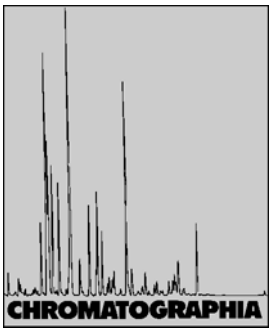
Application of Headspace Solid Phase Microextraction and Gas Chromatography to the Screening of Volatile Compounds from some Brazilian Aromatic Plants



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Key Words

Gas chromatography
Headspace sampling
Solid pahse microextraction
Biogenic organic volatile compounds
Aromatic and medicinal plants

Summary

An HS-SPME method was developed and applied for the isolation of volatile organic compounds from plants native or acclimatized to Brazil. Method optimization was performed using typical analytes from the target samples; fibers coated with 100 μm PDMS and 75 μm Carboxen/PDMS were tested. Using PDMS 100 μm fibers and GC-MS for separation and identification, up to 99.9% of the peak area in the chromatograms from plants were identified. The method was also applied to quantify the major volatile components of one of the samples (*Aloysia gratissima*) with results comparable to those from the conventional steam distillation method.

Introduction

Since its introduction [1] Solid Phase Microextraction (SPME) has been extensively applied for extraction and pre-concentration [2]. Its use for the chemical characterization of aromas and fragrances is now favored over alternative procedures due to its simplicity, speed, low cost and solventless operation [3]. For example, the use of headspace solid phase microextraction (HS-SPME) has been reported in several papers dealing with chemical analysis of food flavors. Using headspace extractions with 65 μm PDMS-DVB (polydimethylsiloxane/divinylbenzene) SPME fibers, Roberts et al. [4] were able to detect and quantify several odorants in prepared coffee, including β-damascenone (0.2 mg · L^{-1}) and 2,3-diethyl-5-methylpyrazine (0.4 mg · L^{-1}). Augusto et al. [5] compared several different SPME fibers for characterization of aromas from tropical fruits; for the target analytes higher extraction efficiencies were obtained with Carboxen – PDMS fibers. Other recent examples of the use of HS-SPME for food aroma analyses include samples such as kiwi [6], cantaloupe [7], wine [8, 9], sugar cane spirits [10] and cheese [11].

Recently, HS-SPME has been proposed for analysis of aroma and aroma-related biogenic volatile organic compounds (BVOC) released from live vegetables or detached parts of plants. Using HS-SPME with 100 μm PDMS fibers and analysis of the extracts with GC-FID and GC-MS, MacTavish et al. [12] determined the BVOC emission profiles from live flowering *Boronia megastigma* plants, identifying compounds such as α-pinene, 5-acetoxylinalool, dodecyl acetate and *Z*-*n*-heptadec-8-ene in the samples. Other HS-SPME plant BVOC studies include species such as Fraser firs (*Abies fraseri*) [13] and eucalyptus (*Eucalyptus citriodora*) [14]. Bicchi et al. [15] performed a systematic study comparing different SPME fibers to extract volatile compounds released by powdered aromatic and medicinal plants. Fibers coated with mixed phases, as Carboxen – PDMS and PDMS-DVB, were found to be the most convenient for such procedures. Nonetheless, 100 μm PDMS fibers still are the most used for in vitro HS-SPME analysis of plant volatiles. Cornu et al. [16] detected and identified more than 60 different BVOC (mainly mono- and sesquiterpenoids), released by sliced aerial parts from French natural grassland plants such as *Meum athamanticu*m, *Pimpinella saxifrag*a, *Achillea millefolium,* and *Thymus pulegioide*s. HS-SPME also has been helpful in studies such as that performed by Cremer and Eichner [17], on the kinetic and thermodynamic aspects of the production of volatile aroma compounds by paprika (*Capsicum annuum*) during its heating.

In this work, a general procedure based on HS-SPME coupled to GC-MS and

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GC-FID for characterization of the volatile fraction from aromatic and medicinal plants is presented. Qualitative and quantitative data resulting from the application of this method to several plants, either natural to or acclimated to Brazil, are presented.

Experimental

Materials

Chemicals

Geraniol, geranyl acetate, *cis*-nerolidol, *trans*-nerolidol, α-pinene, β-pinene and limonene with no less than 98% purity were employed (Sigma, St. Louis, MO and Aldrich, Milwaukee, WI, USA), as well as analytical grade ethanol and sodium chloride (Synth, São Paulo, Brazil) and *n*-alkanes from C_9 to C_{25} (Sigma and Aldrich). All aqueous solutions were prepared using deionized water generated by a Milli-Q system (Millipore, Bedford, MA, USA).

Plant Samples

Leaves from the following aromatic and medicinal plants were used: *Aloysia gratissima* (whitebrush or Brazilian lavender), *Cymbopogun martinii* (palmarosa), *Mentha spicata* (spearmint or Vilhoça mint), *Mentha piperita* var. *citrata* (peppermint), *Ocimum basilicum* (basil), *Ocimum gratissimum* (clove basil), *Origanum vulgare* (wild marjoran) and *Origanum applii* (origanum). Samples were collected from specimens grown at the Experimental Agricultural Fields of the CPQBA – Unicamp, finely ground and carefully stored before use.

Other Materials

SPME fibers coated with 100 μm PDMS (P100) and 75 μm Carboxen – PDMS (CAR) and a manual SPME holder were employed (Supelco, Bellefont, PA, USA). For all extractions, samples were kept in 25 mL vials sealed with Teflon/silicone septa (Pierce, Rockford, IL, USA). Fibers were conditioned prior use according to the instructions provided by the supplier.

Gas Chromatography

For the optimization of the extraction procedure and for quantitative analysis an HP-5890 GC-FID (Hewlett-Packard, Palo Alto, CA, USA), fitted with a 30 m × 0.25 mm × 0.25 μm HP-5 capillary column, was employed. The operating temperatures were: injector 220 °C; detector 250 °C and column oven 2 min at 80 °C → 3 °C · min^{-1} up to 120 °C → 7 °C · min^{-1} up to 200 °C → 7 min at 200 °C. Qualitative analysis was performed in a HP-5890 II CG coupled to a HP-5971 Mass Selective Detector and fitted with a 25 m × 0.20 mm × 0.33 μm HP-5 capillary column. The operating temperatures were: injector 220 °C; detector 280 °C and column oven 60 °C → 3 °C · min^{-1} up to 240 °C → 7 min at 240 °C. Chromatographic grade helium (1.0 mL · min^{-1}) was used as carrier gas for both GC-FID and GC-MS. The desorption time was 3 min for all HS-SPME extractions; no carry-over or memory effects were observed in blank runs performed between extractions.

Methods

Extraction Time and SPME Fiber Selection

The selection of the fiber to be adopted for the further experiments was performed using aqueous solutions containing 1 mg · mL^{-1} of α- and β-pinene, geraniol, limonene, geranyl acetate and *cis*- and *trans*-nerolidol (expected to be significant components of the plant materials) as test samples. Headspace extractions of 15 mL of these solutions were performed using CAR-PDMS and P100 fibers at 55 °C, with sample/headspace equilibration time of 30 min (parameters determined in preliminary experiments) and under magnetic stirring (1200 rpm). The solutions also contained NaCl (36% *m/v*, corresponding to a saturated solution), to increase the extraction efficiency. Experiments with extraction times ranging from 10 min to 60 min were carried out with both fibers under evaluation.

Repeatability and Reproductibility

The reproducibility and repeatability of the HS-SPME procedures were evaluated through extractions of aqueous solutions of geraniol and geranyl acetate. Experiments used test samples containing 1 mg · mL^{-1} geraniol and 0.2 mg · mL^{-1} of geranyl acetate (Solution A) and 0.1 mg · mL^{-1} geraniol and 0.02 mg · mL^{-1} of geranyl acetate (Solution B). During 5 consecutive days, these test samples were extracted 5 times per day using the same P100 fiber; extraction time was 20 min and the other parameters were the same as already described.

Screening of BVOC from Aromatic Plants

To identify the volatile components of the plants, 200.0 mg of powdered leaves were suspended in 14.0 mL aqueous saturated NaCl in a sample vial to which 1.0 mL of 50 μg · mL^{-1} ethanolic solution of the *n*-alkanes C_9 to C_{25} (for the evaluation of the retention indexes of the analytes) was added. The headspace of the suspensions was extracted with P100 fibers. The extracted materials were immediately desorbed and analysed by GC-MS. Identification of the detected analytes was accomplished by matching their mass spectra with the available databases (NIST-98 and Wiley-198), and confirmed by comparing their retention indexes with literature data [18] and by co-injection of the pure compounds, when available.

Quantitation of Selected BVOC

The two major analytes in *C. martinii* – geraniol and geranyl acetate – were quantified using HS-SPME. Extraction was conducted using the same procedure already described. The corresponding calibration curves were generated by extracting aqueous standard solutions of geraniol and geranyl acetate over a range of concentrations. The results were compared to the analysis of the same sample using conventional steam distillation: 40.0 g of pulverized sample was suspended in 700 mL deionized water and the essential oil separated after distillation for 3 h in a Clevenger apparatus. The amounts of geraniol and geranyl acetate present in the essential oil were estimated by direct injection of the essential oil, using *n*-nonane as internal standard.

Results and Discussion

Selection of Fiber and Extraction Time

Figure 1 shows the extraction time profiles. To simplify the examination of these profiles, peak areas for each analyte, extraction time and fiber were normalized according to Eq. (1):

$$N = 100 \times \frac{A_{X,t}}{A_{X,10}} \quad (1)$$

where N is the normalized area, $A_{X,t}$ and $A_{X,10}$ are respectively the peak areas ob-

tained for the analyte with extraction times of t min and of 10 min (used as reference).

For the CAR fiber, the areas for geranyl acetate and *cis*- and *trans*-nerolidol are maximized after 20 min extraction. However, there is a significant decrease in areas of the other analytes for extraction times greater than 10 min: *e.g.*, for 20 min extraction the limonene peak area is only 63% of the value observed after 10 min extraction. Thus, considering the analytes tested here, it is not possible to define a single optimum extraction time for this fiber. For the P100 fiber, areas for geraniol, geranyl acetate, limonene, α- and β-pinene are maximized with 20 min extraction. For *cis*- and *trans*-nerolidol, there is a sharp increase on the extracted efficiency up to 20 min; further increments in the extraction time lead to less pronounced area increases.

Inspection of Figure 2 allows comparison of the extraction efficiency of the P100 and CAR fibers. It can be seen that:

- For limonene and α- and β-pinene the differences between the amounts extracted with P100 fibers is higher: the average ratios between the P100 and CAR peak areas for each of these analytes are respectively (15 ± 4), (36 ± 4) and (65 ± 7). These compounds are isomeric monoterpenes ($C_{10}H_{16}$), and can be assumed to be highly apolar; therefore, their affinity for the non-polar coating of the P100 fiber is also expected to be high.
- For geraniol (a monoterpenol, $C_{10}H_{18}O$), its derivative geranyl acetate and for the isomeric sesquiterpenols *cis*- and *trans*-nerolidol ($C_{15}H_{26}O$), the difference between the extraction efficiencies is smaller, although still favorable towards P100. Compared to the monoterpenes above, these compounds are expected to be more polar due to the presence of hydroxyl and carbonyl groups in their structures. The smaller differences between P100 and CAR fibers for isolation of these more polar analytes was expected, since in the CAR fiber the process responsible for the extraction also is, at least in part, adsorption.

Due to its higher extraction capacity towards the tested analytes (which were selected to represent the samples that are expected to be routinely analysed by this methodology), 20 min extraction with P100 fiber was adopted for the further experiments.

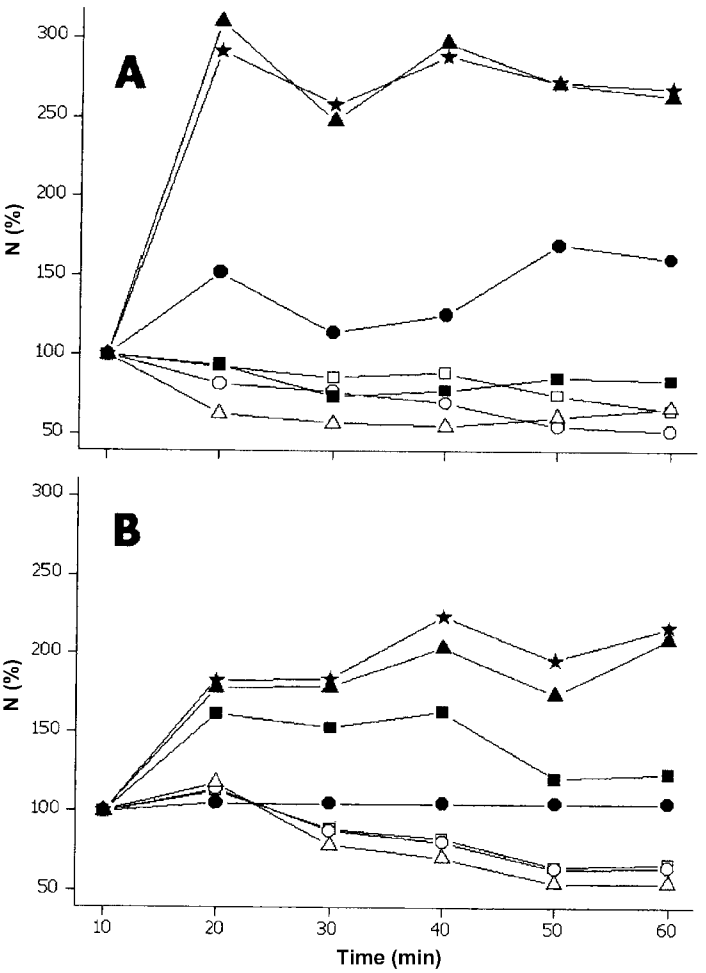

Figure 1. HS-SPME extraction profiles for CAR (**A**) and P100 (**B**) fibers. Analytes: □ = α-pinene; ○ = β-pinene; △ = limonene; ■ = geraniol; ● = geranyl acetate; ▲ = *cis*-nerolidol and ★ = *trans*-nerolidol.

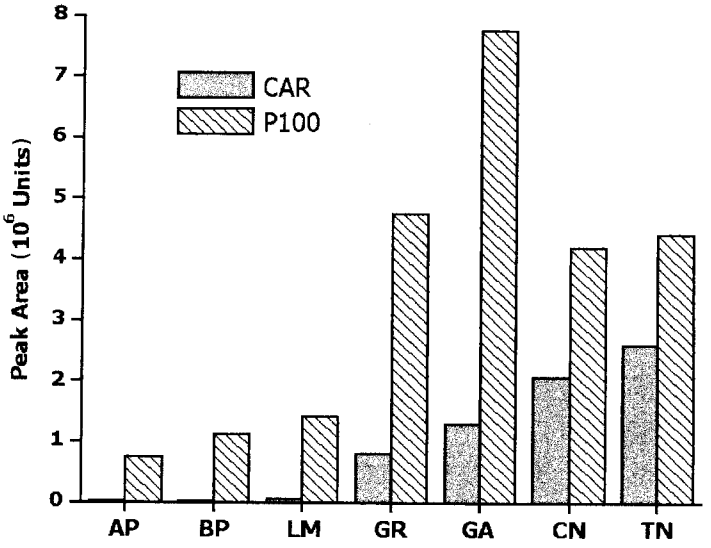

Figure 2. Peak areas with 20 min extraction for α-pinene (**AP**), β-pinene (**BP**), limonene (**LM**), geraniol (**GR**), geranyl acetate (**GA**), *cis*-nerolidol (**CN**) and *trans*-nerolidol (**TN**).

The discussions above point to an important issue related to the application of SPME to quantitative studies of complex samples such as the blends of plant volatile compounds studied in this work. The analytes present on these samples normally have a broad range of physical and chemical properties, such as volatility, po-

Table I. Estimates of relative standard deviation, s_R (%), obtained for geraniol and geranyl acetate peak areas.

	Analyte	s_R %[a]				
		Day #1	Day #2	Day #3	Day #4	Day #5
Solution A[b]	geraniol	8.3	8.6	9.5	8.4	7.4
	geranyl acetate	9.2	4.5	9.8	7.0	8.6
Solution B[c]	geraniol	7.4	8.9	6.8	9.7	9.2
	geranyl acetate	6.0	8.5	4.7	7.0	7.9

[a] Five replicate extractions per day. [b] 1 mg · mL^{-1} geraniol and 0.2 mg · mL^{-1} of geranyl acetate; [c] 0.1 mg · mL^{-1} geraniol and 0.02 mg · mL^{-1} of geranyl acetate.

Table II. ANOVA analysis of the estimates of relative standard deviation from Table I.

Source of Variation ↓	*SS*	*v*	*MS*	*F*	F^{95}
Analyte & Concentration	8.82	3	2.94	1.04	3.49
Day	1.14	4	0.286	0.101	3.26
Error	34.0	12	2.83		
Total	43.9	19			

SS = sum of squares; *v* = degrees of freedom; *MS* = mean square; *F* = F-test parameter and F^{95} = 95 % significance critical value for the *F*-test parameter.

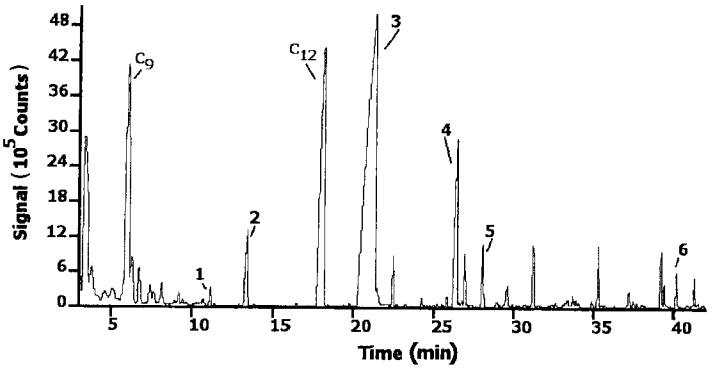

Figure 3. Section of a typical HS-SPME-GC-MS chromatogram for *C. martinii* (palmarosa). Peaks: **1** = β-ocimene; **2** = linalool; **3** = geraniol; **4** = geranyl acetate; **5** = *trans*-caryophillene and **6** = *cis*-farnesol; $\mathbf{C_9}$ and $\mathbf{C_{12}}$ = *n*-alkanes (RI markers).

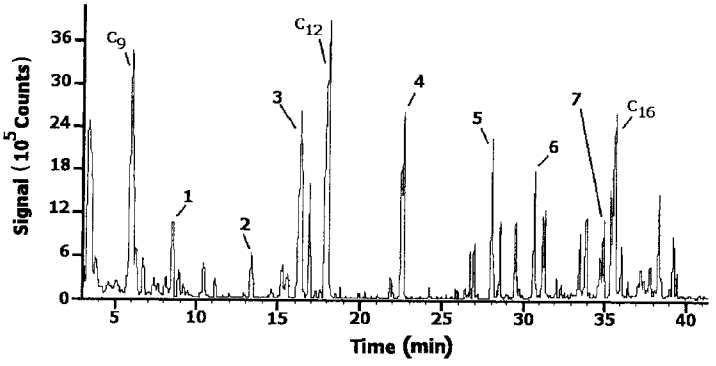

Figure 4. Section of a typical HS-SPME-GC-MS chromatogram for *A. gratissima* (Brazilian lavender). Peaks: **1** = β-pinene; **2** = linalool; **3** = *trans*-pinocamphene; **4** = *trans*-pinocarveyl acetate; **5** = *trans*-caryophillene; **6** = germacrene D and **7** = germacrene B; $\mathbf{C_9}$, $\mathbf{C_{12}}$ and $\mathbf{C_{12}}$ = *n*-alkanes (RI markers).

larity, etc. In consequence, for these samples it is almost impossible to have a unique set of experimental conditions (sample/headspace and headspace/fiber equilibration times, fiber coating, etc) where parameters such as extraction efficiency and analytical sensitivity are simultaneously optimized for all possible target analytes. The same considerations are also valid for some other similar equilibrium-based extraction techniques such as SPE and micro-LLE.

Analysis of Reproductibility and Repeatability

Table I lists the results of the systematic evaluation of reproductibility and repeatability. For each day, 5 replicate extractions were performed with test solutions containing two different levels of the analytes, and the relative estimates of standard deviations for each analyte, concentration and daily set of replicates was calculated. Values for s_R ranged from 4.5% to 9.8%, which suggests that the precision of the methodology is adequate for quantitative applications. Additionally, these data was submitted to univariate ANOVA analysis [19] (Table II), to find out if there was dependence of the precision on the day of sampling, analyte nature and level. Both estimated *F*-test parameters – representing variations with s_R associated to analyte nature and level (1.04) and representing variations on s_R associated to the day of sampling (0.101) – were inferior to the corresponding 95% significance critical values (3.49 and 3.26, respectively). Therefore, it can be assumed that the precision of the methodology is independent of the nature of the analyte or its level, for the tested substances and concentrations. Also, there was no significant change in the precision during the time span of the experiment. Since the same P100 fiber was employed for all the assays (being applied to *ca.* 50 headspace extractions during the period, including those used to obtain these data), it can be concluded that no degradation or alteration on the fiber coating that could affect the precision of the experiments occurred.

Screening and Quantitation of BVOC from Aromatic Plants

The BVOC found in the various plant samples are listed in Table III. Figures 3 and 4 show HS-SPME-GC-MS chromatograms obtained for *C. martinii* and *A. gratissima*. As can be seen in Table III, it was possible to identify most of detected analytes: for six out of eight of the evaluated species, the total area of the identified peaks was higher than 90% of the total area; for the other two plants, the sum of the identified peaks was 77.2% (*A. gratissima*) and 86.7% (*C. martinii*).

The production of the organic compounds by plants is highly dependent on environmental and seasonal factors [20]: completely different blends of volatile substances can be produced by plants of the same species [21]. Other possible

Table III. BVOC identified after HS-SPME-GC-MS analysis of aromatic plants. AG = *A. gratissima*; CM = *C. martinii*; MS = *M. spicata*; MP = *M. piperita*; OB = *O. basilicum*; OG = *O. gratissimum*; OV = *O. vulgare* and OA = *O. applii.*

Analyte	*RI*[a]	Sample[b]							
		AG	CM	MP	MS	OV	OA	OB	OG
α-pinene	934	0.18							
1-octen-3-ol	978					0.68			
β-pinene	979	3.65							
β-mircene	991	1.07							
3-octanol	998			10.1					
p-cimene	1024					1.50			
limonene	1028	1.80							
1,8-cineol	1031							1.05	
trans-β-ocimene	1035					0.39			
cis-β-ocimene	1043	0.67	0.73						
γ-terpinene	1057					1.99			
isopentyl *n*-butirate	1068			0.67					
α-terpinolene	1083	0.18				0.31			
fenchone	1088							0.53	
linalool	1093	2.15	4.10	51.0			4.92	32.6	
cis-*p*-menth-2-en-1-ol	1122			0.52		1.47			
trans-pinocarveol	1136	1.77							
trans-*p*-menth-2-en-1-ol	1140					0.86			
camphor	1150							10.1	
trans-pinocamphone	1161	10.86							
borneol	1169					2.52			
cis-pinocamphone	1173	4.02							
terpin-4-ol	1176			8.00		33.3	3.08	0.99	0.26
cimen-8-ol	1182	0.23					0.64		
α-terpineol	1188	0.27		1.31		4.25		3.90	
trans-piperitol	1208					0.16			
trans-carveol	1216	0.22							
thimol methyl ether	1231					0.24	1.47		
carvacrol methyl ether	1241					1.33	5.91		
carvone	1241	0.12		23.42					
trans-geraniol	1262		68.2						
bornyl acetate	1282	0.51				0.20			
thimol	1294					38.0	64.5		
trans-pinocarveyl acetate	1303	9.27							
eugenol	1358							28.1	93.9
piperitone oxide	1373				94.8				
α-copaene	1378	0.15							
geranyl acetate	1379		10.3			0.33			
β-bourbonene	1385	0.31		0.18			1.35	0.23	
β-elemene	1393	1.19						1.92	
methyleugenol	1405	0.19							
trans-caryophillene	1422	5.95	2.31	2.31		2.66		2.00	1.08
β-gurjunene	1430						0.36		
β-bergamotene	1435							1.66	
γ-elemene	1437	2.18							
α-guaiene	1438							0.29	
β-farnesene	1454				0.76				
α-humulene	1455	2.65						0.77	
allo-aromadendrene	1461						0.28		
germacrene D	1482	4.56		0.44		1.47	4.79	5.49	4.23
γ-muurolene	1484				1.06				
δ-guaiene	1506							0.68	
β-bisabolene	1508					1.05	1.98		
γ-cadinene	1516						0.89	1.27	
δ-cadinene	1524	0.40						0.29	0.19
elemol	1555	2.31							
germacrene B	1564	2.95							
espatulenol	1580	1.63				1.44	1.62		
caryophillene oxide	1586	2.49				1.07			
unknown, MW = 222	1607	9.19							
epi-α-muurolol	1643						0.72	5.81	
α-eudesmol	1655							0.24	
α-cadinol	1656					0.29	1.53	0.38	0.16
unknown, MW = 222	1678	4.12							
cis-farnesol	1724		1.10						
SUM OF IDENTIFIED PEAKS		77.2	86.7	98.0	96.6	95.5	94.1	98.3	99.9

[a] *RI* = retention index. [b]Results expressed as % Area.

Table IV. Concentrations (% m/m) of the major BVOC found in *C. martinii* after HS-SPME or steam distillation (SteDist) analyses.

Analyte	SteDist	HS-SPME
geraniol	0.65 ± 0.05	0.75 ± 0.06
geranyl acetate	0.06 ± 0.01	0.05 ± 0.01

causes for variation of the composition of the volatiles produced by plants can be the existence of different chemotypes within the same species. For example, the literature [22, 23] mentions several chemotypes for *O. vulgare*, regarding the different composition of the volatile fraction of the leaves. However, in the samples of *O. vulgare* inspected here, the main compounds were thymol (38.0% peak area) and terpin-4-ol (33.3%) – even after an extensive search, no description of chemotypes with this particular composition was found in the literature for this species.

Table IV compares the concentrations on the major components of the *C. martinii* volatile fraction, geraniol and geranyl acetate, found by HS-SPME and steam distillation. A simple *t*-test analysis of these data shows that the amounts found using both techniques are statistically equivalent. However, the HS-SPME method uses only 0.5% of the sample quantity needed for steam distillation and is considerably faster than this technique (the total extraction time in the present work is 50 min, which compares favorably to the 3 h needed for hydrodistillation).

Conclusions

SPME has proved to be a useful tool for routine qualitative analysis of plant BVOC examined in this work, allowing the identification of the main constituents of these samples. PDMS was found to be the most suitable fiber coating for the examined samples, providing higher recoveries than Carboxen-based fibers. When compared to the standard hydrodistillation procedure, HS-SPME provided comparable quantitative results. The small amounts of plant material needed for the HS-SPME procedure is an important advantage, minimizing damage and stress to the plant specimens caused by the sample collection – which is especially important for rare specimens and in studies monitoring the conditions of the same individual over a long period of time. This methodology is starting to be applied as a routine procedure in several aromatic and medicinal plant studies being conducted at the CPQBA-Unicamp.

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