Investigation of the Variation in Chemical Composition of *Tasmannia lanceolata* Solvent Extracts

Valerie A. Dragar,* Sandra M. Garland, and Robert C. Menary

School of Agricultural Science, University of Tasmania, G.P.O. Box 252-54, Hobart, 7000 Tasmania, Australia

The solvent extract of *Tasmannia lanceolata* from a small community of plants was examined over 230 days of the growing season. The results from gas chromatography were analyzed using cluster analysis and principal coordinate analysis. Two distinct groups of plants were identified, based on polygodial content and the entire range of components detected. The use of as many components as possible is recommended for the analysis, to give the most complete representation of the composition of the extracts. Within the low-polygodial group, the tendency was for polygodial levels to remain constant over time, whereas in the high-polygodial group, concentrations varied.

INTRODUCTION

Tasmannia lanceolata (Poir.) ACSmith (mountain pepper) is described as a much branched shrub, up to 5 m high, with dark green glabrous aromatic leaves and distinctive, crimson, young stems. This dioecious plant bears black, berry-like fruit, ≈5 mm in diamenter, containing numerous small seeds (Curtis and Morris, 1975). The plant grows in cool wet habitats from sea level to ~1200 m in Tasmania, preferring disturbed sites in which it is an early colonizer, preceding wet eucalypt forest and *Nothofagus* rainforest (Reid and Hill, 1983). It is also found in similar situations in Victoria and at high altitudes in New South Wales, as far north as the Hastings River.

The solvent extract of *T. lanceolata* is being investigated as a potentially useful flavor product. For most essential oil crops, the timing of harvest and the selection of suitable populations of plants are two fundamentally important aspects of commercialization. Both of these factors have a bearing on extract quality. Ideally, the extract of a commercial population would be chemically homogeneous and would be harvested at a time of optimal yield.

It is recognized that *T. lanceolata* has a great range of morphologies from one area to another. Its range of extract compositions is likewise varied.

Some preliminary work has been done on seasonal changes in oil composition (Read, 1996), and this has shown that there is little variation in oil quality due to the position of the leaf sampled.

Variation in oil quality due to plant gender was considered. Unpublished data have revealed that there is no significant difference between the extracts from male and female plants. Consequently, sex was not a factor that was taken into consideration in the selection of experimental plant material.

The focus of the present work was to examine the total extract composition in a group of plants from a localized geographic area, to determine the extent of compositional differences and their changes over time. Included were the levels of polygodial (the major contributor to the extract's peppery character), and the percentage of monoterpenes was studied over a period of 230 days. Cluster analysis of GC data has been used to select superior cultivars, rich in flavoring potency, in Japanese pepper (Shimoda et al., 1997).

MATERIALS AND METHODS

Six plants were selected at random at the Arve Loop site. The plants were located along a 3.5 km length of the roadway. Plants 1, 4, 5, and 6 were of roughly the same age and were ~2 m in height. Plants 2 and 3 were about half the height of the other four. Plant 6 was not as vigorous as the other five, being somewhat afflicted by sooty mold, with its habitat being more shaded and cooler than the more exposed sites of the other five plants. Leaf position does not affect oil quality (Read, 1996), but for reasons of uniformity of physiological age, three recently fully expanded leaf pairs were taken on seven separate occasions [August 16, 1996 (0 days), November 14, 1996 (91 days), December 4, 1996 (111 days), December 30, 1996 (137 days), January 16, 1997 (154 days), February 20, 1997 (189 days), and April 2, 1997 (230 days)].

The extraction method used was that developed by Read (1996) as being efficient for small scale applications. The fresh leaves were dried at 35 °C in a thermostatically controlled oven for 48 h. The leaves were then ground to a fine powder with a mortar and pestle in preparation for extraction. The samples were transferred to 20 mL glass vials with 5 mL of redistilled petroleum ether (Shell Australia Ltd., bp 40−60 °C) containing 1 mg of octadecane (Sigma, 99%) as an internal standard. The vials were sonicated in a Branson 5200 sonication bath for 20 min. The samples were allowed to settle before the solvent solution was trasnferred to a GC vial for analysis.

Gas chromatography (GC) was performed using a Hewlett-Packard (Palo Alto Ca. USA), HP 5890 unit fitted with an HP 7673A automatic injector and an FID detector, with control and data analysis by HP/Chemstation 3365 software. A 15 m HP1 column (cross linked methyl silicone gum, USA) was used, (i.d. 0.22 mm, phase thickness, 0.33 μm). The carrier gas was high purity nitrogen, run at a pressure of 17 psi. The column flow rate was 2 mL/min. The injector temperature was 250 °C and the detector temperature was 280 °C. The temperature program was 50 °C (1 min), 20 °C/min to 150 °C, 5 °C/min to 260 °C (5 min). The injection volume was 1 μL, with a split ratio of 50:1.

* Author to whom correspondence should be addressed (fax 61 3 6226 7609; e-mail valerie@utas.edu.au).

Table 1. Mean over Seven Samples of Total Percentage Polygodial and Monoterpenes in Extracts from Each Plant[a]

plant	% polygodial	% monoterpenes
1	25.241 a	2.321 b
2	3.242 b	2.797ab
3	1.005 b	1.954 b
4	1.321 b	1.821 b
5	37.507 a	3.569 a
6	40.977 a	3.749 a
LSD	7.917	1.164

[a] Means with the same letter are not significantly different (p = 0.05).

Integration of the peaks used a rejection area of 500. Analysis of the extract from each plant, at each sampling time, resulted in a chromatogram that consisted of between 70 and 120 peaks. The complete profiles from all 42 of these runs were consolidated into a single file that represented each of the components as a number from 1 to 183. Where there were peaks that were not present in other traces, they were assigned a unique number.

Compositional data are reported as peak area percents, and, as such, one may assume that the detector response is equivalent from one component to the next (Clark and Menary, 1984).

Variability of the above extraction and analysis method was determined through four repetitions of one sample. The standard error of the polygodial peak area percent was 0.099.

The techniques used for analysis of the data included clustering, principal coordinate (PC) analysis, and correlation coefficient determinations. The package TAXON (Ross and Shields, 1993) was used for cluster and PC work. Similarity coefficients were calculated using standardized Euclidean distance. Clustering was performed by an agglomerative hierarchical procedure using the "incremental sum of squares" sorting strategy (Burr, 1970). The PC analysis was applied to the 42-member matrix [6 plants × 7 times], each with their 183 components (Gower, 1966).

SAS v. 6.12 (SAS Institute Inc., Cary, NC) was used for correlation coefficients and regression analysis of the peak areas of polygodial, with time being the explanatory variable.

RESULTS AND DISCUSSION

The GC analyses of essential oil samples produced peak area data that were converted to percentage of the total peak area. These percentages were then used in the PC and cluster analyses. Absolute values of con-centration were not determined, because it is the relative concentrations that are of interest.

The level of monoterpenes present in the extracts was determined by summing the percentage concentrations of all components that eluted before α-copaene (retention time = 10.5 min). The percentage sesquiterpenes is equivalent to [100 − (% monoterpenes)], and analyses were performed only on the monoterpene data, because the inverse would be reflected in the analysis of sesquiterpene results. The results are shown in Table 1. Extracts from plants 5 and 6 had significantly higher levels of monoterpenes than those from plants 1, 3, and 4. The extract from plant 2 had an intermediate percentage of monoterpenes, which was not significantly different from either of the other two groups. Overall, the percentage of recovered monoterpenes is below the population average of 7.95%, derived from samples of plants within the Arve Loop and the adjacent Hartz Mountain Road area.

The changes in percentage polygodial content for each plant are shown as averages in Table 1 and as individual values over the course of the experiment in Figure 1. It is clear that there are two distinct groups of plants, one with a relatively higher content of polygodial than the other. Plants 2−4 form a group with a polygodial content of <5%. The regression equations for these plants do not have a significant slope, so total polygodial is not dependent upon the number of days elapsed.

Plants 1, 5, and 6 have a polygodial content of >20%. Of these, plant 6 does not have a significant regression with time, whereas plants 1 and 5 do.

The outlines shown within Table 2 are derived from the four-group solution of the 42 member plant × days matrix by the TAXON analysis. The numbers 1−42 were used to represent the plant × day combinations. The clustering broadly shows that plants 5 and 6 differ from all of the other plants. In both groups, the later sampling times were significantly different from the early ones, with the exception of the fourth sample from plant 1.

The question of whether the six plants were the same, in a chemical sense, and whether the chemical composition was affected by the passage of time was thought to be answerable by the use of PC analysis. The results of the PC analysis are shown in Figure 2, in which the
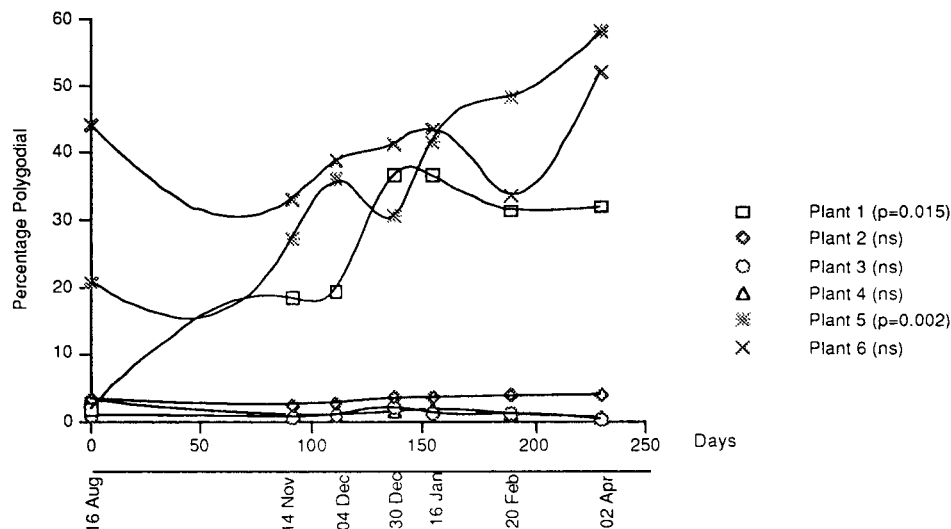

Figure 1. Variation in percentage polygodial over time.

Table 2. Result of Cluster Analysis[a]

Time (Days) \ Plant	1	2	3	4	5	6
1 (0)	1	8	15	22	29	36
2 (91)	2	9	16	23	30	37
3 (111)	3	10	17	24	31	38
4 (137)	4	11	18	25	32	39
5 (154)	5	12	19	26	33	40
6 (189)	6	13	20	27	34	41
7 (230)	7	14	21	28	35	42

[a] The four group solution is shown by the outlines. The numbers 1−42 represent the plant × day combinations as shown.
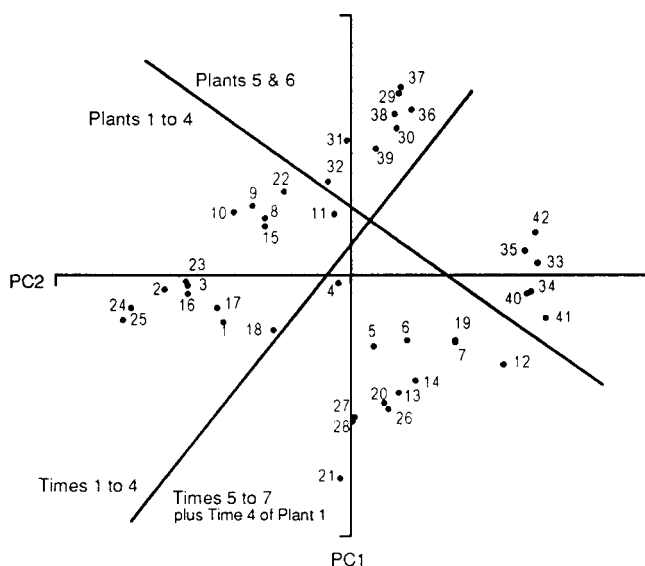

Figure 2. PC analysis. Numbers indicate the individual plant/ time samples.

first two PCs are represented by the axes PC1 and PC2. The lines drawn on the diagram delimit the four-group solution from the cluster analysis. The four groups are as follows: times 5−7 plus time 4 of plant 1; times 1−4 of plants 1−4, excluding time 4 of plant 1; plants 5 and 6 at times 1−4; and plants 5 and 6 at times 5−7.

The seven most influential components contributed 20% to the separation of the early times from the later ones. Among these were calamenene and germacrene D, with the remainder being unknown sesquiterpene components. The remaining 80% of the separation was effected by the remaining components, with no single component contributing more than just a few percent to the separation.

Similarly, only 20% of the separation of plants 5 and 6 from plants 1−4, within times 1−4, was accounted for by the six most influential components. Among these was polygodial, the others being unknown sesquiterpenes. None of the remaining components contributed more than a few percent to the separation.

Within the later three times, plants 5 and 6 were separated using six components with a similar contribution to the separation as in the early times. Polygodial, germacrene D, and eugenol were the identified components.

If polygodial alone is used as the criterion for separation, the obvious solution would put plants 2−4 in one group and plants 1, 5, and 6 in another, as shown in Figure 1 and Table 1. By including as many components as are available, the grouping is refined to plants 5 and 6 being separate from the remaining four. The analysis must include as many of the components as possible when contemplating the question of chemical similarity, to give a complete representation of the extract. This would be particularly true when using this sort of information for taxonomic purposes. Southwell and Brophy (1992) state that *T. lanceolata* extracts are predominantly monoterpenic, with 1,8-cineole predominating. This study, and other unpublished data associated with this project, indicates that sesquiterpenes are predominant in the Tasmanian plants, with monoterpenes contributing only 5% to the total extract. In addition, the concentration of polygodial approached 40%, in some instances. This compound has been isolated and purified and was found to be a principal contributor to the peppery taste of the leaves (Loder, 1962). Polygodial has also been isolated in our laboratories, confirming its hot, spicy character.

The behavior of polygodial content over the growing period shows the follwing:

(a) There are two patterns of response. Polygodial levels may either increase or stay constant over time. Groups of plants were found that exhibited each of these trends.
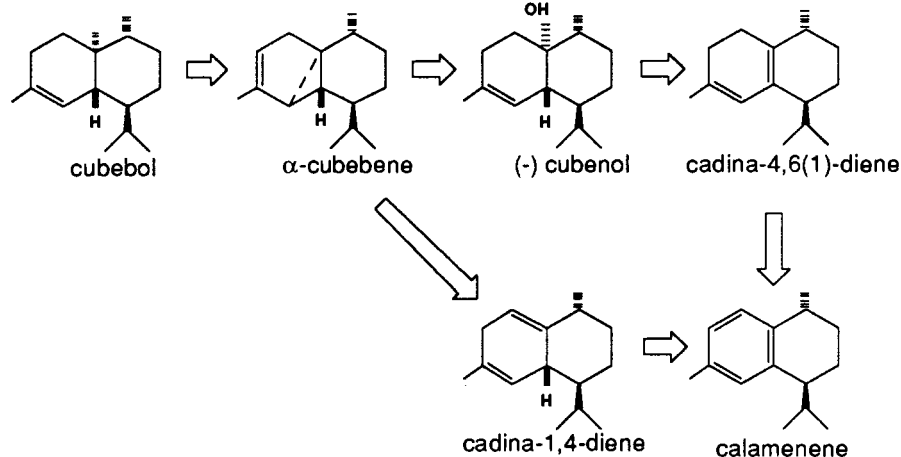

Figure 3. Biosynthetic pathways to calamenene. Correlation coefficients: α-cubebene−calamenene, −0.74; α-cubebene−cadina-1,4-diene, 0.81.

(b) There are plants that have levels of polygodial with a mean of ~30% and others that have a much lower mean, 2−3%.

It should, therefore, be possible to select plants either having a constant high level of polygodial or in which the level increases during the season. However, the overall balance of flavor components will ultimately determine which plant types are selected for commercial purposes.

The correlation analysis of component peak areas produces an array of relationships between the components of the extract. The strongest correlations were unknown peak 38 with peak 84 (−0.80), peak 82 with peak 15 (0.89), δ-cadinol with peak 61 (0.88), and peak 177 with peak 183 (0.87). α-Cubebene is negatively correlated with both calamenene and unknown peak 84 (−0.74 and −0.73, respectively). α-Cubebene is the precursor for calamenene, via one of two pathways, as shown in Figure 3. One path requires the conversion of α-cubebene to (−)-cubenol, to cadina-4,6(1)diene, and then to calamenene. The other path involves cadina-1,4-diene as the intermediate. The negative correlation may be a result of this duality, which gives calamenene production the potential to be independent of cadina-1,4-diene synthesis. Calamenene is also negatively correlated with components 93, 80, and 85 (−0.76, −0.74, and −0.60, respectively).

Among the important positive correlations are α-cubebene with cadina-1,4-diene (0.81). From the schematic of the biosynthetic pathways, α-cubebene is the precursor for cadina-1,4-diene. The regulation of these pathways produces the situation in which there is a negative correlation between the quantities of α-cubebene and calamenene and a strong positive correlation between α-cubebene and a possible precursor of calamenene, cadina-1,4-diene. The correlation between cadina-1,4-diene and calamenene is −0.55, which may point to the fact that the production of calamenene can be achieved via an alternate route.

No strong correlations with the polygodial peak were observed.

There are many relationships between components that could be studied further. However, a prerequisite for such work would be the identification of the unknown compounds in question.

In summary, the variations observed within a small population of *Tasmannia* sp. were demonstrated through the use of PC analysis and clustering. The polygodial content of the extract may be either high or low. At low polygodial content, there is no significant change in concentrations with time. In the high-polygodial group, the concentration may increase or remain constant over the course of the growing season.

LITERATURE CITED

Burr, E. J. Cluster sorting with mixed character types. I. Fusion strategy. *Aust. Comput. J.* **1970**, *2*, 98−103.

Clark, R. J.; Menary, R. C. The effect of two harvests per year on the yield and composition of Tasmanian peppermint oil (*Mentha piperita* L.). *J. Sci. Food Agric.* **1984**, *35*, 1191−1195.

Curtis, W. M.; Morris, D. I. *The Student's Flora of Tasmania*, 2nd ed.; T. J. Hughes, Government Printer: Tasmaniaa, 1975; Vol. 1, p 25.

Gower, J. C. Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrika* **1966**, *53*, 325−328.

Loder, J. W. Occurrence of the sesquiterpenes polygodial and guaiol in the leaves of *Drymis lanceolata* (Poir.) Baill. *Aust. J. Chem.* **1962**, *15*, 389−390.

Read, C. Aspects of Leaf and Extract Production from *Tasmannia lanceolata.* Ph.D. Thesis, University of Tasmania, 1996.

Reid, J.; Hill, R. S. Rainforest invasion onto Tasmanian old fields. *Aust. J. Ecol.* **1983**, *8*, 149−161.

Ross, D.; Shields, K. *TAXON Users' Manual*, personal computer version 1.0; Mt. Ommaney, Australia, 1993.

Shimoda, M.; Wu, Y.; Nonaka, S.; Osajima, Y. Cluster analysis of GC data on oxygenated terpenes of young leaf and green fruit samples of Japanese pepper (*Xanthoxyum piperitum* D.C.). *J. Agric. Food Chem.* **1997**, *45*, 1325−1328.

Southwell, I. A.; Brophy, J. J. Differentiation within the Australian *Tasmannia* by essential oil comparison. *Phytochemistry* **1992**, *31*, 3073−3081.