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Solid-Phase Microextraction of Volatile Compounds from the Chopped Leaves of Three Species of *Eucalyptus*

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Headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography and ion-trap mass spectrometry has been used to identify biogenic volatile organic compounds present in the headspace of chopped leaves of *Eucalyptus* (*E.*) *dunnii*, *E. citriodora*, and *E. saligna*. A simple HS-SPME method entailing 30 min of extraction at 30 °C was developed for this purpose. Thirty compounds were identified in the headspace of 60 juvenile chopped *Eucalyptus* leaves, and another 30 were tentatively identified. The presence of compounds such as (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMNT), (*E*,*E*)-α-farnesene, (*E*,*E*,*E*)-3,7,11,15-tetramethyl-1,3,6,10,14-hexadecapentaene (TMHP), β-caryophyllene, α-humulene, germacrene D, and β-cubebene in the headspace of the leaves but not in the essential oils from the same *Eucalyptus* trees and information about the infochemical roles of some of these compounds in other living plant systems suggest they might play a bioactive role in *Eucalyptus* leaves.

KEYWORDS: Solid-phase microextraction (SPME); *Eucalyptus dunnii*; *Eucalyptus citriodora*; *Eucalyptus saligna*; biogenic volatile organic compounds (BVOC)

INTRODUCTION

Headspace solid-phase microextraction (HS-SPME) is an excellent technique for the extraction of volatile compounds from dried or living (fresh) plants (*1*, *2*). Especially for dried plants HS-SPME has been successfully used as a technique for screening complex volatile mixtures and for complementing information obtained from other methods of extraction, especially because of its simplicity and reliability (*2*, *3*). Some of the techniques conventionally used to extract volatile compounds from plants are dynamic and static headspace, supercritical fluid extraction, several variations of distillation, and solvent extraction. The first two of these require the use of additional devices, distillation is usually performed at high temperatures, and distillation and solvent extraction are time-consuming processes.

Plant biogenic volatile organic compounds (BVOC) are involved in multiple interorganism interactions and are also important in the flavor and fragrance, pesticide, and perfumery industries (*4*−*7*). As far as the authors are aware only two publications have been devoted to the BVOC present in the fresh parts of *Eucalyptus*: volatile compounds from the cut stem tips of six species of *Eucalyptus* (different from the species investigated in this work) and from the chopped leaves of three other *Eucalyptus* species (including *E. citriodora*) (*8*, *9*). Investigation of the BVOC present in the fresh chopped leaves of *E. dunnii* and *E. saligna* and more data on *E. citriodora* are still lacking.

Investigation of BVOC can be conducted by using the macerated or cut parts of a plant or can be performed in situ. The volatile fraction obtained by use of each method might be different and furnish complementary information about the plant (*10*). Results from previous investigations have shown in situ HS-SPME to be a very useful analytical tool for the study of the circadian profiles of the BVOC from three species of living *Eucalyptus* plants (*1*, *11*). This work is a continuation of the study of living *Eucalyptus* BVOC, but this time using chopped leaves. Taking advantage of the differences between the two techniques used (distillation is an exhaustive technique that employs high temperatures, whereas HS-SPME is an equilibrium process that enables study of fresh chopped leaves at mild temperatures), our aim was to investigate qualitative differences between the composition of the headspace of *Eucalyptus* leaves and that of the volatile oils from the same trees. Data obtained in this study of fresh chopped leaves and results previously obtained from leaves in situ are discussed in terms of their implications in plant biology. Changes in the chromatographic profile of chopped *E. dunnii* leaves have, moreover, been followed by PDMS extraction some minutes and 24 h after cutting.

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EXPERIMENTAL PROCEDURES

Plants. Juvenile leaves from one tree of *E. citriodora* and *E. saligna* and two trees, A and B, of *E. dunnii* (21−22 months old) were sampled during July and August 2000 and submitted to HS-SPME. Fifteen leaves from *E. saligna*, 12 from *E. dunnii* A, 12 from *E. dunnii* B, and 21 from *E. citriodora* were submitted to HS-SPME. Although most of the leaves tested were totally expanded (leaf that reached the maximum size genetically defined), two to five leaves of each set of leaves were only partially expanded. Leaves were collected from different positions on the tree, ensuring variable and comprehensive sampling.

Leaves used for hydrodistillation were randomly sampled from the canopy of the tree and included both totally and partially expanded leaves. Whereas leaves used for HS-SPME and hydrodistillation were taken from the same *E. dunnii* and *E. saligna* trees, leaves from five *E. citriodora* trees were employed for hydrodistillation (leaves from one of these five trees were used for HS-SPME). All of the trees came from the Barba Negra tree farm (Guaíba, RS, Brazil) when they were 6 months old and were cultivated in the greenhouse of the University of Waterloo.

SPME Materials. The SPME holder and fibers coated with 100, 30, and 7 μm poly(dimethylsiloxane) (PDMS), 85 μm polyacrylate (PA), and 65 μm PDMS/divinylbenzene (DVB) were supplied by Supelco (Oakville, ON, Canada). Before use, fibers were conditioned according to the supplier's instructions.

Gas Chromatography—Ion-Trap Mass Spectrometry (GC-ITMS). Chromatographic analysis was performed with a Saturn 4D GC-ITMS system (Varian Associates, Sunnyvale, CA) fitted with a 30 m × 0.25 mm × 0.25 μm HP-5MS column (Hewlett-Packard, Avondale, PA) and a septum-equipped programmable injector (SPI). TAG-grade helium (Praxair, Kitchener, ON, Canada) was used as carrier gas. Mass spectra recorded by the ITMS system were used to produce a total ion chromatogram (TIC). For chromatography of *Eucalyptus* leaf essential oils and headspace from the chopped leaves, the column oven was programmed from 60 to 280 °C at 5 min^{-1}. The only exception to this was for chromatography of the green leaf volatiles (GLV) after the preliminary experiments (see Experimental Procedure) when the oven temperature was maintained at 50 °C for 1 min, then programmed at 1 °C min^{-1} to 60 °C, and finally programmed at 30 °C min^{-1} to 280 °C; the first ramp was slower to make it easier to observe the GLV peaks. The temperature of the SPI was 250 °C, the carrier gas was helium at 15 psig (1.5 mL min^{-1}), the ion-trap and transfer line temperatures were 150 and 240 °C, respectively, and the electron multiplier potential was from 1710 to 1860 V (optimized daily). The PDMS fibers were left in the injector for 30 min. Compounds were identified by comparison of their retention data obtained using the HP-5MS column with the ones of standard compounds and by comparison of their mass spectra with those in a laboratory-produced mass spectral database previously collected from chromatographic runs of pure compounds (Sigma, St. Louis, MO; Aldrich, Milwaukee, WI; and Fluka, Buchs, Switzerland) performed with the same equipment and under the same conditions (compounds labeled "b" in **Table 1**). Whenever necessary, a 30 m × 0.25 mm × 0.50 μm HP-Innowax column or a 60 m × 0.25 mm × 0.25 μm HP-1MS column (Agilent) was employed to further confirm the identity of compounds. Three compounds, octane, citronellyl acetate, and *n*-eicosane, were used as chromatographic markers (*12*). When pure compounds were not available, comparison with retention data and linear temperature programmed retention indices (LTPRI) reported in the literature was also used for tentative identification of some of the compounds (*12*−*19*). The LTPRI of some of the volatile components of *Eucalyptus* leaves headspace were determined by use of a GC 3400C_x/FID (Varian Associates) equipped with an HP-5MS column, under experimental conditions described above. The FID temperature was 250 °C (compounds labeled "c" in **Table 1**). Finally, compounds labeled "a" in **Table 1** were the ones tentatively identified using *Eucalyptus* volatile mass spectra compared to NIST 98, Varian MS library, and literature data (*12*).

Experimental Procedure. The performance of the several coatings (100, 30, and 7 μm PDMS, 85 μm PA, and 65 μm PDMS/DVB) was tested during preliminary experiments on 0.05 g of chopped leaves in 4 mL amber glass vials and with extraction for 1 min at 24 °C. Experiments with and without addition of buffer solution (0.025 M Na_2HPO_4−KH_2PO_4) and stirring at 300−1000 rpm (10 mL clear glass vials) were performed under the same conditions. The same applied to experiments used to monitor GLV, which were performed in quadruplicate. Preliminary experiments were performed manually, and a temperature-controlled block (Multiblock Labline Instruments) was employed during experiments. Leaves from *E. dunnii* were used for these experiments because this species is rich in monoterpenes and sesquiterpenes.

In the general procedure *Eucalyptus* leaves were cut with stainless steel scissors, and 0.05 g of the pieces (~5 mm^2) was placed inside 10 mL clear glass vials. Buffer solution (pH 7, 2 mL) was added to each vial, which was then immediately capped and placed on a temperature-controlled tray (30 °C) for a minimum of 24 h. Extraction of headspace BVOC was performed automatically (Combipal autosampler, CTC Analytics, Basel, Switzerland) for 30 min, using the 7 μm PDMS fiber.

Extraction time profiles were investigated for 7 and 30 μm PDMS at 30 °C and for 30 μm PDMS at 45 °C, with stirring at 300 rpm, for extraction times of 1, 5, 15, 30, 45, and 90 min. Two totally expanded leaves of *E. dunnii* A were chopped and mixed to homogeneity, and the pieces were distributed among six 10 mL clear glass vials. Experiments were repeated in triplicate for each extraction time and for each set of experimental conditions.

Results obtained by HS-SPME with 7 and 30 μm PDMS were compared for five leaves of *E. citriodora* and for the same number of leaves of *E. dunnii* A and B. Each leaf was submitted to the general procedure described above, placed in two different vials, and submitted to headspace extraction with the 7 and 30 μm PDMS fibers in the different vials.

Repeatability of the area counts of BVOC peaks obtained by HS-SPME of chopped leaves was assessed by use of two fully expanded *E. dunnii* leaves. These leaves were submitted to the general procedure already described and placed in five vials. After extraction and analysis, the relative standard deviation of the area counts obtained for 80% of the BVOC peaks was <20%.

Extraction of the headspace of chopped leaves was performed twice or three times with each leaf (totally expanded leaf) unless there was insufficient green tissue (partially expanded leaf).

Hydrodistillation was performed in a modified Clevenger apparatus for 5 h with ~300 g of fresh leaves and 1 L of deionized water. The refrigeration system was maintained between −4 and 4 °C by use of a mixture of water and ethylene glycol, to avoid losses of volatile compounds. The hydrodistilled oil was dried over sodium sulfate.

Although quantitative analysis of the BVOC detected was not intended in this work, relative amounts of the same compounds were compared in different experiments. For this reason detector response (GC-ITMS) was investigated in the working range of 5−500 ng and proved to be linear for the compounds 2-carene, 2,5-dimethylstyrene, 1,8-cineole, myrtenal, linalyl acetate, β-caryophyllene, and caryophyllene oxide. These compounds were chosen because they are representative of the terpenoid classes hydrocarbon monoterpenes, oxygenated monoterpenes, hydrocarbon sesquiterpenes, and oxygenated sesquiterpenes, classes that represent some of the most common compounds among the BVOC present in *Eucalyptus* oils (*5*).

RESULTS AND DISCUSSION

Method Development. The SPME coatings 7 μm PDMS, 65 μm PDMS/DVB, and 85 μm PA were tested for extraction of *E. dunnii* leaves during 1 min at 24 °C. Although the same numbers of compounds were extracted by use of the three coatings, the best results were obtained with 7 μm PDMS. A larger amount of low molecular weight compounds was extracted by PDMS/DVB, but the chromatographic column was overloaded by the main compounds of the volatile mixture, resulting in poor resolution of some peaks. The high performance of PDMS/DVB in the extraction of low molecular weight compounds has previously been reported, as have competition among analytes and displacement effects (*20*). Peak areas for compounds with molecular masses of ~204 g mol^{-1} (sesquiterpene hydrocarbons) were, however, larger when 7 μm PDMS

Table 1. Some Compounds Detected in the Headspace of the Chopped Leaves or in the Hydrodistilled Oils from *E. citriodora*, *E. dunnii*, and *E. saligna* and Their Relative Abundances[a]

		E. citriodora		*E. dunnii* A (B)		*E. saligna*		identification
no.	compound	HD	SPME	HD	SPME	HD	SPME	procedure
1	α-thujene	x	x	x	xi			a
2	α-pinene	x	x	+	++/+	++	++	a
3	camphene			x	x	+	+	a
4	β-pinene	x	x	x	x	x	xi	a
5	β-myrcene	x	x	x	xi			a
6	α-phellandrene			x	xi	x	xi	a
7	α-terpinene						xi	a
8	*p*-cymene	x	x/+	x	++/+ (+)	+	++/+	a
9	limonene	x	xi	+	+	+	+	a
10	1,8-cineole	+	x	++	++	+	+	a
11	(*Z*)-β-ocimene	x	x/+	+	+			a
12	(*E*)-β-ocimene			x	x/+		x/+i	a
13	γ-terpinene	x	x/+	+	++/+ (x)	+	x	a
14	terpinolene	x	x	x	xi (x)	x	xi	a
15	linalool	x	x			x		c/b (*14*, *18*, *19*, *40*)
16	isoamyl isovalerate					x		a
17	*cis*-rose oxide	x	x/+					a
18	*endo*-fenchol			x		x		c/b (*19*, *40*, *41*)
19	DMNT				x/++		xi	c/d
20	campholenal					+		c/b (*17*, *19*, *40*)
21	*trans*-rose oxide	x	x					a
22	*trans*-pinocarveol			x (+)		x		c/b (*18*, *19*, *40*)
23	isopulegol	+	x/+					a
24	citronellal	++	++					a
25	isopinocamphone					x		c/b (*17*)
26	pinocarvone			x		x		c/b (*18*, *19*)
27	borneol					+		c/b (*13*, *17*, *19*, *40*)
28	terpinen-4-ol	x		x	xi	x		a
29	α-terpineol	x		+	x	+		a
30	*trans*-carveol			x		x		c/b (*16*, *18*, *40*)
31	citronellol	++	++					a
32	*cis*-carveol			x		x		c/b (*16*)
33	α-cubebene	x	xi					c/b (*18*, *42*)
34	citronellyl acetate	x	x/+					a
35	α-copaene	x	xi	x	x/+	x	x/+	a
36	β-bourbonene		xi					c/b (*13*, *17*, *18*, *42*)
37	β-elemene		x					c/b (*15*, *18*)
38	β-cubebene				xi (x)			c/b (*13*, *14*, *16*, *41*)
39	longifolene			x	x			c/b (*16*)
40	α-gurjunene		xi	x	x/+ (x)	x	x	c/b (*16*, *40*)
41	β-caryophyllene	+	++		x (x/+)	x	x/+	a
42	calarene			x	x/+			a
43	aromadendrene	x	xi	+	++ (+/++)	x	x/+	a
44	α-guaiene	x	x	x	x			c/b (*15*)
45	α-humulene	x	x/+		xi (x)	x	x	a
46	*allo*-aromadendrene	x	xi	+	+	x	x/+	a
47	γ-muurolene			x	x			c/b (*15*)
48	germacrene D	x	x/+		xi (x/+)			c/b (*13*–*15*, *17*, *18*)
49	valencene			x	x			c/b (*16*, *18*)
50	(*E*,*E*)-α-farnesene		x/+		xi (x/+)		x/+i	c/b (*13*, *14*)
51	γ-cadinene			x	x			c/b (*13*, *17*, *18*)
52	(1*S*)-*cis*-calamenene	x	x	x	x	x	x	c/b (*13*, *15*, *17*–*19*, *42*)
53	δ-cadinene	x	x	x	x	x	x	c/b (*13*, *15*, *17*–*19*, *42*)
54	TMTT				x (x/+)		x/++i	c/b/d (*13*, *16*, *18*, *40*)
55	spathulenol	x		x		x		c/b (*13*, *16*, *18*, *40*)
56	caryophyllene oxide	x	x/+					c/b (*17*, *40*)
57	globulol			+	+	x	x/+	a
58	guaiol			+	+	x	x/+	c/b (*16*)
59	α-eudesmol			x	x	x	xi	c/b (*16*)
60	TMHP				x (x/+)			c/d

[a] Compounds are listed in order of elution from an HP-5MS column. Procedures employed for identification or tentative identification of BVOC and for estimation of the relative abundance of each compound, among other information: a, co-injection with pure compound and comparison with its retention data in one to three GC columns; b, comparison with literature LTPRI; c, comparison with mass spectra from the NIST-98 and Saturn (Varian) libraries and literature retention data (*12*); d, comparison with mass spectra in Dr. M. Posthumus' mass spectra library from the University of Wagenigen; e, ++ = peak area usually 10.0% or more of the total area of all detected compounds; + = peak area usually between 1.0 and 10.0% of the total area of all detected compounds and x = peak area usually <1.0% of the total area of all detected compounds; i, compound detected in the headspace of leaves investigated but its presence is not constant. Empty cells indicate the compound was not detected. Different contributions found in the headspace of *E. dunnii* B are given in parentheses in the *E. dunnii* columns. Compounds were considered to be present in the headspace of a tree if they were detected in at least five leaves from the tree.

was used; this afforded better quality mass spectra. There was no significant qualitative difference between results obtained with PDMS and PA, even though the affinity of the PA coating is higher for the most polar compounds. Bleed peaks were predominant in the second part of the chromatogram obtained after extraction with PA (from 13 min), and the abundance of the target analytes peaks was extremely low. Because of these results, the PDMS coating was chosen for subsequent work.

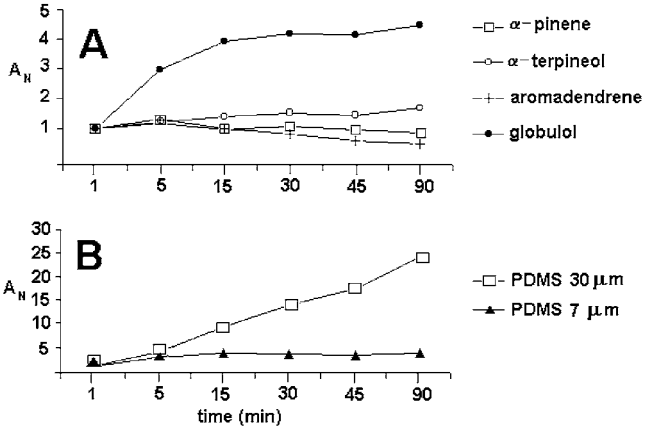

Figure 1. Extraction time profiles at 30 °C for (A) extraction of α-pinene, α-terpineol, aromadendrene, and globulol with PDMS 7 μm and (B) extraction of globulol with PDMS 7 μm and PDMS 30 μm.

The terpenoid compounds usually present in *Eucalyptus* leaves oils are prone to chemical transformation promoted by temperature, light, and pH. For this reason 30 °C was chosen as the extraction temperature to enable extraction of the BVOC present in the natural composition of the headspace of the leaves and to prevent artifact formation (*21*). To choose the thickness of the PDMS coating used in this work, the time needed to achieve equilibrium between the volatile mixture in the headspace and the fiber coating was taken into consideration. The extraction time profile was investigated for 7 μm PDMS after 1, 5, 15, 30, 45, and 90 min; the results are shown in **Figure 1A**. The compounds chosen for this investigation represent some of the major classes of compound present in *Eucalyptus* oils, that is, monoterpene hydrocarbons (α-pinene), oxygenated monoterpenes (α-terpineol), sesquiterpene hydrocarbons (aromadendrene), and oxygenated sesquiterpenes (globulol). Normalized areas shown in **Figure 1** were obtained by use of the equation

$$A_N = A_x/A_{1min} \quad (1)$$

where A_N is the normalized peak area after extraction for x min, A_x is the peak area after extraction for x min, and A_{1min} is the peak area after extraction for 1 min. Equilibrium was achieved after ~5−15 min for α-pinene, α-terpineol, and aromadendrene and after 15−30 min for globulol. When the same experiments were performed with 30 μm PDMS, similar behavior was observed for α-pinene, α-terpineol, and aromadendrene, but globulol took longer (>90 min) to reach equilibrium, as is apparent from **Figure 1B**. Another trial was performed with 30 μm PDMS at 45 °C and stirring at 300 rpm to accelerate the time necessary for globulol to reach equilibrium between the headspace and the coating. Equilibrium was reached after 60−90 min, which is longer than the time needed for the chromatographic run (44 min) and would make the whole analytical process inconveniently long. Finally, at 30 °C there were no significant differences between the chromatographic profiles obtained by use of both PDMS fiber coatings; 7 μm PDMS afforded smaller peak areas but also less disturbance of the equilibrium between the living sample and its headspace. Other reasons for not using the 30 μm coating were the distortion of the shapes of the major peaks in the chromatograms due to the overloading of the chormatographic column and extraction of amounts outside the range of linearity of the ITMS. Bleed peaks from PDMS coatings were also less intense for the 7 μm coating than for the 30 μm coating. The same applies to 100 μm PDMS, for which the same issues were also observed with greater intensity.

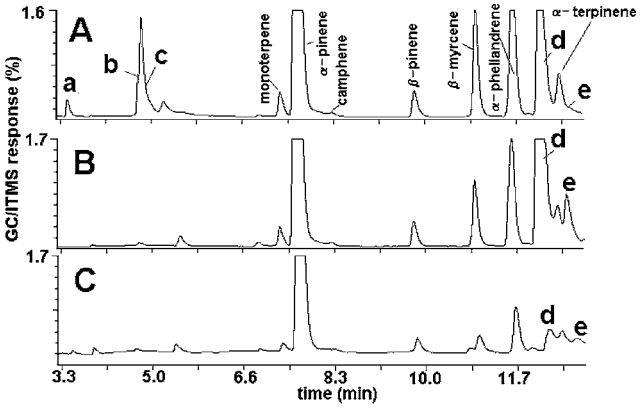

Figure 2. Total ion current chromatograms showing elution of GLV extracted from the headspace of chopped *E. dunnii* leaves (A) 28 min, (B) 3.3 h, and (C) 24 h after chopping of the leaves: (a, b) C6-GLV; (c) 3-(*Z*)-hexen-1-ol; (d) 3-(*Z*)-hexenol acetate; (e) hexyl acetate.

For these reasons, and because use of 7 μm PDMS would result in a shorter equilibrium time for globulol (30 min) and enable use of a lower extraction temperature and because the extraction process would take less time than the chromatographic run (44 min), 7 μm PDMS was considered to be an advantageous choice over other PDMS coating thicknesses for this work.

Green Leaf Volatiles (GLV). From the moment the green tissue is disrupted, enzymatic processes are initiated and the so-called GLV are emitted to the headspace (*22*). These enzymatic processes generate a complex analytical situation in which the concentration of analytes varies during the extraction. **Figure 2** depicts results from monitoring of the dynamic chromatographic profile of the headspace of *E. dunnii* leaves during the 24 h after leaf chopping. Twenty-eight minutes after chopping (**Figure 2A**) the peaks of C6-GLV (green leaf volatiles presenting six carbons in the molecular structure) can be seen (a−c), as can that of the green leaf ester peak, 3-(*Z*)-hexenol acetate (d). Esters such as this are synthesized from their alcohols (*22*). A second ester—hexyl acetate (e)—starts to appear under the α-terpinene peak tail (**Figure 2A**), as is shown by its mass spectrum; the level of this compound is highest 3.3 h after chopping (**Figure 2B**) and is decreasing by 24 h (**Figure 2C**). Similar behavior is observed for 3-(*Z*)-hexenol acetate (d), whereas the contributions of the other terpenoid peaks remain similar throughout the sampling period. Repetition of this experiment under different experimental conditions such as temperature, season of the year, and solar radiation (data not shown) revealed the presence of the same compounds in the headspace of chopped leaves, although the kinetics of formation and fading was different. Similar GLV were also present in *E. citriodora* and *E. saligna*, as is shown in **Figure 3**. These results show the simplicity and potential of HS-SPME for the study of dynamic processes in living systems such as chopped leaves. In a recent publication in vitro experiments with tomato plants (*Lycopersicon esculentum* Mill.) by HS-SPME revealed changes in 3-(*Z*)-hexenal and 3-(*Z*)-hexenol emissions during an 80 min period; monitoring of *Eucalyptus* leaf GLV has not, however, yet been reported (*23*).

Comparison of SPME and HD. Table 1 shows 30 BVOC identified and another 30 tentatively identified in the headspace of 60 chopped *Eucalyptus* leaves and in the *Eucalyptus* hydrodistilled oils, by the procedures described under Experimental Procedures. Another remaining 60 compounds were also

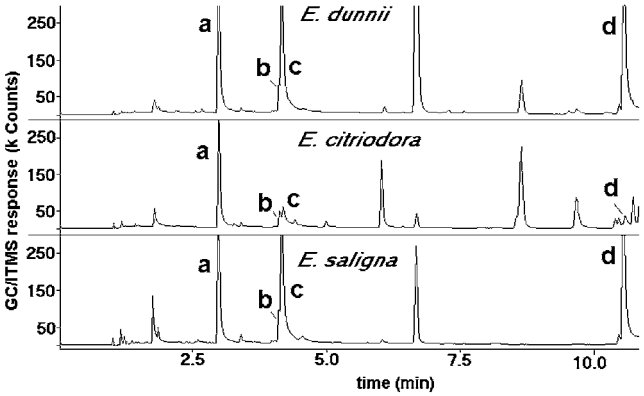

Figure 3. Total ion current chromatograms showing elution of GLV obtained from the headspace of chopped *E. dunnii*, *E. citriodora*, and *E. saligna* leaves: (a, b) C6-GLV; (c) 3-(*Z*)-hexen-1-ol; (d) 3-(*Z*)-hexenol acetate.

detected but not included in **Table 1** as their identification was not possible. The best option results from the ITMS libraries search (>90% purity match) for these compounds combined with the investigation of their elution order in the literature (*11*) are as follows: 3 butanoic or propanoic esters, 15 terpenoid compounds, *p*-cymenene, (*E*)-β-terpineol, isoborneol, *p*-cymen-8-ol, neral, geranial, *cis*-isopulegyl acetate, thymol, thymol isomer, jasmone, eugenol, geranyl isobutyrate, 10 sesquiterpene hydrocarbons, calacorene, viridiflorene, 3 oxygenated sesquiterpenes, β-eudesmol, 9 sesquiterpenes, and another 4 compounds that could not be assigned at all. The individual contribution of these compounds to the total area of all detected compounds was less than 1.0%, and they were detected in at least one of the experimental trees.

In general, the major compounds found in the headspace of chopped leaves were also found in the essential oils, although minor monoterpene oxygenated compounds were found only by HD and some significant peaks were found only by SPME. These cases will be discussed later in the text. Discussion of the HS-SPME results will be restricted to compounds present in two of the *Eucalyptus* species or to compounds having peak areas contributing close to or more than 1% of the total peak area.

Comparison of results from totally and partially expanded juvenile leaves by use of HS-SPME revealed no qualitative differences, although the percentage of the total peak area contributed by several peaks varied for totally and partially expanded leaves. There were no qualitative differences between the essential oils of *E. dunnii* trees A and B.

Direct quantitative comparison of results obtained by HS-SPME and hydrodistillation is not possible because the techniques are based on fundamentally different principles and extract different matrices. SPME is an equilibrium extraction process, and hydrodistillation is an exhaustive process; the latter extracts the essential oil, whereas the former extracts the volatile compounds present in the headspace of the leaves. This can be used to advantage—because of the difference between the two techniques used in this investigation, qualitative differences between results obtained from hydrodistillation and from HS-SPME might lead to interesting findings, because the SPME method employed in this work exploits mild experimental conditions, whereas hydrodistillation occurs at approximately the boiling point of water. **Table 2** shows a summary of the numerical results obtained by applying both techniques to *Eucalyptus* leaves. In an investigation of the oil of rhizomes of *Rhodiola rosea* L., the number of volatile compounds found in

Table 2. Number of Compounds Detected in *Eucalyptus* Leaves by Headspace SPME and in the Hydrodistilled Oil from the Leaves

	E. citriodora	*E. dunnii*	*E. saligna*
HD	57	59	58
HS-SPME	48	59	35
only by HD	14	8	27
only by HS-SPME	5	8	4

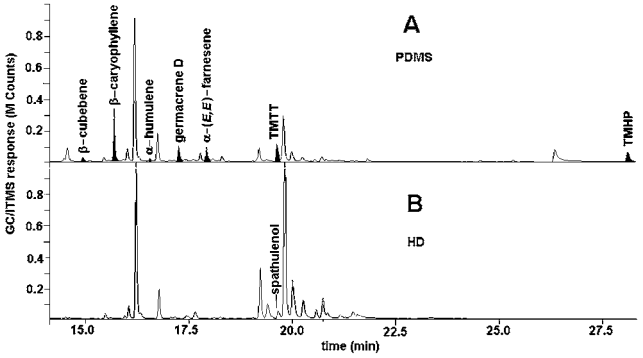

Figure 4. Sections of the total ion current chromatograms obtained by (A) HS-SPME and (B) hydrodistillation of *E. dunnii* leaves (filled black peaks indicate sesquiterpenes detected by HS-SPME only).

the distilled oil was higher than that found by HS-SPME (*24*). In contrast, the number of volatile compounds found in the flowers and leaves of sachalinmint (*Mentha sachalinensis* Briq. Kudô) was approximately the same (25), and, according to Rohloff, HS-SPME gave additional detailed information about less important terpenic compounds in sachalinmint volatiles. In the analysis of chopped *Eucalyptus* leaves some homoterpenes and sesquiterpenes detected by use of HS-SPME were not found in the hydrodistilled oil and might play a biological role in the leaves, because different amounts are obtained by analysis of different leaves and they are mentioned in the literature as potential infochemicals in several other living plants. It is very likely that these findings are because of the extraction of a living sample (freshly cut leaves) in which enzymatic processes are still occurring, whereas the study of sachalinmint was performed with dead (dried) matrices (*25*). Another study of *E. citriodora*, *E. nicholli*, and *E. globulus* found a richer fraction of sesquiterpene hydrocarbons in the headspace of freshly cut leaves than in the essential oils of the same species, although the reported composition of the oil was taken from the literature and did not correspond to the same experimental trees used for HS-SPME (*9*). In our work, a larger number of hydrocarbon sesquiterpenes and some homoterpenes were found by HS-SPME only and were identified, or tentatively identified (**Table 1**), as β-cubebene (**38**), β-caryophyllene (**41**), α-humulene (**45**), germacrene D (**48**), (*E*,*E*)-α-farnesene (**50**), (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) (**19**), (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) (**54**), and (*E*,*E*,*E*)-3,7,11,15-tetramethyl-1,3,6,10,14-hexadecapentaene (TMHP) (**60**). Numbers in parentheses correspond to those given in **Table 1**. **Figures 3** and **4** show the peaks corresponding to these compounds in two parts of the chromatographic profile of the BVOC obtained from *E. dunnii* leaves. Additional data obtained in previous work in which the BVOC of *E. dunnii*, *E. citriodora*, and *E. saligna* leaves were extracted in situ for 6−9 days are also included in this discussion, because they are related to the results found in this work. Further details of the experimental procedure used for in situ extraction of BVOC have been published elsewhere (*1*, *11*).

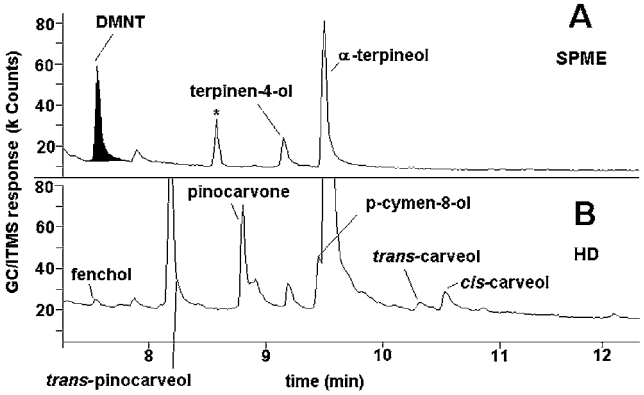

Figure 5. Sections of the total ion current chromatograms obtained by (A) HS-SPME and (B) hydrodistillation of *E. dunnii* leaves (filled black peak indicates DMNT detected only by use of HS-SPME).

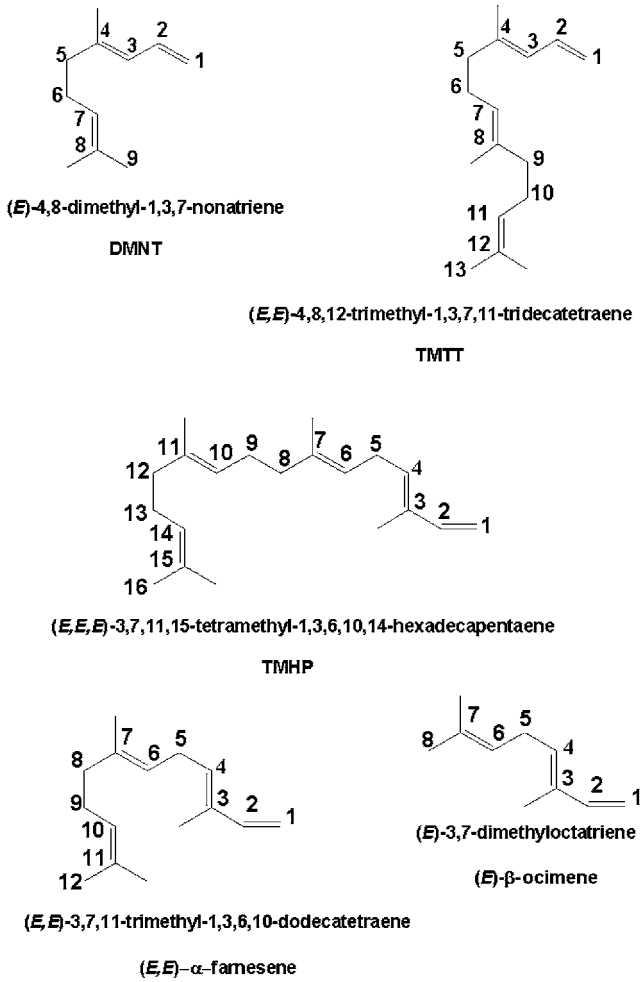

Figure 6. Structural formulas of some of the compounds present in the headspace of *Eucalyptus* leaves.

Intact plants emit DMNT and its higher homologue TMTT (**Figure 6**) and their emission has on several occasions been found to increase after herbivore attack (*4*, *26*). In combination with other volatile compounds DMNT and TMTT are used as "alarm calls" to attract insects that reduce the herbivore population on the host plant (*27*). Both compounds, and β-ocimene, are part of a volatile mixture emitted by defoliated black alders (*Alnus glutinosa*), which possibly makes these trees and their neighbors more resistant to *Agelastica alni* herbivores by contributing to interplant communication (*28*). DMNT and TMTT are also emitted by several herbivore-infested plants, such as lima beans (*29*), green walnuts (Hartley var.) (*26*), and cotton plants (*4*).

DMNT and TMTT have been found in the headspace of the chopped leaves of *E. dunnii* and *E. saligna* and in the headspace of their leaves in situ when sampled in natural light. Traces of these compounds have been detected by in situ sampling of *E. dunnii* leaves. Similarly, on the first day of in situ sampling of *E. saligna* leaves, only traces of these compounds were found, whereas in the two subsequent days the peak areas of these compounds increased during the sampling periods of 8 h per day. Similar results were obtained for cotton plants, for which DMNT had a circadian profile with two emission peaks when the plant was intact and also when damaged by caterpillars, although in the experiment performed on the damaged plant the emissions were higher. Another compound found in cotton plant emissions was (*E*,*E*)-α-farnesene, which had the same circadian profile as DMNT (*4*). Interestingly, (*E*,*E*)-α-farnesene was also found in the chopped leaves of the three *Eucalyptus* species studied, and its emission behavior during in situ sampling of *E. saligna* and *E. dunnii leaves* was similar to that described for DMNT and TMTT emissions during in situ *E. saligna* sampling. Although mechanical damage of the *Eucalyptus* tissue was very small (only six small holes in the green tissue at the beginning of 8 h of sampling) compared with the damage inflicted on cotton plants (continuous caterpillar feeding during the sampling period after starving for 6 h), similarity among emissions from cotton plants and *Eucalyptus* leaves suggests that the triggering mechanism for synthesis and/or emission of these compounds might be similar in both situations. DMNT is also known as a predator attractant in agricultural plants (*28*). An example of its action is its emission from lima bean plants infested with *T. urticae*—DMNT was found to attract the predatory mite *Phytoseiulus persimilis* (*29*). Similarly, Takabayashi and co-workers reported an increase in (*E*,*E*)-α-farnesene emissions after infestation of apple leaves by *T. urticae*. When DMNT was present in the headspace of chopped *E. dunnii* and *E. saligna* leaves, and in situ leaves, mild infestation with *T. urticae* was noticed on a branch distal from the sampling point. This presence of *T. urticae* on *Eucalyptus* leaves and infested lima bean plants is worthy of comment. It seems reasonable to hypothesize that the presence of DMNT in the headspace of *Eucalyptus* leaves might be a systemic response of the tree, and not only to mechanical damage. It is, furthermore, also possible to suppose that an incipient spider mite infestation could be present on the tested leaves (chopped and in situ), even though they were not apparent to inspection by the human eye. In this instance emission of DMNT could also be interpreted as a local response of the tree or a result of spider mite activity (*29*). All of this reasoning requires further investigation, however, and a final conclusion on this issue is beyond the scope of this work.

In a recent publication it was suggested that the presence of DMNT indicates to the *Eucalyptus* woodborer *Phorocantha semipunctata* that *Olea europeae* is a nonhost tree. Barata and co-workers even suggest the use of DMNT as a possible aid in integrated strategies for control of *P. semipunctata* (*30*).

It seems relevant to mention that (*E*,*E*)-α-farnesene, present in the headspace of chopped *Eucalyptus* leaves from the three species studied, is accompanied by the presence of its higher homologue TMHP (tentatively identified) in the headspace of chopped *E. dunnii* leaves. Emission of (*E*)-β-ocimene, a lower member of this homologous series, has already been detected by in situ headspace analysis of the leaves of the same three

Eucalyptus species (and the same trees) investigated in this work. The circadian profile of (*E*)-β-ocimene emissions in a greenhouse environment is the same for the three species studied in situ; one emission peak was observed during 8−10 h of sampling. The presence of this homologous series [(*E*)-β-ocimene, (*E*,*E*)-α-farnesene, and TMHP] in the headspace of the three species of *Eucalyptus* in different experiments (chopped and in situ leaves) is, at the very least, noteworthy and might be indicative of a possible biochemical relationship among these compounds (*1*).

Although several components were detected by HS-SPME only, some oxygenated compounds were detected in the hydrodistilled oil only. Spathulenol, a common oxygenated sesquiterpene of *Eucalyptus* essential oils, was detected only in the hydrodistilled oil from the leaves of the three *Eucalyptus* species under study. During investigation of liverworts (*Pagiochila fruticose* and others), (−)-*ent*-spathulenol was found to be an artifact produced during the extraction process. The compound (−)-*ent*-bicyclogermacrene was transformed into (−)-*ent*-spathulenol at ambient temperature (*31*). Bicyclogermacrene was not detected in the *Eucalyptus* oils obtained in this work or in the headspace of *Eucalyptus* chopped leaves; the absence of spathulenol from the headspace of chopped leaves might, however, possibly indicate that spathulenol is somehow generated during the HD process.

Other oxygenated compounds, for example, *endo*-fenchol, campholenal, *trans*-pinocarveol, pinocarvone, borneol, and other minor components (see **Table 1** for their contribution to total peak area), were also found in the hydrodistilled oil only. Although it was not possible to reach a final conclusion about the reasons these oxygenated compounds were not detected in the headspace of chopped leaves, two hypotheses were proposed: (1) The compounds might be artifacts resulting from the oxidation of other terpenes, because of the high temperature used during HD (*21*). (2) The low affinity of the PDMS coating for more polar compounds might have restricted their extraction, especially those present in small amounts (*32*). Because campholenal was detected in the in situ headspace of *E. saligna* leaves in previous experiments with PDMS/DVB, the first proposition does not seem to be plausible, at least for this specific compound (*1*). Preliminary experiments with the PA coating (which has a greater affinity for polar compounds) did not reveal the presence of any of these analytes. Perhaps the leaves employed in the preliminary experiments did not have these oxygenated monoterpenes in their headspace composition for ontogenic or other reasons. A final conclusion about these specific experimental results is not possible and would require further investigation.

Sesquiterpenes such as germacrene D and α-humulene were found only in the headspace of chopped *E. dunnii* leaves. Neither were these compounds detected in the essential oil from the same tree in previous studies of this species reported in the literature (*5*, *33*, *34*). Interestingly, germacrene D and α-humulene also were detected only in the headspace of freshly cut leaves of *E. globulus* and *E. nicholii* (*9*), even though literature data about the components of the essential oils of these species do not report the presence of these compounds (*5*).

Similarly to other compounds found in the headspace of *Eucalyptus* chopped leaves, germacrene D and α-humulene have also been found in emissions from damaged host plants. Damaged *Adenostyles alliariae* leaves emitted more germacrene D and α-humulene and were more attractive than intact leaves to *Oreina cacaliae* herbivores (*35*). Both compounds were also found in volatile emissions from infected nasturtium (*Tropaeolum majus* cv. Mahogany) (*36*). In a recent investigation of the Eurasian cotton bollworm moth *Helicoverpa armigera*, a major type of receptor neuron with high sensitivity and selectivity to germacrene was identified (*37*). Its levorotatory enantiomer has also been recognized as a masking substance, inhibiting locomotory movement of the cerambycid beetle, *Monochamus alternatus*, toward healthy pines, *Pinus densiflora* (*38*).

The sesquiterpene β-caryophyllene was also detected only by HS-SPME of both *E. dunnii* leaves investigated and not by HD. The amount of β-caryophyllene extracted from the headspace of *E. dunnii* B was higher than that from *E. dunnii* A. Previously published data showed that the circadian profile of β-caryophyllene emissions during in situ sampling of the same *E. dunnii* tree (B) followed the same pattern as that described for (*E*)-β-ocimene, whereas β-caryophyllene was not detected in the volatile emissions from *E. dunnii* A (*1*). That β-caryophyllene plays infochemical roles in several plants and is present in chopped *E. dunnii* leaves but absent from the hydrodistilled oil of the same trees reinforces previous hypotheses of a possible infochemical role for this compound in *Eucalyptus* (*1*, *28*, *39*).

Because of the multiple biological activity, reported in the literature, of the seven compounds [(*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMNT), (*E*,*E*)-α-farnesene, (*E*,*E*,*E*)-3,7,11,15-tetramethyl-1,3,6,10,14-hexadecapentaene (TMHP), β-caryophyllene, α-humulene, and germacrene D] detected only by HS-SPME of *Eucalyptus* chopped leaves (and sometimes also in the in situ headspace of the leaves) and not in the essential oil of the same trees, possible infochemical roles can be suggested for these compounds in *Eucalyptus* leaves.

On the basis of this study it can be concluded that HS-SPME is a simple and reliable means of obtaining complementary information about BVOC in the fresh parts of a plant, ex situ, without the use of the special devices or temperature conditions required by other techniques. This work with chopped *Eucalyptus* leaves demonstrates the use of HS-SPME not only for screening of BVOC but also for extracting compounds that are, perhaps, important in the physiology of the living plant and are not found by hydrodistillation. Results obtained in this work suggest that application of this technique to a vast range of other living matrices might lead to the discovery of compounds which might play important bioactive roles in living systems.

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