Volatile Constituents of Essential Oils Obtained from Newly Developed Tea Tree (*Melaleuca alternifolia*) Clones

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Volatile constituents of essential oils of eight different clones propagated from the Australian tea tree (*Melaleuca alternifolia*) were analyzed by gas chromatography and gas chromatography/mass spectrometry. The oils isolated by a simultaneous purging and solvent extraction method (SPE) contained high levels of monoterpenes, including α-thujene, sabinene, α-terpinene, γ-terpinene, 1,8-cineole, terpinolene, and limonene. The volatile composition of oils prepared by a simultaneous steam distillation and solvent extraction method (SDE) varied significantly among different clones. Six oils contained 1,8-cineole as the major constituent, whereas two oils had terpinen-4-ol as the major component of the SDE samples. More compounds were found in SDE samples than in SPE samples. A principal component analysis on volatile compositions of eight oils conducted by computer indicated that sabinene and α-thujene in SPE samples and terpinen-4-ol and α-terpinene in SDE samples are highly correlated with each other.

Melaleuca alternifolia, commonly known as Australian tea tree, is a perennial shrub species native to northern New South Wales, Australia. The essential oil from this tree has a characteristic strong greenish aroma. It possesses some antifungal and antibacterial activities. The oil contains high concentrations of terpinen-4-ol, which is known to have germicidal activity (Williams et al., 1988). In addition to terpinen-4-ol, major constituents of this oil are monoterpenes, including γ-terpinene, α-terpinene, 1,8-cineole, and α-terpineol (Guenther, 1968). The first comprehensive analysis of the oil was conducted by Swords and Hunter (1978), who reported 40 components, including a novel sesquiterpene viridiflorene. More recently, Brophy et al. (1989) reported compositional differences of the oils prepared from trees of various ages. Because of its pharmaceutical activities, the oil has been used in skin-care products to treat skin infections or disorders. However, the oils with low cineole content are recommended for medicinal use because this compound irritates mucous membranes and skin (Lassak and McCarthy, 1983). Therefore, there is a strong need to breed tea trees that produce high-quality oil. Moreover, the market for tea tree oil in the United States is increasing, with the demand currently exceeding supply. In the present study, the volatile composition of eight newly propagated tea tree clones was investigated.

EXPERIMENTAL PROCEDURES

Cultivation of Tea Tree Seedlings and Clonal Propagation of Selected Seedlings. A plantation of 250 seedlings, planted at approximately 12 500 seedlings/ha, was established at the University of California, Davis, in March 1986. Leaf oil analyses made in August 1987 indicated that eight of these seedlings would be of interest for commercial exploitation (Sachs et al., 1989). Hence, clonal propagation of these eight seedlings was begun in September 1987 with a view to establishing clonal plantations in 1988. Stem cuttings approximately 10 cm long were made from lateral branches of mother plants of each of the selected seedlings. The basal ends of the cuttings were dipped in a 4000 mg/L indolebutyric acid solution for 10 s and then stuck in a perlite/vermiculite (50/50) mix in flats. The flats were placed under mist benches with 23 °C bottom heat for 2–3 weeks after which time root initiation had begun on most cuttings. Rooting percentage was in excess of 70% during the April–July period. Rooted cuttings were transplanted to peat pots in a peat/sand potting mix, maintained in a greenhouse for 1–2 weeks, and then moved into a lathhouse for hardening before field planting. Clones were field planted in April 1988; plants were on 1-m centers (about 12 500 plants/ha) in 28–35 plant blocks, each block with double borders. Samples were removed for analysis in October 1988 and, after having been cut back at least once, again in September 1989.

Sample Preparations. Branches with leaves of each clone were stripped from the main stem and cut into approximately 10-cm lengths. Then the essential oil was prepared by two methods.

(1) *Simultaneous Purging and Extraction Method (SPE).* Branches from each clone (100 g) were placed in 500-mL round-bottom flasks, and a headspace was purged with a purified nitrogen stream into 250 mL of deionized water, which was simultaneously extracted with 70 mL of dichloromethane for 4 h by using an apparatus prepared by Umano and Shibamoto (1988). The sample flasks were maintained at 15 °C, and the water trap was maintained at 8 °C. The extracts were dried over anhydrous sodium sulfate for 12 h. After removal of sodium sulfate and solvent, approximately 2.4 mg of light yellow oil was obtained.

(2) *Simultaneous Steam Distillation and Solvent Extraction Method (SDE).* Branches (100 g) were placed in 2-L round-bottom flasks with 1 L of deionized water, and steam distillate was extracted with 50 mL of dichloromethane simultaneously for 1 h by using a modified Likens-Nickerson apparatus (Schultz et al., 1977). The extracts were dried over anhydrous sodium sulfate for 12 h. After sodium sulfate was filtered out, solvent was removed by using a Kuderna-Danish evaporative concentrator. A yellow essential oil (3 g) was obtained. Samples prepared by both methods were analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS).

Instrumental Methods. The GC Kovats retention index (*I*) and the MS fragmentation pattern of each component were

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Table I. Volatile Constituents Identified in the Oils of *M. alternifolia* Obtained by SPE

		GC peak area % of oils							
I^a	compd	I	II	III	IV	V	VI	VII	VIII
1005	α-pinene	0.98	4.18	5.64	5.91	7.28	4.85	5.99	2.81
1007	α-thujene	13.38	3.50	0.02	7.03	4.12	4.56	2.53	1.80
1078	β-pinene	1.05	0.70	1.48	2.48	3.63	3.45	2.97	1.57
1103	sabinene	49.14	12.34	4.62	18.70	7.53	10.55	7.89	1.65
1131	myrcene	2.20	3.47	6.43	3.53	5.61	6.03	5.38	6.69
1148	α-terpinene	9.24	19.82	7.51	8.84	8.01	7.53	8.94	0.95
1160	limonene	1.87	2.83	6.83	6.67	9.21	10.55	9.91	9.12
1172	β-phellandrene	0.63	0.50	1.55	1.91	1.27	1.33	1.40	1.83
1192	1,8-cineole	2.57	8.81	13.72	20.15	32.20	33.47	33.94	52.37
1215	γ-terpinene	13.54	24.63	11.79	10.92	12.07	9.39	12.64	0.99
1232	*p*-cymene	0.83	9.97	2.44	6.97	0.95	2.25	1.94	*b*
1239	terpinolene	1.92	4.71	33.12	2.28	1.77	1.99	2.24	18.31
1265	cyclohexanone	0.06	*c*	0.17	*b*	0.02	*b*	*b*	0.14
1292	(*Z*)-3-hexenyl acetate	0.11	*c*	0.29	*b*	*c*	*b*	*b*	*c*
1433	*trans*-sabinene hydrate	0.42	*b*	0.16	*b*	0.39	0.21	0.48	*c*
1458	β-cubebene	0.09	*c*	*c*	*c*	0.12	*c*	*c*	*c*
1507	linalool	*c*	*c*	*c*	*c*	*c*	*c*	*c*	0.14
1518	*cis*-sabinene hydrate	0.20	*b*	0.44	0.04	0.81	0.57	1.14	*c*
1526	*trans*-menth-2-en-1-ol	*c*	*c*	*c*	*c*	0.08	*c*	*c*	*c*
1538	β-caryophyllene	0.09	*c*	*c*	*c*	0.12	*c*	*c*	*c*
1572	terpinen-4-ol	0.69	3.15	1.27	2.29	2.05	2.00	2.04	0.16
1578	alloaromadendrene	0.05	*c*	0.10	0.06	0.08	*b*	*b*	*c*
1657	α-terpineol	0.26	1.16	0.55	0.83	0.86	0.83	0.90	0.79
1663	γ-elemene	0.04	*c*	*c*	*c*	0.20	*c*	*c*	*b*
1679	δ-cadinene	0.18	*b*	0.21	*b*	0.18	*b*	*b*	*c*

[a] Kovats index on Carbowax 20M. [b] Value less than 0.01. [c] Not detected.

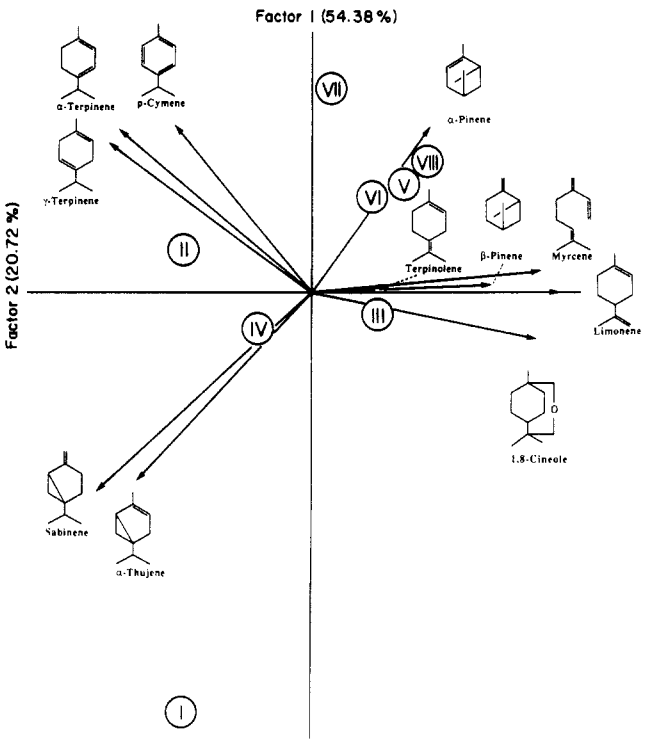

Figure 1. Principal component analysis of oils (Roman numerals) obtained by SPE.

compared to those of the authentic compound to identify the volatiles in the samples.

A Hewlett-Packard Model 5890A GC equipped with a flame ionization detector (FID) and a 50 m × 0.25 mm i.d. Carbowax 20M fused silica capillary column were used. Peak area was integrated by using a Spectra-Physics 4290 integrator. The oven temperature was held at 60 °C for 4 min and then programmed to 180 °C at 2 °C/min. The helium carrier gas flow rate was 30 cm/s. The injector and detector temperatures were 200 and 210 °C, respectively. A VG Trio-2 mass spectrometer interfaced to an HP 5890 GC was used for MS identification of the GC components under the following conditions: filament current, 167 mA; multiplier voltage, −2.5 kV; electron energy, 70 eV. The GC column and oven conditions were as described for the Hewlett-Packard instrument.

RESULTS AND DISCUSSION

Two methods, SPE and SDE, were used to obtain essential oils from tea tree leaves (*M. alternifolia*) in the present study because the isolation techniques greatly influence the composition of the resulting oils (Fischer et al., 1987). The SPE samples most closely resemble the natural forms because the mild treatments applied minimized alteration of constituents during sample preparation (Shibamoto, 1981). On the other hand, the oils obtained by the SDE method might have similar volatile compositions to these found in commercial oils because steam distillation is most commonly used to obtain commercial tea tree oils. The recovery of oils by the SDE method was much higher (2.3–3.4 g/100 g of fresh leaves) than that obtained by the SPE method (2–10 mg/100 g of fresh leaves) in the present study. Recovery efficiencies of SPE and SDE have been reported previously (Umano and Shibamoto, 1988; Leagy and Reineccius, 1984). The compounds identified in the oils obtained from the eight tea tree clones by SPE and SDE are shown in Tables I and II, respectively.

Headspace samples (SPE) contained high levels of monoterpenes, including α-thujene, sabinene, α-terpinene, γ-terpinene, 1,8-cineole, terpinolene, and limonene. Other monoterpenes, including α-pinene, β-pinene, myrcene, and *p*-cymene, were present in low levels. Terpinen-4-ol and α-terpineol were also identified. The presence of *trans*- and *cis*-sabinene hydrates, which were reported as precursors of terpinen-4-ol, α-terpinene, α-thujene, or sabinene (Southwell and Stiff, 1989), was recognized.

The composition of oils obtained by SDE varied significantly among different clone samples. Two terpene alcohols, 1,8-cineole and terpinen-4-ol, exhibited the greatest variations in quantity among clones. α-Thujene, sabinene, and sabinene hydrate in *M. alternifolia* were hypothesized to change to terpinen-4-ol or terpinenes during steam distillation (Southwell and Stiff, 1989). The levels of 1,8-cineole and terpinen-4-ol may play an

Table II. Volatile Constituents Identified in the Oils of *M. alternifolia* Obtained by SDE

		GC peak area % of oils							
I^a	compd	I	II	III	IV	V	VI	VII	VIII
1005	α-pinene	2.00	2.13	1.55	2.44	2.90	3.71	3.69	2.91
1007	α-thujene	4.24	1.30	1.18	1.30	1.00	0.03	0.03	0.05
1078	β-pinene	0.53	0.76	0.72	1.29	1.30	1.28	1.19	1.07
1103	sabinene	10.93	1.60	0.70	1.48	1.12	1.40	1.43	0.23
1131	myrcene	1.57	1.26	3.12	2.56	2.47	2.46	2.21	3.57
1148	α-terpinene	10.09	10.37	5.15	5.29	5.48	5.02	5.50	0.63
1160	limonene	1.09	0.61	2.95	4.80	4.78	5.02	4.81	6.97
1172	β-phellandrene	0.82	0.62	0.81	0.93	0.75	0.81	0.29	0.43
1192	1,8-cineole	5.96	0.54	22.09	39.45	37.13	39.13	35.86	56.10
1215	γ-terpinene	17.16	19.36	9.31	8.65	9.34	8.69	8.69	0.94
1232	*p*-cymene	0.82	3.82	0.31	0.37	0.68	0.64	0.53	0.05
1239	terpinolene	3.46	4.06	23.98	1.92	1.94	1.78	1.94	12.60
1433	*trans*-sabinene hydrate	0.28	0.18	*b*	0.10	0.07	0.11	0.23	*c*
1458	β-cubebene	*b*	0.09	0.06	0.10	0.14	0.11	*c*	0.03
1465	α-gurjenene	*c*	0.09	0.15	0.02	0.14	0.11	0.16	0.03
1507	linalool	*c*	0.12	0.48	0.08	0.09	0.07	0.14	0.34
1518	*cis*-sabinene hydrate	0.21	0.12	*b*	0.10	0.15	0.07	0.74	*c*
1526	*trans*-menth-2-en-1-ol	1.41	1.04	0.17	0.63	*c*	0.65	0.32	0.03
1538	β-caryophyllene	*c*	0.20	0.31	0.63	0.36	0.65	0.51	0.11
1572	terpinen-4-ol	32.06	42.85	14.94	17.13	17.13	16.59	17.48	1.07
1578	alloaromadendrene	*b*	0.20	0.17	0.23	0.16	0.13	0.20	0.07
1586	β-cadinene	*b*	0.10	0.24	0.03	0.17	0.14	0.22	0.05
1593	*cis*-menth-2-en-1-ol	0.90	0.76	0.24	0.34	0.17	0.30	0.34	*b*
1614	viridiflorene	*b*	0.23	0.37	0.22	0.43	0.31	0.43	0.15
1630	*trans*-piperitol	0.39	0.23	*c*	*c*	0.31	0.32	0.37	0.34
1657	α-terpineol	2.65	3.89	5.00	7.06	7.21	6.53	6.79	9.59
1663	γ-elemene	*b*	*b*	0.23	*b*	0.40	0.10	0.50	0.30
1679	δ-cadinene	0.54	0.49	0.91	0.35	0.78	0.55	0.93	0.21
1701	*cis*-piperitol	0.45	0.25	0.11	0.10	0.11	0.04	0.15	0.01
1701	γ-cadina-1,4-diene	*c*	0.12	0.12	0.09	0.10	0.03	0.14	0.03
1751	calamenene	*c*	*b*	*b*	*c*	*c*	*c*	*b*	*c*
1805	*p*-cymen-8-ol	*c*	0.10	0.10	*c*	0.10	*c*	*b*	0.04
2001	epicubenol	0.35	0.35	0.54	0.28	0.38	0.39	0.53	0.21
2052	β-eudesmol	0.10	0.31	0.26	0.19	0.25	0.26	0.32	0.20
2126	cadinol T	0.01	0.12	0.08	0.21	0.13	*c*	*c*	*c*
2149	δ-cadinol	0.22	0.15	0.20	0.47	0.16	*c*	0.19	0.08

[a] Kovats index on Carbowax 20M. [b] Value less than 0.01. [c] Not detected.

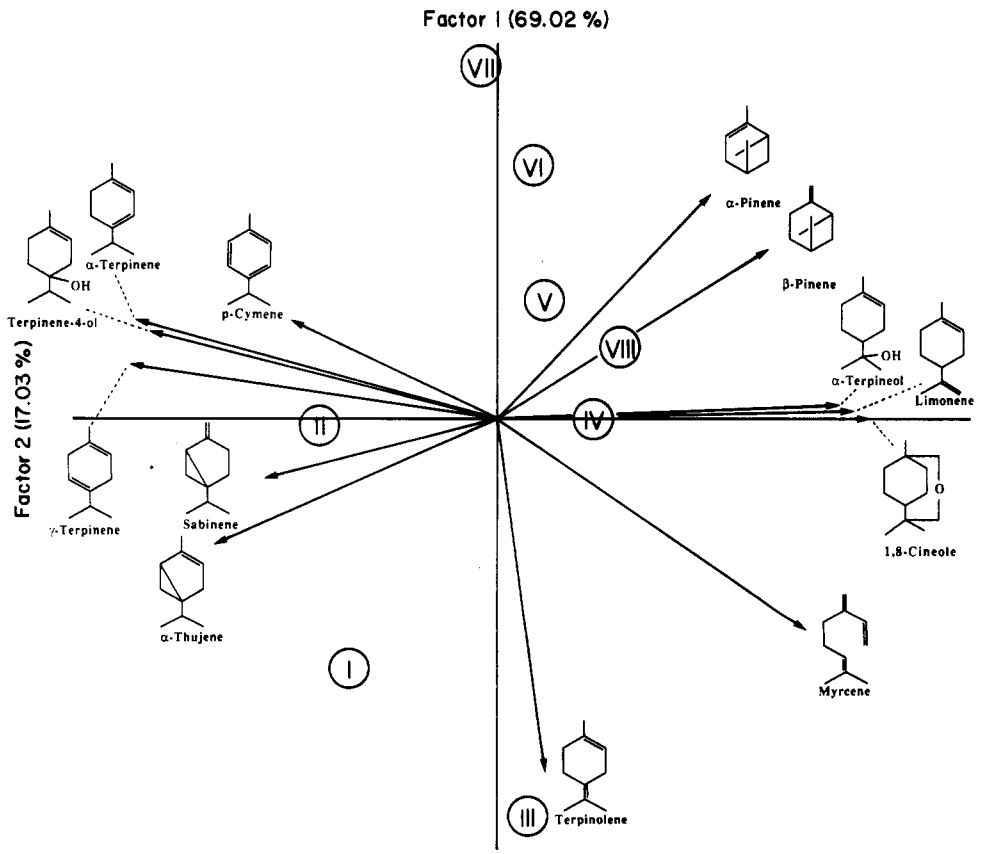

Figure 2. Principal component analysis of oils (Roman numerals) obtained by SDE.

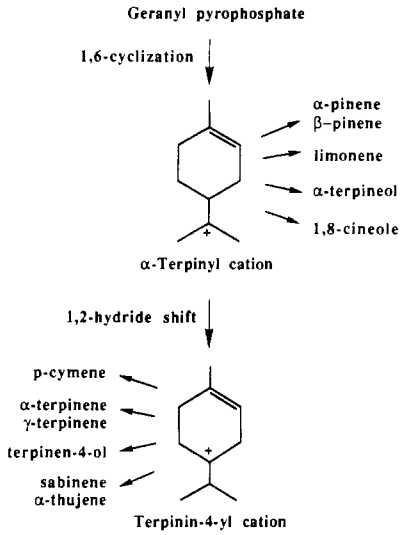

Figure 3. Biosynthesis pathways of monoterpenoids.

important role in the quality of tea tree oils. For example, terpinen-4-ol reportedly has a high biological activity (Williams et al., 1988). In fact, the standard quality of tea tree oils in Australia is specified as a 1,8-cineole content of less than 15% and a terpinen-4-ol content of greater than 30%. Clones I and II are qualified for the Australian specifications.

To investigate characteristics of oils in more detail, 11 predominant components of SPE samples and 13 components of SDE samples were used to perform Principal Component Analysis (PCA) (Statistical Analysis System, 1988). PCA has been widely used to evaluate food flavors (Heymann and Noble, 1989; Peppard, 1989). PCA provided a better understanding of differences and similarities among volatile compositions of clones. Each principal component or axis is a linear combination of the original variables (GC peak area percent of component). A PCA of the volatile composition for eight oils from SPE and SDE is shown in Figures 1 and 2, respectively. The loadings for the components are shown as vectors, the lengths of which roughly indicate their relative importance; generally, variables with short vectors are less important. Conversely, the longest vectors are the most important attributes in explaining the variability among the samples. For example, sabinene and α-thujene in SPE samples and terpinen-4-ol and α-terpinene in SDE samples are highly correlated with each other. Monoterpenoids with a similar structure have close vectors.

Oils obtained from clones V–VIII by SPE have a similar volatile composition. Compounds having a thujane skeleton, α-thujene and sabinene, were characteristic of clone I, which contained α- and γ-terpinene in high levels. Clones III and VIII had high levels of terpinolene and 1,8-cineole, respectively. Clones IV–VII showed characters intermediate between clones I and VIII.

Oils obtained from clones IV–VII by SDE have similar and intermediate characters of volatile compositions among the eight oils. Clone II contained a high amount of terpinen-4-ol and a low amount of 1,8-cineole, whereas clone VIII contained a low amount of terpinen-4-ol and a high amount of 1,8-cineole.

High-quality (according to Australian specifications) clones I and II contained compounds that were hypothesized to form from terpinin-4-yl cation via biosynthetic pathways of monoterpenoids (Figure 3; Ruzicka, 1953). A majority of compounds found in clone VIII seems to form from α-terpinyl cation. The differences in the volatile compositions of the clones seem to be associated with this enzymatic pathway. Therefore, clarification of this pathway may be important for quality control of tea tree oils.

Daly et al. (1958) reported that *trans*-sabinene hydrate was easily isomerized to a mixture of terpinen-4-ol, *p*-cymene, γ-terpinene, sabinene, and α-thujene in acidic media. Southwell and Stiff (1989) reported that terpinenes and terpinen-4-ol in *M. alternifolia* were formed from thujanes, especially from *trans*-sabinene hydrate by steam distillation. Karasawa et al. (1978) also found formation of terpinen-4-ol from *trans*-sabinene hydrate by steam distillation. The same results were observed in steam-distilled sweet majoram oil (Taskinen, 1974) and in Leyland cypress oil (Koedam et al., 1980). Therefore, further investigation on the formation of sabinene hydrate is in order.

When various types of *M. alternifolia* are available for commercial use in the future, control of the biosynthetic pathway between terpinin-4-yl cation and α-terpinyl cation and the clonal propagation method may be effective in obtaining high-quality tea tree oils.

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Registry No. α-Pinene, 80-56-8; α-thujene, 2867-05-2; β-pinene, 127-91-3; sabinene, 3387-41-5; myrcene, 99-86-5; α-terpinene, 138-86-3; limonene, 138-86-3; β-phellandrene, 555-10-2; 1,8-cineole, 470-82-6; γ-terpinene, 99-85-4; *p*-cymene, 99-87-6; terpinolene, 586-62-9; cyclohexanone, 108-94-1; (*Z*)-3-hexenyl acetate, 3681-71-8; *trans*-sabinene hydrate, 17699-16-0; β-cubebene, 13744-15-5; linalool, 78-70-6; *cis*-sabinene hydrate, 15537-55-0; *trans*-menth-2-en-1-ol, 29803-81-4; β-caryophyllene, 87-44-5; terpinen-4-ol, 562-74-3; alloaromadendrene, 25246-27-9; α-terpineol, 98-55-5; α-elemene, 29873-99-2; δ-cadinene, 483-76-1; α-gurjunene, 489-40-7; β-cadinene, 523-47-7; *cis*-menth-2-en-1-ol, 29803-82-5; viridiflorene, 21747-46-6; *trans*-piperitol, 16721-39-4; *cis*-piperitol, 16721-38-3; γ-cadina-1,4-diene, 16728-99-7; calamenene, 483-77-2; *p*-cymen-8-ol, 1197-01-9; epicubenol, 19912-67-5; β-eudesmol, 473-15-4; cadinol T, 5937-11-1; δ-cadinol, 19435-97-3.