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Table: 1, Figs : 2

Comparative performance of different methods used to collect tomato plant volatiles

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ABSTRACT

We compared 3-methods of volatiles collection [simultaneous distillation extract (SDE), dynamic headspace trapping (DHT), and headspace solid phase micro-extraction (HS-SPME)] for their efficacy in collecting the volatiles emitted from tomato plants. 25-components were identified from various volatile fractions and their percentages were calculated in the volatiles collected by each method. Mono- and sesquiterpenes were the major components of volatile blends, among them β-phellandrene (28>50%) was most abundant, followed by β-caryophyllene (4.5-18%) > (+) -2-carene (8-15%) > δ- elemene (2-15%)>α-caryophyllene (1-4%) in the SDE, DHT and HS-SPME volatile fractions. Both qualitative and quantitative differences were found in collections done by three methods. The total number of volatile components in SDE extract were higher than in DHT and HS-SPME extracts. HS-SPME was more efficient than both SDE and DHT, in collecting highly volatile compounds (such as mono- and sesquiterpenic terpenes), whereas, SDE was more efficient than DHT and HS-SPME in collecting low-volatile compounds (such as long chain hydrocarbons, ketones, acids and esters). SDE extraction involved damage to plant, which resulted in releases of green leaf volatiles. These results will be useful for selection of techniques for collection of volatiles from plants.

Key words: Volatile organic compounds, *Solanum lycopersicum*, SDE, DHT, HS-SPME

INTRODUCTION

Plant volatiles play important roles in the interactions between the plants and arthropods (1,6,18,38). Upon herbivore attacks, plants release diverse blends of chemicals that can influence the behaviour of herbivores, their natural enemies and the physiology of nearby plants (7,16,20,27). In recent years, many studies have been conducted to identify the "key" volatiles and understand their physiological, ecological and evolutionary effects in tritrophic systems and their application in pest control (12,46,52). Hence, the study of volatile organic compounds (VOCs) emitted by plants is also of interest to the botanical pesticide industry (15,25,26,53).

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The interest in biochemistry, physiology and ecology of plant VOCs has developed many methods to collect and analyses the plant volatiles (31,46). To collect the volatile blends emitted by living plant or other materials various methods [steam distillation (SD) (24,41,42), solvent extract (22,44,45), simultaneous distillated extract (SDE) (11,45), supercritical fluid extraction (SFE) (24,37), solid phase micro-extract (SPME) (43, 46,50), and dynamic headspace trapping (DHT) (23,46,47)] are used. Each technique has its own advantages and disadvantages (35,46,49). The chromatographic profile of volatile blends isolated from a living plant or other materials often differ significantly depending on the extraction methods. For example, significant qualitative and quantitative differences were observed in volatile profiles of *Valeriana officinalis* var. *latifolia*, when the extraction was done with SFE, SPME and hydrodistillation (24). Differences in volatile profiles owing to extraction methods are known (21,28,32,42,45,49). Therefore, use of more than one method has been suggested to collect the VOCs emitted from a living plant or other materials (46,49). Among the various extraction methods, SDE is superior to solvent extraction, hence, widely used to collect the volatile compounds from numerous materials (11,45). DHT and SPME, which do not require wounding of plant for extraction, are convenient and provide realistic profiles of volatiles emitted from living plants (43,46).

Tomato plants emit intense aroma and the volatiles emitted from its many accessions are highly repellent or toxic to insect and mite pests (3,4). The profiles of volatiles emitted from tomato plants had been studied and some of the volatile components emitted from wild or cultivated species have been isolated by direct solvent extraction and tenax trapping (2,8,48). The co-extraction of non-volatile materials (such as cuticular waxes or chlorophylls) is a main disadvantage of solvent extraction. Besides, solvent extraction also requires large amount of solvents and is time- consuming. In tenax trapping, the mechanical wounding of plants results in additional emission of volatiles, which is a major drawback (8).

In studying the interactions of tomato with its phytophagous insects, it is desirable to collect the volatiles from undamaged plants growing in natural conditions. Although semiochemicals of tomato plants have been studied (2,3,4,8,48), but there is no study to collect the volatiles from intact living plant. This study aimed to (i). compare 3-collection techniques: [SDE, DHT and headspace solid phase micro-extraction (HS-SPME), to collect volatiles from tomato plants (ii). analyze the components of volatile blends, (iii). determine their relative abundance in individual compounds by gas chromatography-mass spectrometry (GC-MS) and (iv). determine the effectiveness of these techniques.

MATERIALS AND METHODS

Tomato (*Solanum lycopersicum* L. cv. Hezuo 903) seeds obtained from the Seed Company of Zhejiang Province in 2006 were grown in the pots (each with 300g soil mixture of peat, perlite, vermiculite and organic compounds). The plants were cultivated in a glasshouse [temperature (25±4°C) and humidity (40-75% RH) conditions with natural lighting]. During cultivation, the plants were protected from biotic and abiotic damage. When plants had 7-9 fully expanded leaves, they were moved to a temperature-controlled room (26±1°C, 60-70% RH and 14L: 10D photoperiod) for volatile trapping or leaf collection.

Commercial standard chemicals: (Z)- 3-Hexenal, α-pinene, benzene, 1-methyl-3-(1-methylethyl), (+) -2-carene, β- phellandrene, δ- elemene, camphene, butylated hydroxytoluene, caryophyllene oxide, 1-hexadecyne, phytol, 1-2-benzene dicarboxylic acid, n- triacontane, n- nonadecane, n- nonadecane and n- hentriacontane, were purchased from Sigma-Alderich (St. Louis, Missouri, USA); β-caryophyllene and α-humulene were obtained from TCI (Tokyo, Japan).

I. Simultaneous distillation extract (SDE): A Likens-Nickerson apparatus modified from Chaintreau (2001) was used in this method. Fresh leaves (100 g) were harvested and cut into small pieces and placed in a 1 L round flask containing 0.4 L of redistilled water connected to one arm of the apparatus. A second round flask containing 40 ml ethyl ether as the extracting solvent was connected to the second arm, and the leaf mixture was boiled under atmospheric pressure for 4 h. After cooling, the extract fractions of solvent flask and the return loop were collected and dried over with anhydrous sodium sulphate and concentrated to 10 ml with a rotary evaporator at atmospheric pressure. The extract was then stored at -20°C prior to analysis.

II. Dynamic headspace trapping (DHT)

(i). Volatile-collection system: Air was first pushed through a charcoal filter to eliminate impurities, then through a glass with water to humidify it, and a flow meter to regulate the air flow. The moist and pure air was pushed at 400 $mL \cdot min^{-1}$ from the top into a glass cylinder (16 cm diam., 35 cm high) in which an intact tomato plant was placed. To create a laminar flow, the air was forced through a glass frit at the top of the cylinder before coming into contact with the plant. Approximately 4 cm above the bottom, there was a horizontal female ground-glass joint outlet for a collection trap. The trap consisted of a glass tube (10 cm long, 5 mm diam.) that contained 30 mg of 80/100 mesh Super-Q (Altech Assoc., USA). The air passing over the plant was pushed through the Super-Q adsorbent, and then vented out. Before use, Super-Q particles were pre-rinsed with methylene chloride in order to remove impurities.

(ii). Volatile collection: Volatile-collection was done in a temperature-controlled room (26±1°C, 60-70% RH and 14L: 10D photoperiod). An intact tomato plant was removed from the pot without damage to the roots and packed with aluminum foil, and then placed in the glass cylinder for volatile collection. Illumination was provided by tow dysprosium lamps (Ningbao-2, 400W, Nanjing, China) 150 cm above the cylinder, simulating daylight (inside the glass cylinder the light intensity was approximately 6280 lux). Collection started at 08:00 in the morning, and lasted for 24 hr. After collection, traps were eluted with 300 ml methylene chloride. The aliquots were stored at -20°C prior to analysis.

III. Headspace solid phase microextract (HS-SPME): A manual SPME sampling unit (Supelco, Bellefonte, PA, USA), in which the SPME fiber was coated with 100 µm thick polydimethylsiloxane (PDMS), was used. Before each session of extraction, the SPME fiber was reconditioned by heating in the hot injection port of a gas chromatograph at 250°C for 30 min. Prior to volatile collection, an intact tomato plant was enclosed in a glass cylinder for 5 hr to diffuse volatile components into the headspace. Then the SPME needle was injected through the septum of the sample container as well as the extended plunger to expose the fiber into the space above the plant. Following equilibration between the fiber and the volatile sample for 120 min, the fiber was retracted into the needle. The SPME device was removed from the container and immediately inserted into the injection port of GC-MS system for desorption and analysis.

Chromatographic analysis

Each sample was analyzed by GC-MS, using a HP 6890 series gas chromatograph (GC) system coupled to HP 5973 mass spectrometry (MS). Chromatographic separations were performed on a HP-5 MS column (30 m × 0.25 mm × 0.25µm film thickness). The temperature of both the injector and GC-MS transfer line was 250°C. Ultrahigh purity helium (99.999%) was used as carrier gas at a constant flow of 1.0ml/min. The temperature program for GC was as follows: the initial temperature was maintained at 40°C, held for 4 min, heating up at 4°C/min to 250°C, and held for 5 min. The electron impact ionization mass spectrometer was operated as follows: ionization voltage, 70eV; ion source temperature, 200°C; scan mode, 35.0-450.0 a.m.v. every 2 s. (mass range).

Volatile compounds were identified by comparing the GC retention times and mass spectra of relevant chromatographic peaks with commercially available standards and/or by matching the mass spectra with spectra of the NIST2005 (National Institute of Standards and Technology, Gaithersbrug MD, USA) and Wiley Libraries.Of each sample collected by SDE and DHT, a 1-µl aliquot was analysed on HP-5 column in splitless mode. The total ion chromatograms were adjusted for the same sample size and the same sensitivity scale. All solvents used were of analytical grade quality and were redistilled before use.

RESULTS AND DISCUSSION

Volatile compounds of tomato by three extract methods

The chromatographic profiles of the volatile fraction of tomato plant isolated by DHT, HS-SPME and SDE are presented in Figure 1. The normalized areas (%) for various peaks in chromatogram of Figure 1 appear in Figure 2. A total of 25 volatile compounds (with a molecular weights from 98 to 436) were detected at > 0.1% relative concentration in volatile blends emitted from the plants using the 3-techniques (Fig 1 and 2). The compounds included 4 hydrocarbons, 2 phenols, 1 aromatic, 1 aldehyde, 12 terpenoids, 1 ketone, 2 acids, 1 alchol and 1 ester and their concentrations with each technique were shown in Figure 2. Mono- and sesquiterpenes were the major components of volatile blends, among which β- phellandrene was the dominating component accounting for 28-50% of the total, followed by β-caryophyllene (4.5-18%), (+)-2-carene (8-15%), δ-elemene (2-15%), α-caryophyllene (1.0-4.0%), α-pinene (0.5-2.5%) and α-muurolene (0.1-1.0%) (Fig 2).

Most of the substances identified by us had already been extracted and identified in previous studies on tomato plants. Volatile components in tomato leaves vary with

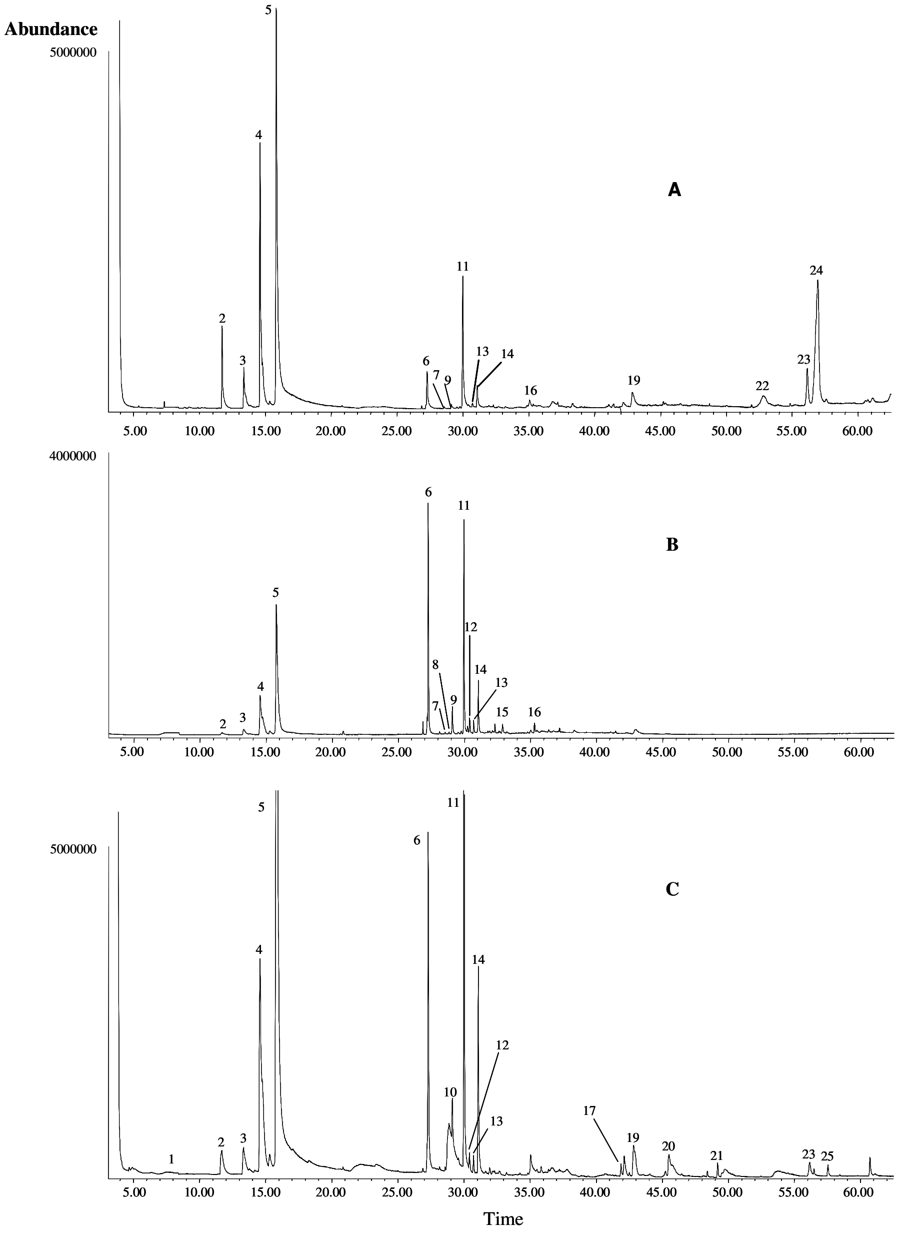

Figure 1. Chromatographic profiles of volatiles collected from *Solanum lycopersicum* leaves by DHT, HS-SPME and SDE extraction followed by GC-MS analysis. The identification numbers correspond to those reported in Figure 2. (A) DHT; (B) HS-SPME; and (C) SDE.

tomato species and cultivars (2, 8, 19, 48). For instance, 12 volatile compounds were identified from 10 tomato cultivars (2), 28 compounds were identified in Red Cherry variety (8), and 25 volatile compounds were identified in Hezuo 903 in this study (Fig 1 and 2). Most sesquiterpenes (such as (+) -2-carene, β- phellandrene, δ- elemene, β-caryophyllene and α-humulene) of cultivar Hezuo 903 were qualitatively similar to other tomato varieties and species (Fig 1 and 2 (2,8,48). Some monoterpenes and aromatic compounds (such as mycene, limonene, benzyl acohol and methyl salicylate) were previously identified as volatiles in some tomato varieties (2, 8, 48). However, zingiberene and curcumene in wild tomato were not detected in odours of cultivars analyzed in this study. One major sesquiterpene α- muurolene, not identified previously, was detected by all 3-methods in this study (Fig 1 and 2). Considering the volatiles mixture obtained from tomato materials by 3-methods in this study, β- phellandrene was most abundant, followed by β-caryophyllene, and these results agree with previous findings (8, 48).

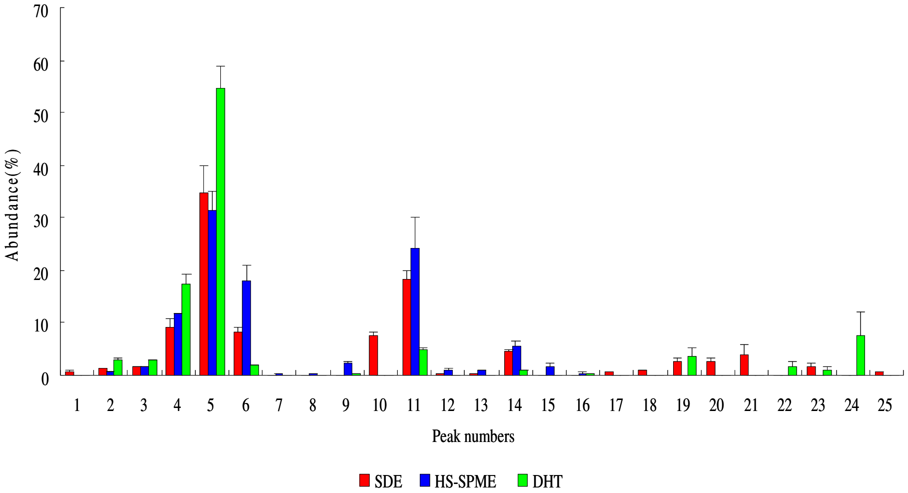

Figure 2. Volatile components and their normalized peak area (%) of *Solanum lycopersicum* plants collected by three extraction methods.

Peak Numbers: 1: (Z)-3-Hexenal, 2: α-Pinene, 3: Benzene, 1-methyl-3-(1-methylethyl), 4: (+) -2-Carene, 5: β-Phellandrene, 6: δ-Elemene, 7: α- Cubebene, 8: Camphene, 9: β-Elemene, 10: Eugenol, 11: β-Caryophyllene, 12: γ-Elemene, 13: α-Muurolene, 14: α-Humulene, 15: Butylated hydroxytoluene, 16: Caryophyllene oxide, 17: 1-Hexadecyne, 18: 6,10,14-Trimethyl-2-pentadecanone, 19: 1-2-Benzenedicarboxylic acid, 20: Tetradecanoic acid, 21: Phytol, 22: n- Triacontane, 23: n- Triacontane, 24: n-Hentriacontane, 25: Bis (2-ehtylhexyl) phthalate

Comparison of 3-Volatiles collection methods

As expected, the volatile profile of a tomato plant was obtained, but it varied with the extraction method. We observed both qualitative and quantitative differences in the chromatographic profiles identified after extraction/ collection with SDE, DHT and HS-SPME methods (Fig 1 and 2). Using SDE, DHT and HS-SPME 19, 16 and 15 volatile components were obtained respectively at concentration above 0.1% (Table 1 and Fig 1). SDE detected relatively wide spectrum of chemical compounds than other methods (11). It allowed better evaluation of high molecular weight compounds with low volatility (such as ketones, acids and esters) better than SPME (9,21) and DHT (41). In this study, volatile compounds in families of alcohols, aldehydes, ketones, and esters were only detected in SDE extract (Fig 2). SDE extracted 2 acids and 1 phenol in higher relative amount i.e. 5.44% and 7.60%, respectively than both DHT (3.75% and not detected, respectively) and HS-SPME (not detected and 1.66%, respectively). HS-SPME proved more efficient in collecting the highly volatile compounds, (such as mono-and sesquiterpenic terpenes) than DHT and SDE. While DHT was more efficient in obtaining hydrocarbons and aromatics than the other two methods. In this study, HS-SPME proved more efficient in collecting terpenes and attained more mono- and sesquiterpenic compounds (11 components) and a higher percentage (96.40%) of these compounds than did both SDE (8, 77.18%) and DHT (9, 82.97%). Finally, DHT gained a richer hydrocarbons (10%) and aromatics (2.92%), than both SDE (2.24% and 1.57%, respectively) and HS-SPME (not detected and 1.53%, respectively). A similar trend had been found in previous studies for analysis of volatile secondary metabolites obtained by SDE, DHT and HS-SPME from *Lippia alba* (41).

Regarding terpenes emitted from *S. lycopersicum* leaves, (+) -2-carene, β-phellandrene, δ- elemene and β- caryophyllene were the predominant volatiles extracted by the three methods (Fig 2). However, percentages of the main families of terpenes differed significantly between the methods (Table 1 and Fig 2). The relative amount of the most volatile compounds, monoterpenes, in the blends accounted for 74.68%, 45.09% and 43.92% for the DHT, SDE and HS-SPME methods respectively, whereas the sesquiterpenes accounted for 8.29%, 32.09%, and 52.48% for the DHT, SDE and HS-SPME methods respectively (Table 1). β- Phellandrene was the richest odour component in all the volatile fractions collected by SDE (34.64 ± 5.35%), HS-SPME (31.43 ± 3.48%) and DHT (54.56 ± 4.18%). Camphene (0.20 ± 0.02%) was detected only by HS-SPME. In particular, most monoterpenes [α-pinene, (+) -2-carene and β-phellandrene] showed higher percentages in DHT extract and most sesquiterpenic compounds [δ-elemene, α-cubebene, β-elemene, β-caryophyllene, γ-elemene, α-muurolene, and α-humulene] showed higher contents in the HS-SPME extract (Fig 2).

The composition of volatile extracts collected from detached or damaged plants can differ significantly from those emitted by live, undamaged specimen (5,46). The C_6 green leaf volatiles, which play an important role in protecting plants against mechanical wounding, herbivore damage, and pathogen attack, are produced by the lipoxygenase pathway and released only from damaged leaves (19,33,39,54). This was also the case in this study. A relatively low concentration (0.73 ± 0.33%) of (Z)- 3-Hexenal, which had previously been identified by Buttery et al. (1987), was only detected by SDE (Fig 1 and 2) among the three methods used. (Z)- 3-Hexenal was induced by the mechanical damage of the plant material during SDE process, and no green leaf volatiles were detected by DHT and HS-SPME, apparently due to the non-destructive nature of these two methods. The C_6 aldehydes, (E)-2-hexanal, (Z)- 3-hexenal and (Z)- 3- hexenol detected in previous studies were likely induced by damage to the tomato leaves (8,48).

Table 1. Summary of volatile compounds, their relative abundance in different groups and the number of compounds in each group as extracted by SDE, HS-SPME and DHT from *Solanum lycopersicum* plants

	Chemical Groups	Total areas [a] (%)			Number of compounds [b]			All [c]
		SDE	HS-SPME	DHT	SDE	HS-SPME	DHT	
1	Alcohols	3.85	– [d]	–	1	–	–	1
2	Aldehydes	0.73	–	–	1	–	–	1
3	Ketones	0.85	–	–	1	–	–	1
4	Acids	5.44	–	3.75	2	–	1	2
5	Esters	0.58	–	–	1	–	–	1
6	Phenols	7.60	1.66	–	1	1	–	2
7	Hydrocarbons	2.24	-	10.0	2	–	3	4
8	Aromatics	1.57	1.53	2.92	1	1	1	1
9	Monoterpenes	45.09	43.92	74.68	3	4	3	4
10	Sesquiterpenes	32.09	52.48	8.29	5	7	6	7
11	Oxygenated terpenoids	–	0.42	0.36	–	1	1	1
	Total				18	14	15	25

[a] : the total percentage of areas of each group based on the data in Figure 2.

[b] : the number of compounds in each group detected by different methods.

[c] : the total number of compounds detected by the three methods.

[d] : the groups or compounds not detected.

Released volatile organic compounds are produced in different plant tissues (leaves, flowers and stems) and their chemical composition varies qualitatively and quantitatively among plant tissues (14,34,36,40). In the present study, detached leaves of tomato plant were used in SDE and intact tomato plants were used in DHT and HS-SPME for volatile collection, and some volatile compounds, such as α- cubebene, β-elemene and caryophyllene oxide, were only detected from the volatiles emitted from the undamaged plants (Fig 1 and 2). We speculated that the differences were caused in part by the different plant materials used in different techniques.

Some volatile compounds of tomato plants play important role in defence against herbivores. For instance, some sesquiterpens and methyl ketones (including zingiberene, curcumene and 2-tridecanone), the volatiles of wild tomato accessions provide resistance against herbivores by many mechanisms including toxicity and repellency (3,4,10,13,17,51). β-Caryophyllene (a major volatile compound from tomato leaves) is repellent against two-spotted spider mite, *Tetranychus urtica*e Koch (4) and it is also highly toxic to *Spodoptera exigua* neonate larvae (17,29,30). We found 11 mono- and sesquiterpenes (such as β- phellandrene, δ- elemene, β-caryophyllene and α-humulene) emitted from *S. lycopersicum* cv. Hezuo 903, among which β-caryophyllene was repellent to *Plutella xylostella* adults and α-humulene was repellent against *Diaphania indica* adults (J. P. Shu, unpublished data). The biological roles of other terpenes deserve further investigations.

CONCLUSIONS

Although the 3-methods of collection (SDE, DHT and HS-SPME) extracted similar major components of tomato plant volatiles, but their volatile profiles varied. The volatile profiles showed both qualitative and quantitative differences among the 3-techniques. Of the three methods, traditional SDE detected more families of compounds, but it also included some components (such as C_6 green leave volatile) induced by damage during the preparation of plant sample. HS-SPME and DHT collected volatiles from intact plants provide the realistic picture of volatile profiles emitted by live plants. Among the 3-methods, HS-SPME proved most efficient in extracting the terpenoid compounds and DHT most efficient in extracting the hydrocarbons and aromatics. The choice of extraction technique depends on the purposes of study. SDE is convenient in obtaining essential oils for bioassays that require high concentrations of plant volatiles. DHT and HS-SPME are more suitable for analysis of volatiles emitted from undamaged plants (27, 46). In some cases, more than one method may be required for a thorough analysis of VOCs emitted from a live plant.

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