parameters (not reviewed herein) (e.g., liner

Table 3. Ranking of plant volatile extraction efficiencies obtained by different methods. A higher efficiency is denoted by a greater number of stars for monoterpenes (MNTs), circles for oxygenated monoterpenes (OX-MNTs) and squares for sesquiterpenes (SQTs). The absence of a symbol denotes that the given group of volatiles was not detected by the respective method

Method	MNTs	OX-MNTs	SQTs	Ref.	Data to establish the efficiency
HD	***	○○○	□	[24]	Relative peak area (%)
SDE	****	○○	□□		
SFE	*	○	□□□□		
MAHD	**	○○○○	□□□		
SD	*	○○○	□	[16]	Relative peak area (%)
Soxhlet	***	○○○	□		
PSE	**	○○○	□□		
SFE	**	○○○	□□		
HD	*	○○		[27]	Area compound/internal standard ratio
Solv Extr(1)	**	○			
SWE	***	○○○	□		
HD	*	○		[26]	Peak area/hydrodistillation peak area ratio
SWE	*	○○			
HD	**	○		[25]	Area compound/ internal standard ratio
SWE	*	○○			
HD	**	○		[22]	Relative peak area (%)
SFME	*	○○			
Soxhlet	***	○○○			
Solv Extr(1)	*	○		[31]	Concentration (mass oil/plant material mass)
UAE	**	○○			
SD	****	○○○○	□□□□	[15]	Concentration (mass oil/plant material mass)
Soxhlet	***	○○○	□□□		
Solv Extr(1)	*	○	□		
UAE	**	○○	□□		
PSE	*****	○○○○○	□□□□□		
HD	**	○	□□	[32]	Relative peak area (%)
UAE	*	○○	□		
SD	*	○○	□	[44]	Relative peak area (%)
Solv Extr(1)	**	○	□□		
HD	**	○○	□□	[12]	Relative peak area (%)
Solv Extr(1)	*	○	□		
HD	**	○○○	□□	[21]	Relative concentration (%)
MAHD	***	○○	□□□		
Improved SFME	****	○	□□□		
Conventional SFME	*	○○○○	□□□□		

(1) Simple Solvent extraction by agitation.

and desorption time in the injector) also influence the method reproducibility and repeatability [29].

2.3. Trapping BVOCs released by living foliage

All the procedures described so far are constrained to cut and ground leaf material. Foliage grinding may nonetheless pose several problems, so some investigations opt for enclosing the living plant and sampling its headspace by SPME (HS-SPME) (Fig. 2c). This technique cannot provide the absolute leaf-BVOC concentration since, in addition to SPME limitations (see sub-section 2.2), the equilibrium concentration in the atmosphere depends on the transfer resistances between the site of compound storage and ambient air. However, HS-SPME is of great interest as a comparative approach and shows roughly equivalent essential oil compositions to HD and PSE extracts [29]. HS-SPME is traditionally used to gain insight into the emission blend of a plant [34,36 and citations

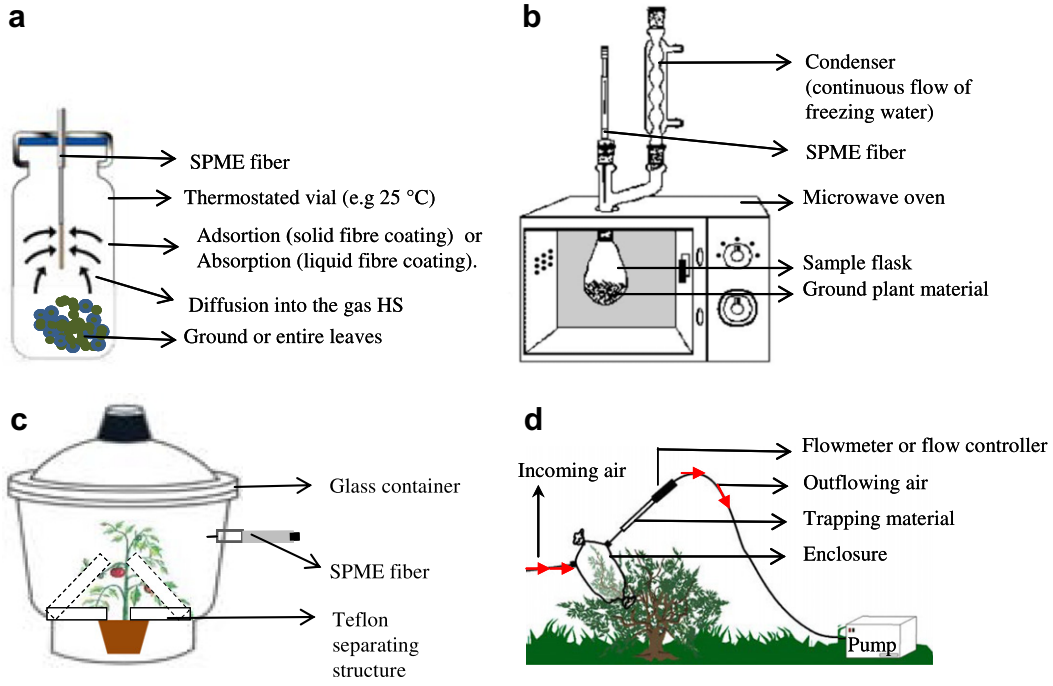

Figure 2. Destructive (a, b) and non-destructive (c, d) modes of sampling the headspace (HS) plant volatiles, as indirect methods to assess the plant terpene content. (a) Exposure of the SPME to the HS of the harvested leaf; (b) Exposure of the SPME to the HS of the plant extract obtained by microwave-assisted solvent extraction (MASE-HS-SPME) (from [14], reproduced with permission) (c) Exposure of the SPME to the HS of the living plant; (d) Dynamic headspace trapping (DHT) system and collection of volatiles by carbon-based adsorbents.

therein] and recent studies claim that it can also be a tool to study the emission rate of BVOCs released by vegetation [37,38]. Thus, some authors argue that HS-SPME is the sampling method that better reproduces the genuine scent one could perceive from fresh plant. An alternative is the static headspace (S-HS) method, where the SPME is replaced by a gas-tight syringe. In this case, relative amounts of compounds should be more representative of the amounts of the headspace, due to the absence of the selectivity effects of specific coatings. However, compounds present in very low concentrations are missed with S-HS due to its lower sensitivity [24].

More rarely, plant BVOC content is estimated through dynamic headspace trapping (DHT) techniques (Fig. 2d) [39] – a technique commonly employed to study plant BVOC-emission rates accurately [40,41]. DHT commonly features lower efficiency, in terms of monoterpene and sesquiterpene signal, than HS-SPME, due to selective adsorption characteristics of adsorbents, but this can be improved by the use of multibed-adsorption traps filled with several adsorbents [24,39].

3. Similarities and discrepancies between BVOC emissions and content

Amounts of specifically stored BVOCs affect the diffusion of volatiles through the cells to the intercellular spaces and the atmosphere [32]. Diffusion occurs along a vapor-pressure gradient from cellular compartments of relatively high concentrations to the air surrounding the leaf, where the concentrations are relatively low because of turbulent transport, extreme atmospheric reactivity and, therefore, brief lifetime of most BVOCs. An important part of qualitative and/or quantitative similarity between emissions and content has been reported in some studies, suggesting that species rich in essential oils are likely to be high BVOC emitters [5]. However, there are undeniable discrepancies between emitted and stored BVOCs [6,42,43], which can be explained by combination of numerous factors described hereafter.

3.1. Technique-dependent factors

The techniques described in this review differ in the efficiency with which they extract or trap different metabolites, and, as a result, there is an unavoidable difference between the volatiles that the plant stores and then releases, and what is present within the plant extract or trapped fraction. Also, the high temperatures applied in some of the previously described techniques, may lead to losses and degradation of the most volatile compounds [26,44]. It must be kept in mind that, in many cases, techniques set up to produce essential oils do not seek to minimize losses of highly volatile compounds, but to attain the specific criteria defined for each essential oil and environmental objectives in terms of solvent and energy consumption. Thus, the essential oil

Table 4. Pre-extraction and post-extraction parameters affecting the resulting BVOC foliage content

Step	Parameters	Recommendation
Pre-extraction	Ground/entire plant material	Grinding increases the contact between leaves and extracting solvent, resulting in increased terpene yields [11,15,62]. However, some studies point out that disintegration of plant material before volatile extraction has adverse effects on yield [9], leads to losses of volatiles, unlike cutting [27] and may result in the over-production of some terpenes and the formation of new non-terpenic compounds possibly reflecting continued enzymatic reactions in the destroyed plant cells [29].
	Fresh or dry material	The impact of the drying technique on terpene extraction must be checked as its effect varies according to the species (due to the type of specific storage organs for accumulating terpenes) and the compound. Freeze-drying is an optimal option compared to fresh and air-dried samples, but freeze-drying at very low temperatures (−198°C instead of -18°C) favors losses of plant volatiles [47,48].
Post-extraction	Storage conditions	Harvested leaves, essential oils and plant extracts are typically stored at least under −20°C under dark conditions. After 30 days of storage, changes in the essential oil are significant [49].
	Oxidation just after extraction and during storage	Light oxidation is usually prevented by using amber vials or aluminum-foil-wrapped vials. Oxidation during storage is avoided using anti-oxidants [e.g., butylated hydroxytoluene (BHT)], often already contained in the purchased organic solvents, and by replacing the air contained in the vial flask by gaseous nitrogen. Oxidation during the drying process does not seem to be a major problem, since plant extracts contain as much oxygenated terpenes before drying, as they do after drying [48].

of *Thymbra spicata* and *Thymus mastichina* must show insignificant amounts of terpenes and a full recovery of specific oxygenated compounds [9,45]. Hence, if these techniques are applied in order to tackle the correlation between BVOC emissions and content, specific analytical conditions (temperature, solvent, extraction times) should be re-examined in order to minimize losses of highly volatile and highly reactive BVOCs.

Grinding is also a potential source of modification of the actual BVOC content, although its effect is not well documented [9,29] (Table 4). If foliage biological activity is not stopped, grinding may lead to enzymatic and non-enzymatic formation of volatiles. As terpene-synthase activities are already very low at temperatures below 5–10°C [46], such a problem can be avoided by homogenizing foliage on ice, or more desirably in liquid nitrogen, to avoid completely enzymatic reactions and vaporization losses of volatile terpenes. Enzymatic reactions involved in terpene formation also occur after foliage harvest, but are considered to be mostly prevented by foliage storage in liquid nitrogen before reaching the laboratory, or under very low temperatures (e.g., −20°C, −80°C) if storage is needed for longer periods (Table 3).

Finally, foliage drying – often performed before extraction and grinding of plant volatiles – can lead to major losses of volatiles. However, it is unclear which the most suitable drying technique is. Some studies claim that freeze-drying leads to satisfactory results [6,47,48] (Table 4), while drying at temperatures of 20–25°C and higher has quantitative and qualitative effects on the essential oil [45,49]. Some others demonstrate that drying at ambient temperature or 45°C is more suitable than freeze-drying at −198°C before extracting BVOCs from plants [48] (Table 4). We suggest that the effect of the drying method should be tested before routine use for any given species. In order to avoid these possible impacts of drying, some studies aim to assess the BVOC content from living plants (Fig. 2c).

3.2. Physico-chemical properties of plant volatiles

Each BVOC species features a certain combination of physico-chemical characteristics [50]. In particular, gas-liquid phase partition coefficients (Henry's law constant, H) and lipid-liquid phase partition coefficient (typically characterized by octanol/water partition coefficient, $K_{O/W}$) vary over four orders of magnitude for key plant volatiles [51]. On the one hand, as H decreases (e.g., for oxygenated volatiles), the volatiles tend to partition in aqueous solutions within the leaf cells, instead of ambient gas phase, especially when liquid-gas phase transfer conductance is small (e.g., closed stomata). On the other hand, compounds with high $K_{O/W}$ have typically high H values, so they are not sensitive to modifications in gas-liquid phase transfer conductance [51,52], and tend to adsorb on the lipophilic surfaces on the leaves as well as the sample apparatus.

Adsorption problems may be particularly significant for larger molecular mass compounds [e.g., sesquiterpenes ($C_{15}H_{24}$) and diterpenes ($C_{20}H_{36}$)]. Essential oils and plant extracts contain large amounts of these intermediate- to low-volatility species. However, they are less frequently found in the emissions. Their low vapor pressure compared with monoterpenes provides one explanation [53,54]. Thus, although they are highly concentrated in specific leaf-storage structures, their release to the atmosphere is greatly restrained. However,

in many cases, it seems more likely that sesquiterpene emissions occur, but their high reactivity and stickiness on gas-collection lines impede them in reaching the analytical detector. Optimizing the sample-chamber and sampling-line design and adsorbent properties can improve the recovery of sesquiterpenes in plant-gas samples [40].

3.3. Biological factors

Passive diffusion is not always sufficient for BVOC volatilization into the atmosphere. This is partly due to a variety of biological factors involving leaf histology, biochemistry and physiology. Stored BVOCs may be retained within specific pools due to the high resistance of cell walls surrounding the storage structures. In addition to these structures, all volatiles need to penetrate the subcellular and cellular membranes to move from the site of their synthesis or storage to the outer surface of cell walls. For example, within some species of the mint family, which exhibit external glandular trichomes, BVOCs have been observed to remain in the specific reservoirs until the cuticle is damaged by abrasion [43]. In *Pinus* species, with internal secretory cavities, the resin ducts, the resistance could be higher than for trichome-featuring species, since BVOCs have to diffuse over several additional barriers (i.e. parenchyma cell layers surrounding the secretory structure, and at least two layers of cells surrounding the cavity, with the innermost layer consisting of secretory epithelial cells, and the outer layers of sheath cells with thick walls).

Another common view is that BVOC emissions do not mirror BVOC content in species with specific storage pools (e.g., conifers), because a fraction of the emissions does not originate from volatilization of compounds stored within resin vessels and synthesized by leucoplasts of the epithelial tissue, but from those *de novo* synthesized in the chloroplasts of the photosynthetic tissue of leaves under light conditions. Although not studied extensively for many conifer species, emissions from *de novo* synthesis seems to be controlled by physiological parameters related to metabolic activity and precursor availability, while emissions from leaf pools depend on physico-chemical properties (H, $K_{O/W}$) [50]. These light-dependent emissions are also typically elicited in response to a variety of biotic and abiotic stresses [55], so these emissions have differing composition than the stored terpenes.

3.4. Abiotic and biotic factors

The resistance to BVOC diffusion flux out of the leaves described above is presumably higher under water deficiency since leaves develop a thick epidermal layer and accumulate waxes in the cuticle to minimize water losses through transpiration [56]. Trichomes also develop a thicker cuticle and cell walls under these conditions [57], and, as a result, volatiles retained in the leaf have to overcome a greater resistance and their release to the atmosphere is restricted despite their high vapor pressure. As discussed in the previous section, *de novo* formation of terpenes in response to biotic and abiotic stresses may also interfere with the emission from storage. In terms of biotic interactions, when the plant is attacked by an herbivore, the stored BVOCs in the secretory structures can burst to the surface of the damaged leaf. Such an exposure of BVOCs to free air will create a high terpene concentration at the point of attack. This sudden release of constitutive BVOCs is unlikely to be proportional to the amounts of BVOCs stored.

4. Conclusions

In the past two decades, some attempts have been made to scale up data on leaf BVOC content to BVOC emissions. These studies, all performed using traditional techniques to estimate leaf BVOC content, have often found a relatively weak correspondence between content and emission. However, in some cases, a fairly good correlation between terpene content and emissions has been found [7]. To shed more light on this complex relationship, we suggest, as a starting point for further studies, exhaustive evaluation of all those techniques that offer special protection for thermolabile constituents, although more traditional techniques (e.g., Soxhlet) should not be neglected. In view of the existing comparative studies, a combination of SFE and SDE could be necessary to achieve the highest sesquiterpene and monoterpene recovery, respectively, for the same plant. Also, SWE seems to be very appropriate for extracting oxygenated BVOCs. Microwave-assisted extraction techniques could also be good options, given the few environmental and biological limitations that they present.

We also highlight that, in many cases, optimum parameters of these novel techniques have been selected to conform to specific industrial requirements for energy cost and pollution generation, and essential oil definitions, so that they should be carefully re-considered from the point of the quantitative recovery of all volatiles that is needed to gain insight into the role of BVOCs in plant-biosphere interactions. However, the effects of biological factors in affecting the equilibrium between leaf interior and ambient atmosphere are still not entirely understood. We suggest that, armed with these new analytical tools, we should be able to achieve rapid progress in understanding the biological controls on emissions.

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