RURAL INDUSTRIES RESEARCH
& DEVELOPMENT CORPORATION

Mountain Pepper Extract

Tasmannia lanceolata

Quality stabilisation and registration

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Mountain Pepper extract (*Tasmannia lanceolata*): Quality Stabilisation and Registration

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Foreword

The essential oils industry in Tasmania is progressing with investigation of novel, uniquely Tasmanian products. To that end, research has continued with *Tasmannia lanceolata* extract, following wide-ranging interest from overseas flavour and fragrance companies.

Market potential has been established in Japan, Europe and the United States. However, the product has remained largely unsaleable due to a lack of formal registration and confirmation of its safety for use in perfumes and flavourings in Europe and the United States.

The work presented here is based on the results of a previous project, (RIRDC Reference No UT-11A), where the need for product registration was clearly identified. Emphasis is on environmental issues, uniformity of product from native stands, clonal selection and requirements for registration.

This project was funded from RIRDC Core Funds, which are provided by the Federal Government, and a contribution of industry funds.

This report, a new addition to RIRDC's diverse range of over 800 research publications, forms part of our Essential Oils and Plant Extracts R&D program, which aims to support the growth of a profitable and sustainable essential oils and natural plant extracts industry in Australia.

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Simon Hearn

Managing Director

Rural Industries Research and Development Corporation

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Contents

Executive Summary

The solvent extract of *Tasmannia lanceolata* (Mountain Pepper), is a dark, lime green product, which has a distinctive and exotic aroma. It possesses fresh, spicy top notes overlying a sharp peppery background. The pungent principle is polygodial, which is the main constituent of the extract.

Currently, throughout the marketplace, there is a great deal of interest in Mountain Pepper extract, due to its unique character and 'natural' status. The greatest impediment to its sale has been the fact that it is not registered with an authorised regulatory body. In the United States, such a body is the Fragrance and Extract Manufacturers' Association (FEMA), which maintains a list of substances that are generally recommended as safe (GRAS). This project focussed on determining the information necessary in order to gain registration on the GRAS list.

A study of the amount of variability in the natural population was undertaken. This provides valuable information on aspects of genetic variation and availability of suitable genetic material for propagation and eventual establishment of clonal plantations. It showed that within a given population, there is the potential for some 65% of trees to have a safrole level of less than 0.0002%. This is important, since there have been restriction placed on the amount of permissible safrole in foodstuffs. The study also demonstrated that there is a large amount of variation within a population, and so, there is scope for selection of particular desirable traits, should plant breeding be attempted.

Environmental degradation of the extract was investigated. Degradation was rapid in soil cultures compared to pure cultures, where relatively little decrease was seen after four weeks. In a soil medium, components of the extract were undetectable in that time. The incorporation of a surfactant into the soil system allowed for even dispersion of the viscous oil through the soil, and enhancing its degradation.

A product sheet has been developed by Essential Oils of Tasmania, in conjunction with the University. This, together with the material safety data sheet, are now available to users of the extract. The extract has a Chemical Abstract Service number: CAS No. 183815-52-3.

A clone library has been established and maintained, which houses 63 of the most distinctive and high yielding of the selections. A further fourteen types are also being maintained as alternative choices.

A pre-submission review was conducted by FEMA. The comments made have been noted and acted upon, where possible. It may be possible to submit the extract as a 'natural' to the expert panel. Previously, this has not been possible, since the GRAS list was only for pure substances. In order to be successful with such a submission, the panel would require the data we have accumulated on composition, as well as the information presented in this report on variation in the source population. Toxicological studies are also required, and negotiations are still in progress between flavour companies which may be interested in funding a registration bid, and toxicology laboratories which would be able to conduct the necessary tests. To that end, a meeting was held with ICP Firefly, which is a NATA approved toxicology and efficacy laboratory based in Sydney. Another company, Citrus and Allied Essences Ltd., in the United States, have also expressed keen interest in progressing with the registration of *T. lanceolata* extract. However, no further progress has been made to date.

The registration situation in Europe has been evaluated, with the conclusion that a FEMA GRAS status would enable straightforward registration elsewhere.

Japan is the only country, so far, where the extract is being used commercially. It has been included in such products as chewing gum, candy and wasabi paste. A United States patent has been issued, detailing the use of polygodial and polygodial containing extracts as a flavour enhancer in a variety of products.

Other marketing feedback from Europe has also been very positive, with potential users being familiar with, and eager to use our product, provided that registration is achieved.

1. Introduction

1.1 General

The solvent extract of *Tasmannia lanceolata* has attracted the attention of a number of major international flavour and fragrance houses. As such, it has the potential to earn export dollars for the community. Since 1994, 50Kg of extract has been used in Japan annually, in a very limited product range. The anticipated volumes following the introduction of broader food categories could be in the order of 250Kg per annum extract in Japan alone, with a current value of AU$ 250-500,000 per annum.

To date, there have been major difficulties in marketing the product to European and United States buyers, since they must comply with more stringent regulations that prevent or hinder the purchase of unregistered raw materials. Although market interest is strong at the user level, that is, amongst flavour and fragrance companies, they must be satisfied that the extract will be safe for the proposed uses and use levels.

In order to ensure such safety and confidence in the product, a formal registration with an internationally recognised body, such as the Flavour and Extract Manufacturers' Association (FEMA), on their Generally Recommended As Safe (GRAS) list, is mandatory.

A direct benefit of registration is the facilitation of marketing efforts.

It also means increased confidence amongst growers who would need to commit themselves in the long term, for successful *Tasmannia* production. This is most effectively communicated through endorsement of product by various regulatory agencies.

Registration also helps to define standards, such that there are benchmarks for quality control.

This will be the first time that the formal process of registration through the FEMA (GRAS) program has been attempted by producers in Australia, and, as such will serve to guide other products through to registration.

Other beneficiaries of this research will be the growers and marketers, in that their product diversity is enhanced. They will also be seen to be promoting an environmentally friendly crop, since *T. lanceolata* is a native species, which will require minimal treatment with pesticides and fertilisers. This will lead to enhanced ecological sustainability and preservation of biodiversity.

The Japanese will be commercialising this extract as a modifier of mint flavours in chewing gum, toothpaste, hard candy, drinks or as a condiment using dextrin as a carrier. These uses have formed the basis of a patent.

1.2 Background

Morphology and development of *Tasmannia lanceolata* (Winteraceae) has been examined by Bailey and Thompson (1918) and Foster and Gifford (1974), who studied the 'vesseless' transport system of *Drymis* and *Tasmannia* species.

Bailey and Nast (1945 a, b and c), Smith (1969), Vink (1970) and Bongers (1973) describe the morphology of the plant in detail, while Gifford (1950), Tucker and Gifford (1964, 1966 a and b) and Sampson (1987) address questions of floral ontogeny and vascular development.

While the taxonomic relationships of members of the genus have been debated in the literature (Vink (1970, 1988), and Smith (1969)), and the family has proved a rich source of novel secondary compounds, (Corbett and Grant (1958), Appel *et al* (1963), Cruz *et al* (1973), Cambie (1976), Sierra *et al* (1986), Vichnewski *et al* (1986)), chemotaxonomic studies of the genus are few (Southwell and Brophy (1992)). The use of the novel compounds present in extracts and oils of Winteraceous species in trials for bioactivity (antibiotic, antifungal, antifeeding and piscicidal) are well reported and polygodial, a dominant component in the extract of *Tasmannia lanceolata* leaf (first reported by Loder (1962), has proved unusually potent in most of these respects (Kubo (1988), Taniguchi *et al* (1988), Himejima and Kubo (1993), review by van Beek and de Groot (1987), Kulakkattolickal (1989)). In particular, work by Kubo and Himejima (1991) in which anethole serves as a powerful synergist for polygodial against filamentous microoganisms may indicate a cost effective future use for the compound.

There is little reference to the use of whole extracts in flavour compounding for any of the Winteraceae species. Reports of medicinal and culinary use amongst indigenous people is usually in the nature of anthropological commentary describing cures for skin and venereal disease, colic, 'cattle itch' and stomach ache or as a possible quinine substitute (Retamar (1986), Salmon (1980)). *Tasmannia lanceolata* bark powder was used as a substitute for herbal remedies prepared from the South American *Drimys wintera*, (LeStrange (1977)) and berries and bark were proposed as allspice or pepper substitutes (Maiden (1899)).

Within Australia there is currently a small trade in preparations of whole, dried and fresh plant parts for the newly fashionable 'indigenous foods' industry.

This group has undertaken preliminary work in the development of basic harvesting and drying techniques, extraction protocol and polygodial purification procedure, and an accurate analytical procedure for assessing yield and composition of extract. Standard physical characteristics of the extract and some preliminary organoleptic procedures have been established. We have conducted a preliminary survey of populations of the plant in Tasmania and established that polygodial content varies from trace levels to over 67% of the extract on a dry matter basis, and found similar variation amongst individuals with respect to other secondary compounds.

Investigation of content, intra species variation and seasonal variation of safrole in the plant extract has been completed. A database containing information on extract, plant and site characteristics from around the state has been developed. This will prove useful in formulating blends to meet specific criteria.

Advances have been made in terms of identification of extract components, which will assist in fulfilling registration requirements. This information is also being used to assist the dried herb industry with quality standards.

1.3 Population variation

Commercial extractions of leaf of Tasmannia lanceolata depend, at the present time, on supplies of leaf material gathered from extensive stands of the species growing on land cleared of rainforest during the middle of the last century for forestry or farm use, and subsequently abandoned.

Leaf is collected, dried and consolidated for many hundreds of trees, before it is milled then extracted to produce a product whose composition reflects an 'average' for the trees selected.

Earlier work (Read and Menary, 2000, Dragar *et al*, 1998) has shown that the natural population of this species in Tasmania shows wide variation in the characteristics of the extract produced from leaf material. In order to assess the extent of variability of extract among trees at one site, the following survey was devised. A random sample of some 320 trees, spread more or less uniformly over an area of approximately 18ha was selected. This area was located in the midst of one of the main sources of leaf material used in the current pre-commercial phase of extract production.

2. Objectives

The major objective of this work is to provide results and information, which will be used to gain registration for a generic extract of *Tasmannia lanceolata* with an internationally recognised body. Thus, the detailed requirements for registration need to be recognised and systematically fulfilled.

As a consequence of the preliminary review by FEMA, a variation study is to be conducted. In addition, the toxicological requirements for registration will be determined.

The maintenance of clones will be an on-going aspect of the project. This part of the program will allow expansion to clonal plantations with predictable yield and quality.

3. Methodology

3.1 Clone collection

The Horticultural Research Centre houses a comprehensive assortment of *T. lanceolata* clones. Over 100 clones were available in all. Of these, 46 were identified with suitable characteristics for replicated planting. A low safrole content of <150ppm, yield of >2.5% and a lantana character to the extract were required for inclusion in the outside holding area. Three blocks were established, containing 3 replications of 46 plants. In addition, the clones not represented here were maintained in pots in a shade tunnel.

Several designs were considered, including blocks of replicates and single tree plots. The latter have several distinct advantages over the former, in that the number of plants (and hence, the area), required are minimised. In addition, the possible confounding of environmental and genetic covariances among members of a genetic unit is virtually removed by single tree plots (Loo-Dinkins *et al*, 1990).

The plants were placed 0.8m apart in double row beds. Each bed being 2m wide, with 3m between beds.

During the course of the project, several clones were lost in the trial and some were replaced by alternative selections.

3.2 Population variation

3.2.1 Field sampling:

Plant material was gathered from a site south of Winneleah, in NE Tasmania, adjacent to a forestry site known as the Star of Peace plantation. The approximate Universal Grid Reference is 55GEQ 715 370. (Easting: 573000, Northing: 5437000)

Sample locations were set out on a 20m grid, oriented magnetic NS, and samples of 10-12 mature twigs produced during the preceding summer were collected at 50-70% of the height of the tree, and where possible from the north-eastern aspect of the canopy. Samples were placed in numbered paper bags, which were then stored in the shade in large plastic bags.

In the case where no tree could be found within 4m of a sampling location a 'blank' was recorded.

Each sampling location was logged using a hand held GPS recording device (Garmin GPS 12) and the tree tagged prominently with pink surveyors tape. The output generated by the GPS system was used to produce the representation of the sampling path shown below, in which three data points (I11, P19 and P20) are missing. The area covered by the sampling path measured approximately 400 x 450m (18 hectares) Figure 3.1.

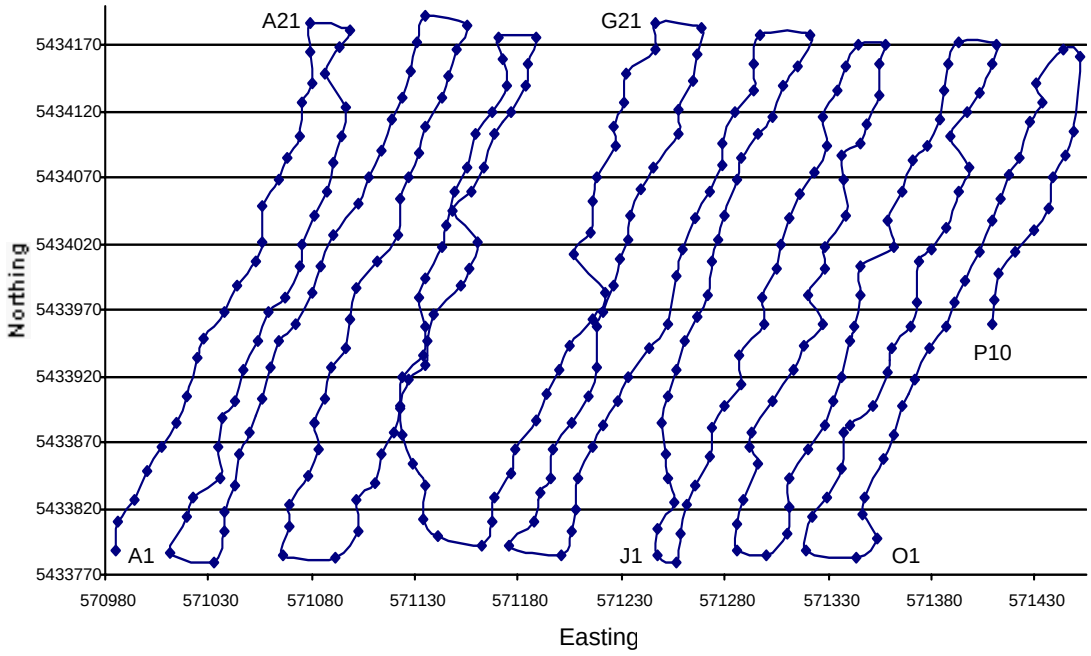


Figure 3.1: Sampling pathway: Rows A-P, each having 21 sample points, numbered from south to north

3.2.2: Analysis

Sample bags were dried in a thermostatically controlled drying cabinet incorporating a circulation fan and set to 35°C.

Eight to ten leaves from each sample were ground to a fine powder, 200mg of which was weighed into a 20ml glass vial, to which was added 5ml of redistilled hexane containing 1mg of C18 standard (octadecane).

Duplicate extraction and analysis was conducted on leaf material of the first ten trees sampled.

The vials were capped and shaken for 2hrs at room temperature, then left to settle before a 1ml aliquot was transferred to a GC vial , and analysed using an HP5890 Series II gas chromatograph equipped with an HP1 column (30m, i.d. 0.32mm, film thickness 0.25µm), operating at head pressure of 8psi, and injector and FID detector temperatures of 250 and 280°C respectively and injections subject to a split ratio of 50:1. Oven temperature for the analysis was programmed: 50°C (1 min) - (20°C min^{-1}) - 140° -(3.5° C min^{-1}) - 200° - (15° C min^{-1}) - 280° (7.02 mins). Sample size was 1µL.

Peak areas for signals between retention times 4.67 and 34.46 min., were integrated, rejecting peaks of area less than 1000, with the exception of a small window accepting peaks down to 100, between 8 and 8.2 minutes, (the approximate retention time for safrole by this method) and corresponding to a safrole content of about 0.0005% of leaf dry weight.

Compositional data are presented in reference to the internal standard by the calculation:

% of Compound Z = {Wt(g) C_{18} Std}/{Wt (g) leaf} x {Area Z}/ {Area Std} x 100
where a response factor of 1 was assumed.

The term 'percentage volatiles' refers here to results calculated using the total peak area for the integration described above.

[A separate determination of the response factors for polygodial (1.51), and safrole enables estimation of % polygodial and % safrole by weight in the leaf sample, although these were not used to prepare the data presented below].

Data entries and calculations were carried out using Excel® 98 spreadsheet and workbook templates.

3.3 Biodegradation of *T. lanceolata* Extract by *Pseudomonas* sp. in Culture

The first seven oil-degrading isolates obtained by enrichment procedures were used for this assay.

Five flasks were set up for each isolate with 0.5%v/v essential oil, together with controls of uninoculated BMS (basal mineral salts medium) plus 0.5%v/v essential oil (for losses to volatilisation), BMS alone and inoculated BMS. The flasks were incubated at 25°C for one to four weeks.

At weekly intervals for a period of one month, one flask of the culture medium was taken and extracted as described below, prior to analysis using gas chromatography (GC).

3.3.1 Method of Oil Extraction from Growth Medium for GC Analysis

i. An internal standard solution of octadecane (C_{18}) was prepared by dissolving 0.1622g of C_{18} in dichloromethane (DCM) in a 100mL volumetric flask. DCM was added up to the bottom of the stem and the flask was then placed in a scintillator until the C_{18} was dissolved. DCM was then added up to 100.0mL.
ii. To each flask of culture and oil, 5mL of the DCM/C_{18} standard was added using a 5mL Eppendorf syringe. The flasks were then swirled and left for 10 minutes covered with aluminium foil.
iii. The DCM layer was removed using a Pasteur pipette into 2mL glass GC vials until approximately 2/3 full, making sure no particulate matter was included. The vials were capped and labelled.

Samples were analysed using a Hewlett Packard GC, HP 5890 unit fitted with an HP 7673A automatic injector and a flame ionisation detector (FID) detector. A 30m BP1 column with 0.22mm ID (internal diameter) and 0.25µm film thickness was used. The carrier gas was high purity nitrogen run at a pressure of 22psi. The column flow rate was 1.2mL/min with an injection volume of 2µL. The split vent flow was 48 mL/min and the purge vent flow was 3 mL/min. The injector temperature was 250°C and the detector temperature was 280°C. The temperature program was an initially 50°C for 1 min, 20°C/min to a final temperature of 150°C, 5°C/min to a final temperature of 215°C and 15°C/min to a final temperature of 280°C. The detector gases were air at 250 mL/min and hydrogen at 25 mL/min with a makeup gas of high purity nitrogen at 30 mL/min.

From the GC chromatograms representative peaks were identified and the amount of each compound relative to the C_{18} internal standard was calculated. Changes in amounts of specific compounds present in the growth medium at weekly time intervals were then determined.

3.4 Soil Microcosms

Microcosms using the following soil types: podzolic on dolerite, black cracking clay, North West red, sandy loam and a soil from a Mt Wellington *T. lanceolata* habitat were set up in sterile 250mL Erlenmeyer flasks. To each flask, 100g dry weight of soil was added aseptically.

The field capacity of each soil type was determined by adding a known volume of distilled water to 10g of soil until the soil was saturated. Excess water was removed by filtration with a Buchner funnel, and the volume in excess was measured and subtracted from the original amount added.

The test microcosms were set up by adding 500mg of oil to the amount of water required to bring the soil to field capacity, in a McCartney bottle. To this, 10 drops of a commercial surfactant (Chemspray Betta Wetta) was added. The bottles were cooled on ice, to prevent volatilisation, before sonication for 60 seconds. This oil/water emulsion was then added to the soil in a sterile flask and aseptically mixed to achieve even dispersion.

Three controls were included; one containing water only and the second containing only the surfactant. The third control comprised of a 100g sample of one soil type (podzolic on dolerite) which was sterilised by autoclave at 121^{o}C for twenty minutes. The microcosm was then set up using an oil and surfactant emulsion as above. This microcosm served as a control for losses to volatilisation. All microcosms and controls were set up in quadruplicate

Flasks were sealed with sterile cotton plugs, covered with aluminium foil and incubated in the dark at 25^{o}C for a period of two months.

A time zero extraction for GC was carried out for each microcosm. The flasks were then weighed before being placed in the incubator. At each subsequent week the flasks were weighed and any water lost during the week was replaced using sterile distilled water. A 4.0g sample of soil was also removed from each microcosm at weekly intervals and tested for oil utilisation using the method described below. The microcosms were reweighed before being returned to the incubator. GC analyses were carried out at weekly intervals for eight weeks.

3.4.1 Method of Extraction of Oil from Soil for GC Analysis

i). Samples of soil (4.0g) were aseptically removed from each microcosm and weighed in sterile 100mL conical flasks.
ii). To each flask, 25µL of DCM containing 1.624mg of C_{18} was added using a glass syringe, followed by 3mL of DCM was added using a 5mL Eppendorf syringe. The flasks were swirled, covered, and allowed to stand for 10 minutes.
iii). The DCM layer was removed into 2mL glass GC vials using a Pasteur pipette, until the vials were 2/3 full.

Samples were assayed using a Hewlett Packard GC, HP 5890 unit fitted with an HP 7673A automatic injector and a flame ionisation detector (FID) detector. A 30m BP1, 0.22mm ID (internal diameter) and 0.25µm film thickness was used, with the same column and settings as used for the pure culture degradation assay. However, the controls were also run splitless to detect any components present in very small amounts. The splitless injection method was the same as used previously but with the purge valve off initially and then turning on at 1min.

From the chromatograms, representative peaks were identified and the amount of each compound relative to the C_{18} internal standard was calculated. A comparison of change in the amount of a compound present over time was carried out to examine patterns of degradation and volatilisation.

3.5 Safrole Level Determination

3.5.1 Extraction

A low safrole clone was identified for use as the matrix for all the standard curve solutions. Approximately 1kg of leaf and stem material was dried in the oven at 35°C for 72 hours. The dried leaf material was ground to a fine powder using a hammer mill.

The weighed sample was extracted with 3 x w/v of petroleum ether, in a lidded vessel. The sample was sonicated for 10min and the solvent filtered through a cotton wool plug into a pre-weighed round bottom flask. The second and third washes were combined and dried down on the rotary evaporator. Final dry down was at 60°C for 5min, before re-weighing.

3.5.2 Analysis

20-30mg of matrix extract was combined with 10µL of 40 mg/mL C_{18} standard solution in each of 5 samples spiked with 0, 0.01, 0.05, 0.1 and 0.2 ppm safrole.

Duplicate samples of the commercial blend were prepared with 30mg of extract. Each sample contained 10µL of 40 mg/mL C_{18} standard solution in order to quantitatively estimate peak areas.

The samples were analysed by GC-MSD at the Central Science Laboratory.

Peaks were initially identified by GC/MS of splitless injections of 1 µL samples on a Hewlett Packard HP 5890 GC coupled via an open split interface to a HP 5970B mass selective detector (MSD). The GC was equipped with an HP1 fused silica capillary column (25 m x 0.32 mm i.d., 0.17 µm film thickness). Oven temp program: 50°C held for one minute, then 30°C/min to 220°C, then 10°C/min to 290°C held for 5min. Injector temp: 250°C. Electron ionisation was undertaken with a source temperature of 200°C and electron energy of 70eV. The ions monitored were 77, 131, 104 and 162 for safrole and 254 for the internal standard.

A standard curve was obtained which relates ppm safrole to the ratio of the response factor, calculated as the areas of the safrole peak 162 relative to the area of octadecane.

Statistical analysis was performed using Excel® 98 spreadsheet statistical functions.

3.6 Analysis of Commercial Extract

A manual on-column injection was performed of the commercial *T. lanceolata* extracts. Samples were obtained from Essential Oils of Tasmania from their bulk holdings. The samples were made up to a concentration of 3mg/L. The composition of the extract was determined using a Hewlett Packard (Palo Alto Ca. USA) HP 5890 unit, with control and data analysis by HP/Chemstation 3365 software. 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 250ºC and the detector temperature was280ºC. The temperature program was 50ºC(1min), ramping by 20ºC/min to 150ºC, then 5ºC/min to 260ºC (5min).

4 Results

4.1 *Tasmannia lanceolata* clone collection

The specimens chosen for inclusion in the replicated holding area are shown in Table 4.1. Their extract aroma characteristics are given, along with levels of polygodial, monoterpenes, sesquiterpenes and safrole. The yield is also shown on a dry matter basis. This area has been maintained as far as possible, in order to provide material for future experimental work. The layout is suitable for any replicated experimental program.

The survival of plants in the open 'plantation' situation was highly variable. The effects of rabbit predation, uneven drainage and/or poor drainage were evident.

By the end of the project over 50% of the trial plants had to be replaced. The surviving types that were not represented in the outdoor holding area were maintained in pots in the shade tunnel.

Of the original selected clones, several were removed from the trial design and replaced with alternatives.. This proved necessary to preserve the integrity of the design when some selections died and identical replacements were not available.

Table 4.1: Characteristics of clones selected for replicated holding area

Clone	% Yield (dmb)	Safrole ppm	Total % Polygodial	% Mono-terpenes	% Sesqui-terpenes
AL1	4.11	0.66	20.14	6.63	93.37
strong lantana spicy, woody lantana balanced, powerful					
AL2	3.66	20.6	11.43	4.70	95.83
lantana; spicy					
AL3	3.52	25.7	27.87	48.3	95.17
spicy; fruit; strong					
BR1	3.50	49.30	41.94	8.38	91.62
spicy nutmeg; medium lantana and spice					
FG1	4.58	50.70	0.25	5.46	94.54
mint; very strong Tasmannia spice; spice lantana					
FG2	5.76	5.20	0.00	5.63	94.37
strong lantana; limey green note; lantana					
FG3	5.19	11.30	0.00	2.04	97.96
Tasmannia; nutmeg, green minty; lantana spice					
FG5	2.68	44.00	0.00	2.26	97.74
sweet; coarse terpine-like with spice; strong lantana spice					
FG6	3.23	18.10	0.00	4.02	95.98
sharp sweet raspberry; dusty cinnamon with fresh floral; strong lantana spice woody (best)					
FG7	3.32	13.60	0.00	2.94	97.06
faint sweet spicy; faint floral; fruity med/strong lantana					
FG8	4.67	9.00	23.09	3.05	96.95
faint sweet floral rose; spice floral; woody lantana					
GL6	2.90	3.40	49.65	2.65	97.35
spicy, citrus; similar to GL5; clear green note, lime					
GL7	2.41	7.50	0.50	6.14	93.86
spicy; citrus, slight lantana; faint, clear, fragrant					
GL11	3.27	17.20	2.53	2.84	97.16
lantana; peppery, dusty, lantana; floral sweet					
HY2	3.74	19.60	44.44	13.71	86.29
lavender strong spicy; rose like spicy lavender; sweet menthol					
HY3	3.92	76.80	57.01	3.36	96.64
lime; flatter greener note with citrus; fruity sweet					
HY4	3.47	64.80	11.41	5.00	95.00
very persistent; sharp spice; sweet floral; spicy lantana					
HY5	3.79	6.60	25.75	4.42	95.58
woody spice sassafras; floral spice sweet; spicy, persistent					
HY6	3.76	12.10	53.75	8.14	91.86
sharp similar to HY5; harsher citrus earthy woody; spicy lantana, persistent					
HY7	3.67	27.40	55.90	1.72	98.28
spicy woody slight lantana; lemony cinnamon; spicy					
HY9	3.12	39.00	47.79	4.24	95.76
fruity sweet persistent; floral; herby					

Clone	% Yield (dmb)	Safrole ppm	Total % Polygodial	% Mono-terpenes	% Sesqui-terpenes
HY10	3.02	52.00	55.42	6.37	93.63
fruity sweet persistent; spice; fruit					
HY11	3.78	40.50	37.52	6.22	93.78
sharp onion; persistent; faint pine harsh solvent-like; citrus lantana					
HY12	4.93	74.30	22.26	5.75	94.25
spicy persistent; lime sweet green, lantana					
HY13	3.43	164.10	20.91	5.43	94.57
woody Tasmannia lantana; high light and spicy					
HY14	3.36	37.50	28.35	5.28	94.72
spicy; herbaceous low; lantana fruity					
HZ1	2.53	1.72	32.73	8.88	91.12
lantana, woody sassafras, strong sweet lantana pepper					
MB1	2.53	7.50	48.31	4.34	95.66
burnt sharp, slight lantana balanced					
MB2	5.20	27.10	23.68	8.80	91.20
tropical fruit spicy, strong lantana (high volatiles) very fruity					
MB5	3.81	69.20	62.26	2.70	97.30
sweet					
MB6	2.64	2.30	21.14	3.67	96.33
fewer top notes, strong lantana, medicinal					
MB8	2.96	29.10	25.45	2.73	97.27
lantana sweet					
MB10	2.69	46.60	50.63	4.80	95.20
rounded sweet fruity spicy, medium lantana (good)					
NW1	4.76	11.70	10.01	11.20	88.80
lemon/lime; floral; lemon; soapy spicy					
NW1	7.55	207.9	54.88	6.51	93.49
woody sassafras pine; pineapple; fishy, oily, slightly lantana					
PP3	3.44	7.20	50.63	5.27	94.73
Tasmannia; strong to medium lantana and spice					
RR1	6.02	2.90	37.77	4.72	95.28
aromatic spicy lantana, (good)					
TR2	3.11	131.20	63.16	4.35	95.65
lantana; fruity lantana; bushy fruity tending to lantana					
TR3	3.98	53.90	44.82	3.96	96.04
sharp spicy; stronger lantana fruity; like TR2 stronger lantana					
UB4	2.56	7.00	63.99	6.03	93.97
sweet fruity spicy; bitter strong; woody/spice some lantana					
WH1	2.45	215.40	51.94	6.58	93.42
strong nutmeg; very spicy, citrus					
WH4	3.17	86.60	16.43	6.49	93.51
fruity sweet; weak; spicy woody					
WH5	3.05	58.40	16.26	5.55	94.45
faint citrus; musty faint; fruit spicy					

4.1.1 Population Variation

Results of the analyses for all trees sampled are included in Appendix 1.

Samples for which low levels of safrole were obtained have been separated and are grouped below, sorted in each case for descending polygodial content. Together the two groups (that is all samples having % safrole <0.002%) account for about 65% of trees sampled, Tables 4.2 and 4.3.

Table 4.2: Trees having Safrole <.0005 (96 trees)

Tree	% volatiles	% polygodial	Tree	% volatiles	% polygodial
G12	6.79	4.97	O12	3.99	2.03
A4	6.70	4.68	G3	3.01	2.03
J8	5.34	3.49	M12	3.24	2.03
I9	4.86	3.45	I18	3.45	1.97
O7	5.61	3.42	I4	3.12	1.96
E8	4.97	3.41	I6	5.14	1.92
N18	4.97	3.34	P15	2.96	1.87
G18	5.35	3.18	L6	4.98	1.81
C19	4.18	3.13	C20	2.57	1.75
F5	4.34	3.03	C16	3.11	1.69
J1	4.67	3.02	L14	2.85	1.68
G20	4.54	3.02	H13	4.30	1.65
H17	4.57	2.98	L13	3.14	1.65
D13	4.56	2.95	K5	3.99	1.63
E12	4.08	2.85	O6	5.20	1.62
H15	4.67	2.83	K15	2.85	1.60
H20	4.05	2.79	F15	4.30	1.55
H16	4.32	2.77	P14	2.29	1.54
H21	4.14	2.75	D4	2.45	1.51
L18	5.15	2.70	D5	3.81	1.50
E15	3.87	2.65	I8	2.63	1.50
H11	3.90	2.58	M2	5.09	1.43
N15	3.88	2.54	M16	8.19	1.43
N7	4.06	2.53	D1	2.17	1.37
P21	3.81	2.47	I10	3.82	1.34
G16	3.96	2.43	K16	2.36	1.34
J5	4.21	2.37	H8	4.72	1.34
M10	3.67	2.37	A21	6.35	1.33
P10	3.71	2.36	B4	3.76	1.21
B7	3.37	2.28	K11	2.56	1.20
D7	3.58	2.27	M20	3.76	1.06
O15	5.22	2.25	L19	3.03	1.02
E2	4.88	2.25	O2	2.97	1.00
E9	3.37	2.24	B21	1.63	0.99
M11	4.30	2.24	K3	4.37	0.98
P11	3.24	2.23	O8	4.34	0.98
M15	3.63	2.23	O13	3.64	0.95
K21	4.11	2.21	J13	4.10	0.95
J12	3.75	2.20	C12	2.41	0.87
B19	3.49	2.19	D18	3.50	0.80
M7	3.64	2.17	K7	3.62	0.79
N5	3.96	2.16	J17	4.87	0.71
I1	3.69	2.16	J3	3.90	0.66
J10	3.54	2.13	K2	1.91	0.57
M21	4.46	2.10	I14	2.06	0.41
D2	3.03	2.09	O14	1.79	0.24
D6	3.34	2.08	J6	1.96	0.21
K6	3.58	2.06	L12	3.85	0.18

Table 4.3: Trees with safrole .0005 <s> .002 (103 trees)

Tree	%polygodial	% volatiles	% safrole	Tree	%polygodial	% volatiles	% safrole
F21	4.51	6.55	0.00077	M5	2.08	3.44	0.00141
N1	4.30	6.25	0.00093	L21	2.04	1.24	0.00123
B15	3.75	5.29	0.00060	E17	1.98	3.46	0.00134
K18	3.72	5.45	0.00163	I3	1.97	3.57	0.00089
F19	3.60	5.22	0.00170	I20	1.96	3.07	0.00167
J15	3.47	5.17	0.00175	A12	1.96	3.13	0.00119
A11	3.46	5.53	0.00185	J20	1.96	3.17	0.00061
F14	3.36	4.72	0.00178	K10	1.92	6.06	0.00095
O20	3.29	4.97	0.00066	L16	1.91	3.37	0.00144
H14	3.27	4.61	0.00094	D3	1.90	2.99	0.00110
O21	3.25	5.29	0.00123	G15	1.84	5.27	0.00185
O17	3.24	4.63	0.00114	N20	1.82	3.08	0.00160
N11	3.24	4.90	0.00196	A10	1.82	2.79	0.00050
L9	3.23	4.86	0.00054	L17	1.74	2.95	0.00124
B13	3.19	5.31	0.00070	G2	1.69	3.40	0.00179
L3	3.12	4.93	0.00067	L15	1.65	4.71	0.00180
G10	3.11	8.04	0.00084	F3	1.55	4.66	0.00054
B10	3.10	4.90	0.00101	D15	1.50	3.87	0.00188
E13	2.89	4.36	0.00106	O19	1.50	5.12	0.00153
L8	2.88	4.87	0.00160	J19	1.45	2.53	0.00071
I11	2.84	4.12	0.00126	H12	1.43	5.77	0.00072
O4	2.80	4.29	0.00152	P18	1.40	4.66	0.00070
I15	2.67	3.99	0.00120	G17	1.36	2.45	0.00167
P20	2.65	4.05	0.00198	M14	1.30	5.21	0.00088
C10	2.64	3.91	0.00146	O16	1.25	2.55	0.00168
O5	2.55	3.95	0.00127	E16	1.22	3.83	0.00122
D20	2.51	3.86	0.00130	E1	1.14	2.01	0.00054
G5	2.48	3.87	0.00077	C6	1.11	4.79	0.00188
G13	2.44	3.80	0.00108	G9	1.10	4.31	0.00057
F18	2.41	4.67	0.00156	J18	1.02	3.49	0.00124
I19	2.39	3.99	0.00153	F10	1.01	4.60	0.00079
N14	2.37	3.78	0.00142	A20	1.01	2.51	0.00120
A7	2.37	5.65	0.00131	I7	1.01	2.01	0.00089
N10	2.35	3.89	0.00139	A3	0.99	2.66	0.00188
L4	2.33	3.44	0.00109	K1	0.96	3.07	0.00085
I2	2.33	3.56	0.00180	C15	0.79	4.42	0.00127
H10	2.31	4.50	0.00062	M8	0.75	4.29	0.00111
G1	2.28	3.59	0.00191	L2	0.73	4.97	0.00130
J14	2.28	3.63	0.00200	J4	0.69	3.18	0.00184
P19	2.25	3.81	0.00133	P17	0.67	2.45	0.00080
K14	2.25	3.46	0.00092	G19	0.65	3.57	0.00085
M19	2.24	3.70	0.00199	M1	0.64	1.72	0.00193
B1	2.22	3.74	0.00181	E3	0.62	2.69	0.00135
A16	2.22	3.45	0.00172	L20	0.61	2.58	0.00076
M17	2.19	3.82	0.00063	B9	0.60	4.04	0.00100
C18	2.17	3.43	0.00067	L5	0.55	2.88	0.00140
J16	2.16	3.37	0.00138	I13	0.52	3.65	0.00103
L11	2.16	3.22	0.00103	C3	0.47	3.62	0.00154
O1	2.13	3.40	0.00091	J7	0.45	5.60	0.00061
H6	2.12	3.50	0.00153	G4	0.38	3.97	0.00138
J11	2.08	3.74	0.00130	B11	0.33	6.11	0.00147
				K8	0.31	2.09	0.00064

Trend in production of GC artefact for polygodial

The percentage of polygodial breakdown product, eluting approximately 3.2 minutes before the polygodial peak during the chromatography run is shown in Figure 4.1 as a ratio with the main polygodial percentage, and shows an interesting trend during the analysis of the more than 300 samples prepared in this trial.

In the assessment of polygodial yield employed here, the percentage of the two peaks is summed, so that the observed trend will not have affected the overall result.

Nevertheless, it appears that some progressive change in conditions in the injection chamber or column is altering the tendency of polygodial to degenerate during the analysis.

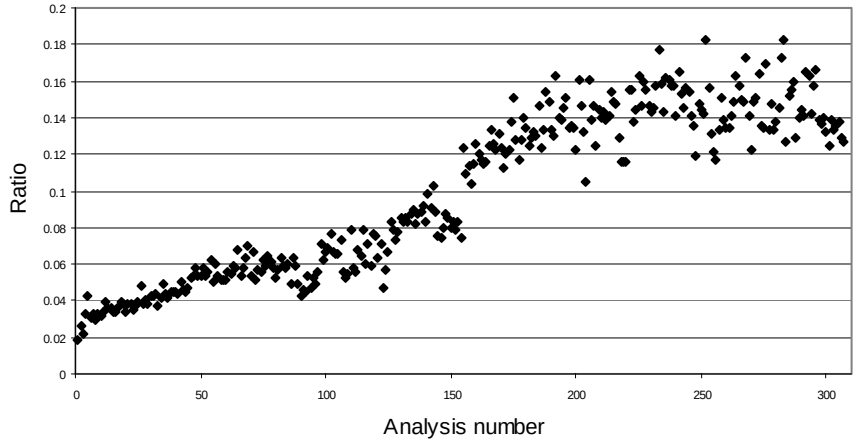


Figure 4.1 Trend in ratio of percentages of polygodial and its GC breakdown product during the conduct of 307 consecutive analyses

The scatter diagrams (Figures 4.2 to 4.4), show the general spread of results for % polygodial, %volatiles and %safrole respectively in the samples.

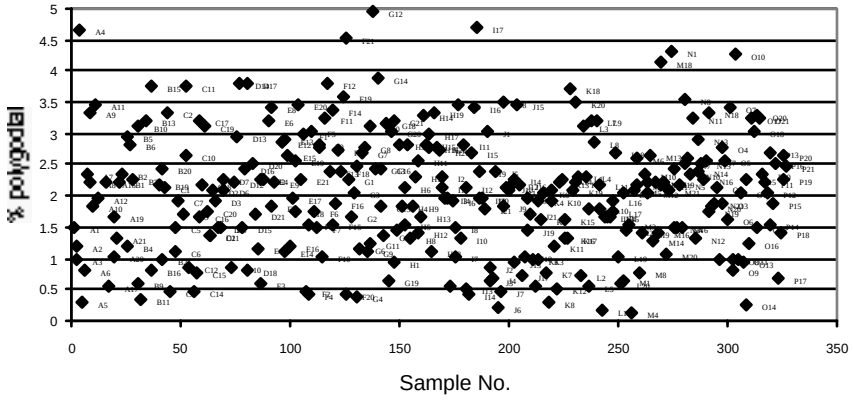


Figure 4.2 Percentage polygodial (total) in leaf samples shown by tree number- samples from transects 'A' to 'P' from left to right

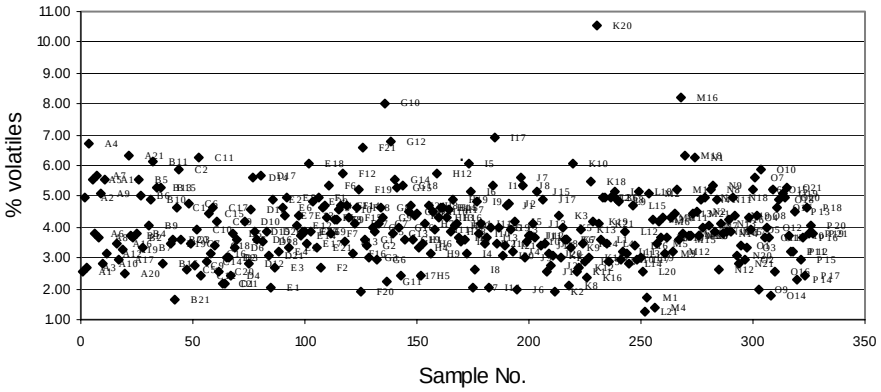


Figure 4.3 Percentage of volatiles, in leaf samples shown by tree number-samples from transects 'A' to 'P' from left to right

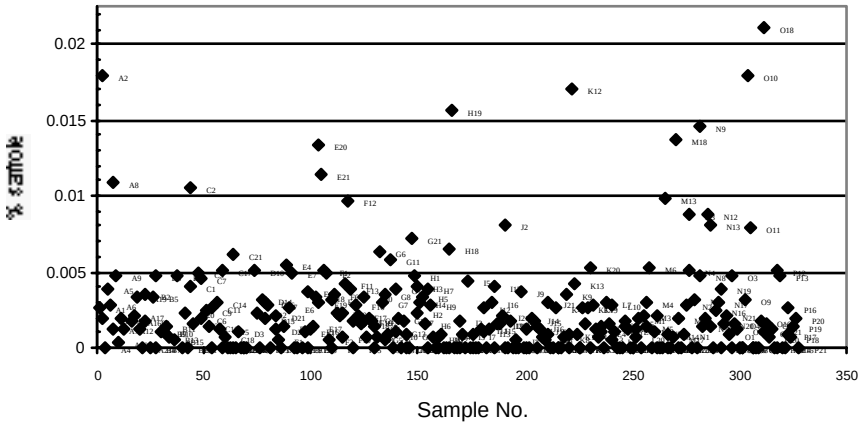


Figure 4.4 Percentage of safrole detected in leaf samples, shown by tree number - samples from ransects 'A' to 'P' from left to right. Zero results indicate 'undetected' at the lower threshold for the analytical procedure.

The distribution of sample results is summarised in the histograms of Figures 4.5 to 4.7, in which the 307 sample results are grouped by category for each component of interest.

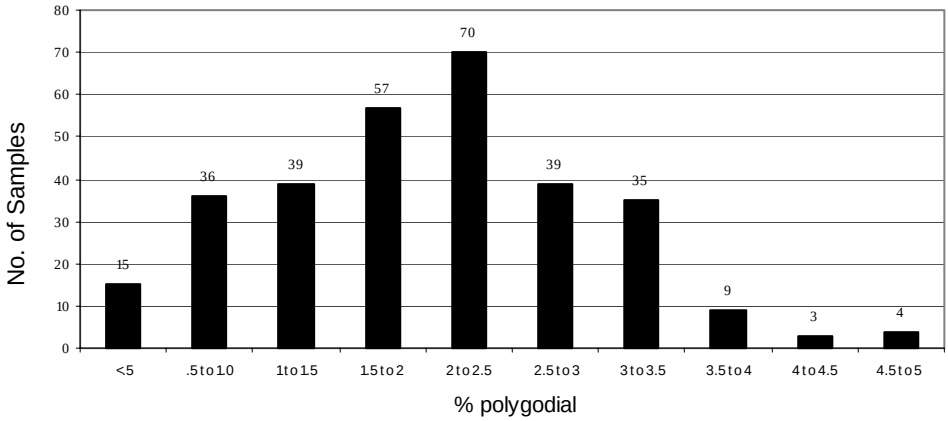


Figure 4.5 Distribution of polygodial content in 307 dry leaf samples

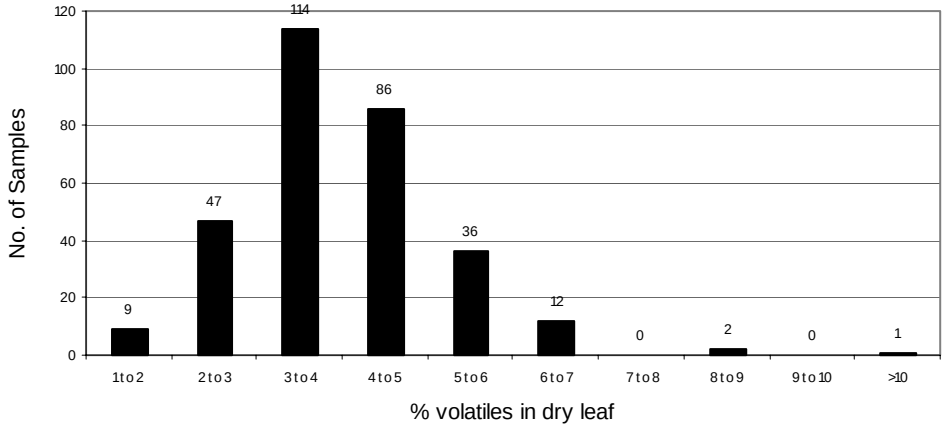


Figure 4.6 Distribution of samples by percentage volatiles

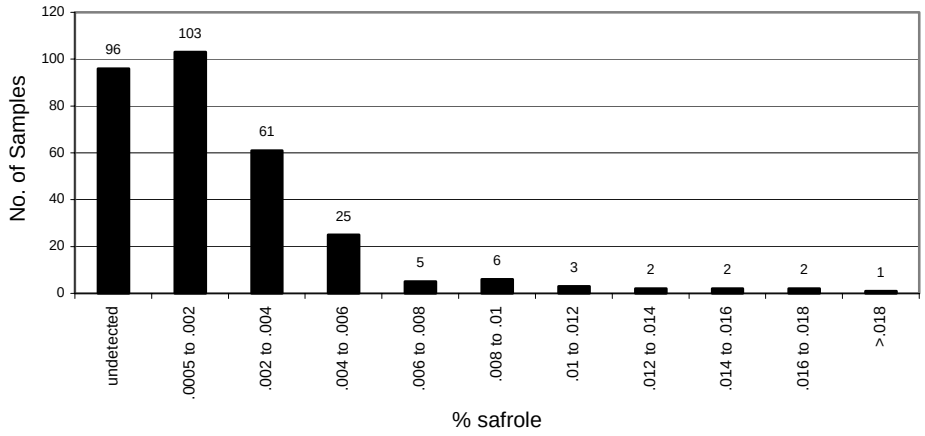


Figure 4.7 Distribution of samples by % safrole

Note: 'Undetected' category refers to all samples having levels less than the lower detection limit of 0.0005%

For all sample data, means, standard deviation and median statistics are shown in Table 4.4 below. Confidence intervals (95 and 99%) for the mean of samples of 20 trees, and 99% for 30 trees taken from this population are also given.

Table 4.4 Statistics for Sampled Population

	% volatiles	% polygodial	% safrole
Mean	4.01	2.04	0.0022
Standard deviation	1.170	0.954	0.0010
Median	3.87	2.08	0.0013
95% confidence interval (n=20)	0.546	0.445	0.0005
99% confidence interval (n=20)	0.745	0.607	0.0006
99% confidence interval (n=30)	0.534	0.436	0.0005

The 'undetected' samples were further analysed by GC-SIM to determine variation in this low safrole region. Some 120 samples with <5ppm safrole were re-analysed. The distribution of safrole levels is shown in Figure 4.8. Appendix 2 shows the results of these analyses.

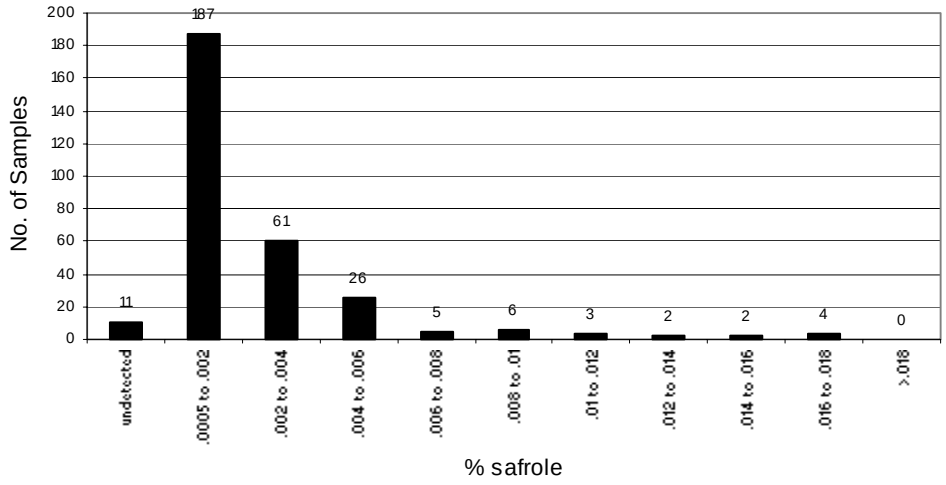


Figure 4.8 Distribution of samples by % safrole

4.2 Product Registration

4.2.1 Toxicology

To date there has been minimal toxicological testing performed on either the extract of *Tasmannia lanceolata* or on its major constituent, polygodial. However, negotiations are continuing with interested flavour companies and testing laboratories to secure an economically effective outcome.

4.3 Environmental Breakdown

4.3.1 Biodegradation of *T. lanceolata* extract by *Pseudomonas* sp. in Culture

The utilisation of the essential oil components of *T. lanceolata* by a number of the degrading isolates is shown in Figures 4.9-4.17. Controls were included to examine losses of components to or potential changes in the absence of either inoculum or oil. No peaks on the chromatograms were detectable for controls of BMS alone and with inoculum only. This assay was performed before further identifications were carried out, subsequently some species were examined more than once. Therefore the results incorporate the identification, with the mean amount of a component present, relative to the C_{18} internal standard, determined for each of the different species of *Pseudomonas.* This gives a clearer picture of the degradation of representative compounds.

The very large number of different compounds present in the essential oil made it difficult to ascertain which compounds were being utilised by degrading microbiota. The relative amounts of representative compounds were however, calculated and the changes plotted for each including losses due to volatilisation. There appeared to be three types of change:

i). The rate of loss could be attributed entirely to volatilisation (this was the case for α-pinene and the combined peak of β-phellandrene plus limonene) (Figures 4.9 and 4.10). For these compounds there is a rapid decrease in amount present from week zero to one for all isolates including the control.

ii). Other compounds such as α-cubebene, cadina-1,4-diene and polygodial showed a little change in the volatilisation control, with probable degradation occurring by all pseudomonads shown in Figure 4.12, 4.14 and 4.16.

iii). The final type of degradation curve is one where there was little volatilisation or degradation indicated for linalool, calamenene, unknown III and 272-diterpene (Figures 4.11, 4.13, 4.15 and 4.17).

There appeared to be little difference between isolates regarding the individual compounds they degraded, this is difficult to confirm without repetition of the experiment to allow statistical analysis of results.

These results gave an indication of the rate of degradation of representative compounds over time relative to the C_{18} internal standard. However in order to have true quantification of the rate of degradation, standards would be required for each compound.

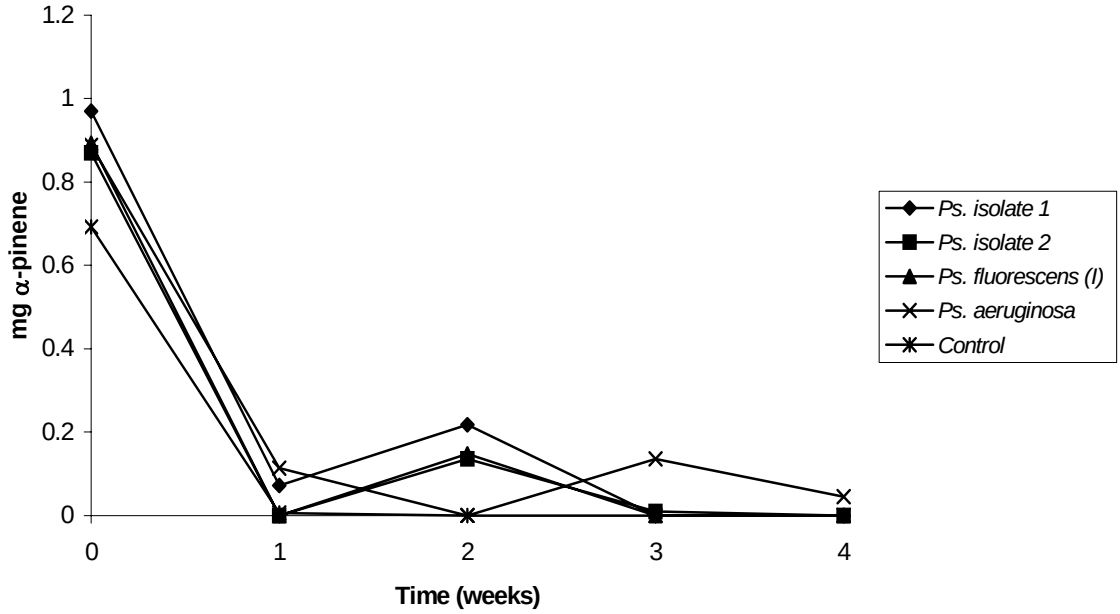


Figure 4.9 Changes in α-pinene levels with or without *Pseudomonas* species

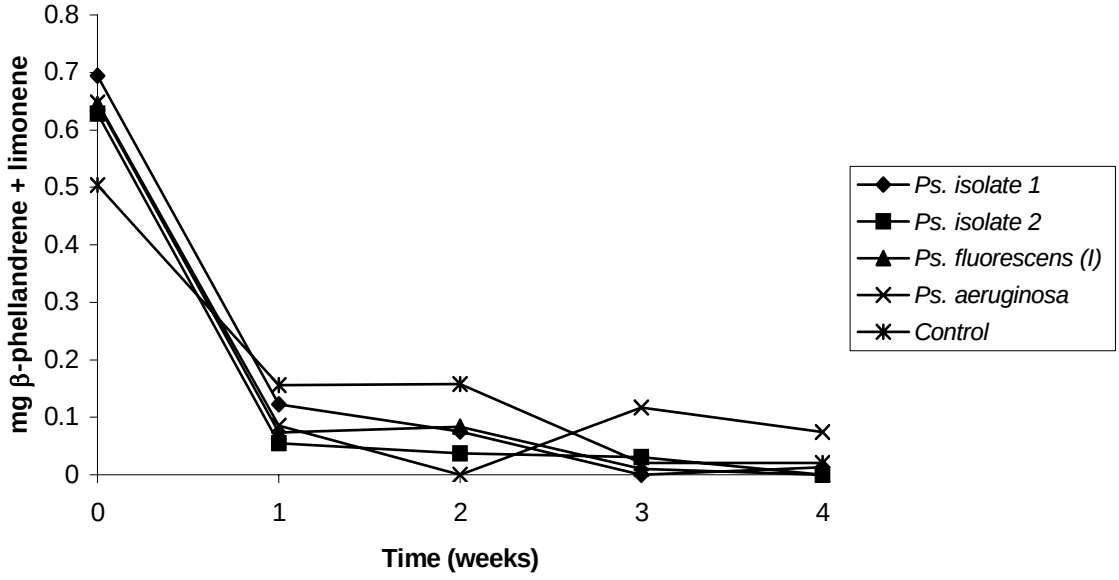


Figure 4.10 Changes in β–phellandrene + limonene levels with or without *Pseudomonas* species

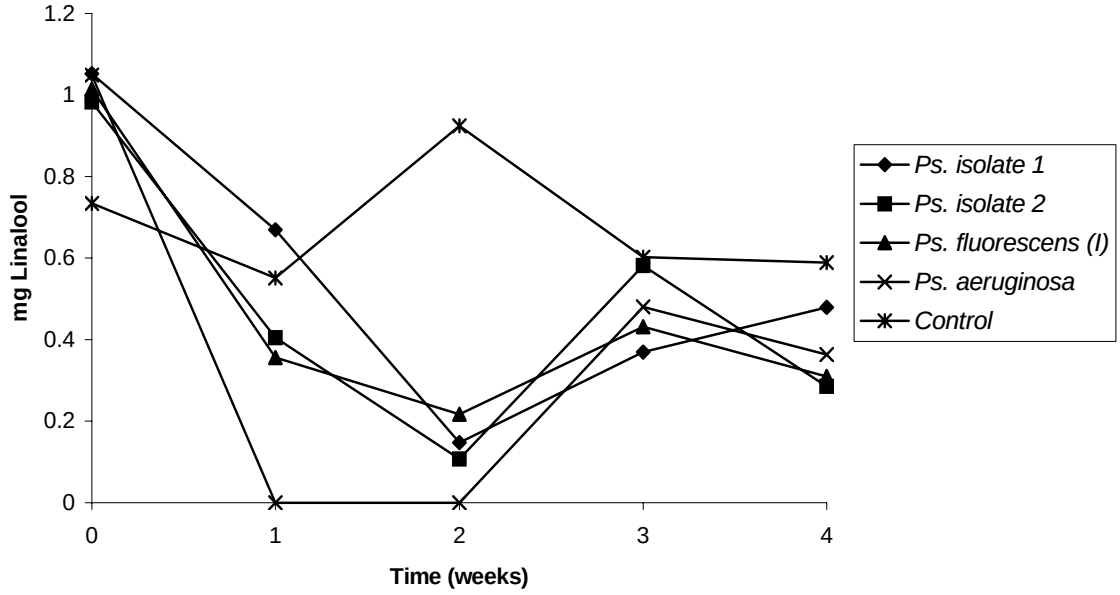


Figure 4.11 Changes in linalool levels with or without *Pseudomonas* species.

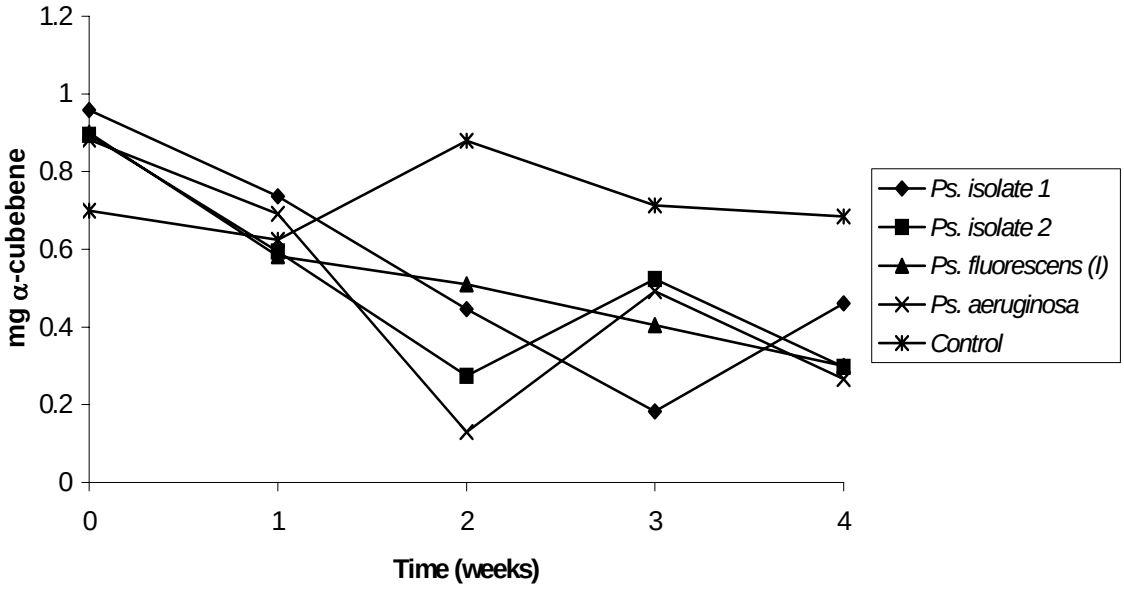


Figure 4.12 Changes in α-cubebene levels with or without *Pseudomonas* species.

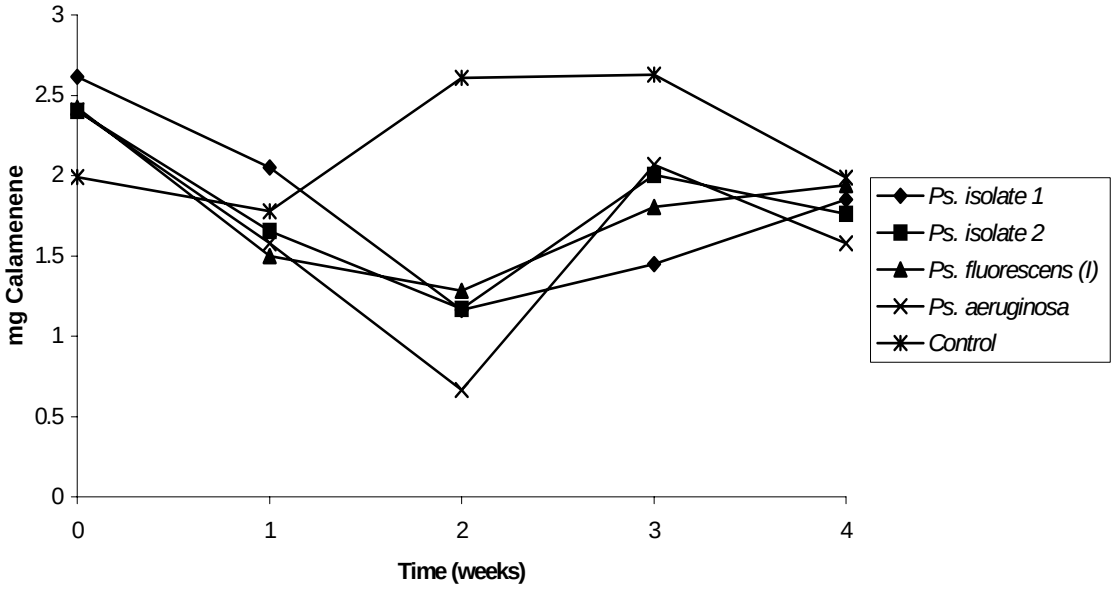


Figure 4.13 Changes in calamenene levels with or without *Pseudomonas* species.

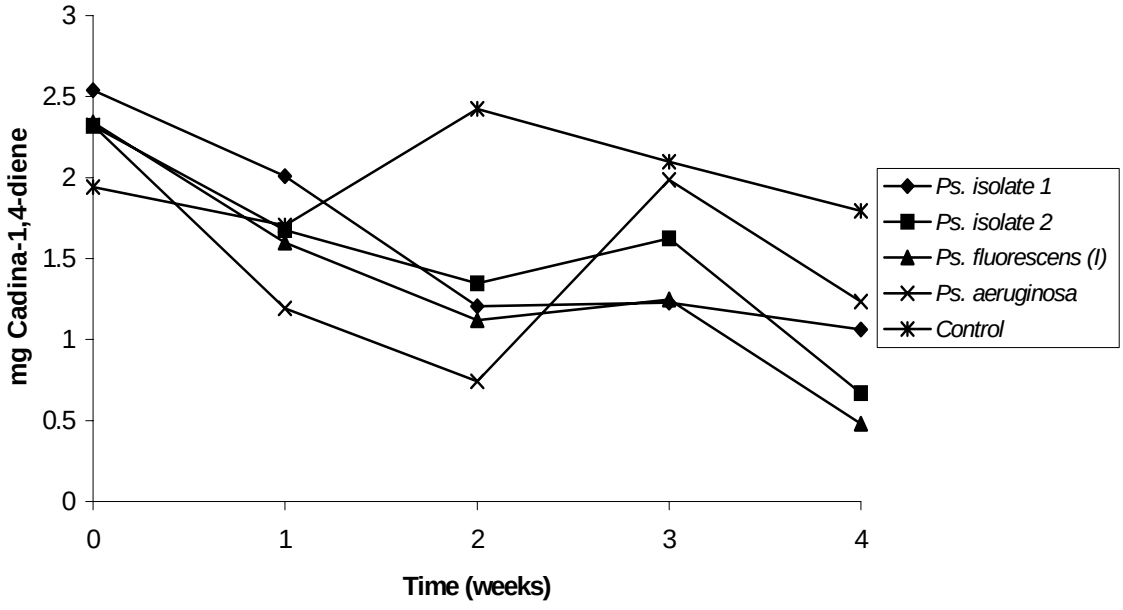


Figure 4.14 Changes in cadina-1,4-diene levels with or without *Pseudomonas* species.

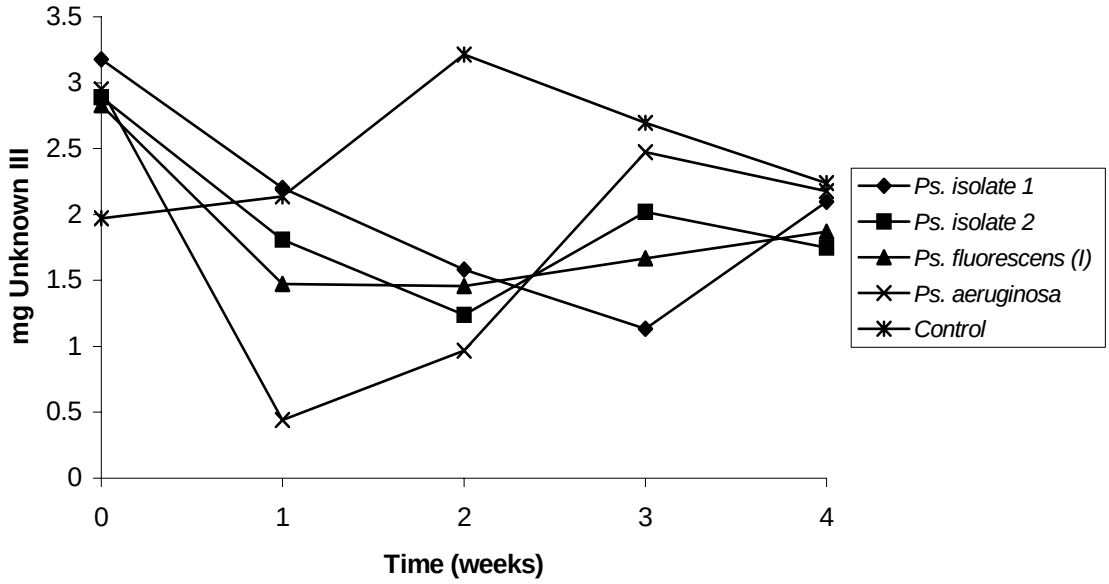


Figure 4.15 Changes in unknown compound III levels with or without *Pseudomonas* species

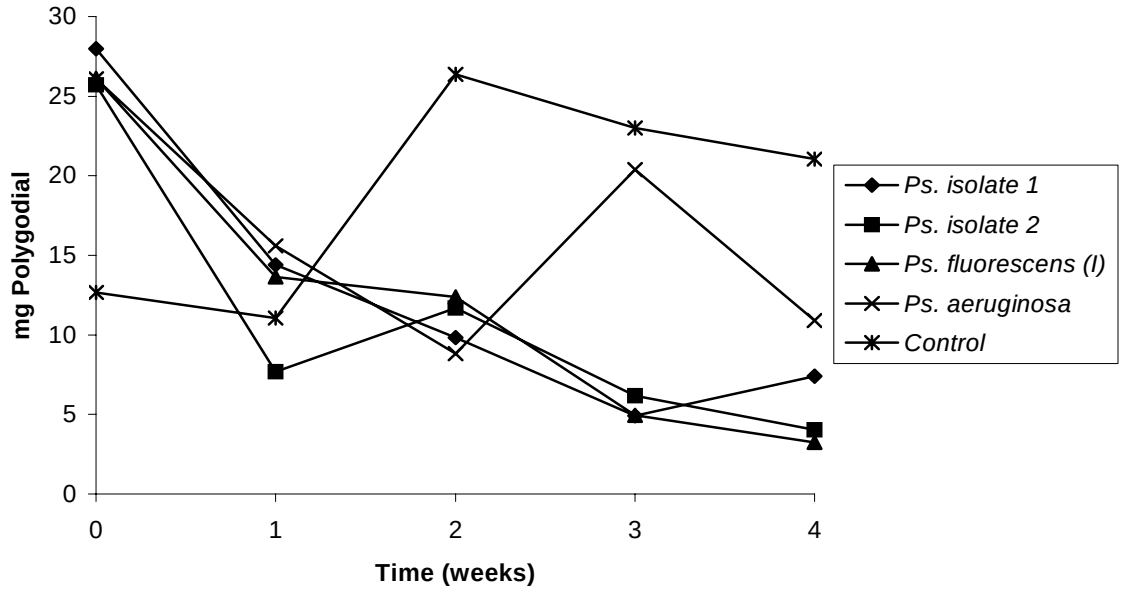


Figure 4.16 Changes in polygodial levels with or without *Pseudomonas* species.

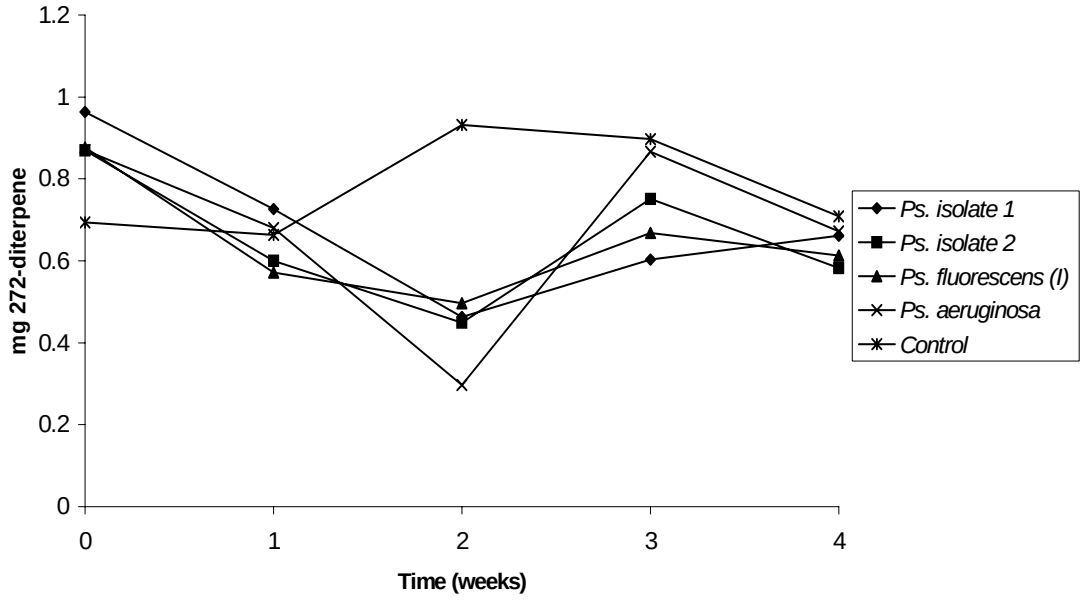


Figure 4.17 Changes in 272-diterpene levels with or without *Pseudomonas* species.

4.3.2 Soil Microcosms

A total of twelve components of the essential oil of *T. lanceolata* were selected for study in this investigation as shown in Table 4.5. Representative gas chromatograms for NW Red and volatilisation control soils in Figures 4.18-4.21 show the decrease in essential oil constituents over the eight-week incubation period. The ability of crude soil microbiota to degrade these components is indicated in Figures 4.22-4.33.

There appeared to be four different types of change occurring:

i). Volatilisation dominates for these compounds and the rate of loss can be attributed entirely to volatilisation. This was true for the monoterpenes α-pinene, limonene and β-phellandrene where volatilisation was indistinguishable from degradation ($p>0.05$).

ii). The second type of change was where there was clear evidence for the degradation of a compound, such as linalool and piperitone. For these compounds there was rapid losses evident relative to controls, with levels dropping to zero in one week. However, there were no significant differences between soil types ($p>0.05$).

iii). For other components such as α-cubebene, cadina-1,4-diene and 272-diterpene, there appears to be little or no degradation, with curves closely following those of the volatilisation control over a two-month period.

iv). The final type of change was one with some evidence of degradation in some soil types however, this was not as significant as for linalool and piperitone. Analysis revealed a significant difference ($p<0.05$) between BCC and NW Red soil from the volatilisation controls over the course of the investigation for calamenene. This may imply evidence for the degradation of calamenene in these soil types. There was a significantly faster rate of decrease of 218-diterpene in BCC and NW Red soils ($p<0.05$) compared to the other soils and the control. Polygodial levels showed a rapid decrease in the first week for all soils except the volatilisation control which was significantly different to all other soils types ($p<0.05$).

Table 4.5 Representative components selected for soil microcosm study

Component Number	*Component Name*	**Compound Type**
1	α-pinene	bicyclic monoterpene
2	β-phellandrene + limonene	monocyclic monoterpenes
3	linalool	acyclic monoterpene alcohol
4	piperitone	monocyclic monoterpene ketone
5	α-cubebene	tricyclic sesquiterpene
6	calamenene	bicyclic sesquiterpene
7	cadina-1,4-diene	bicyclic sesquiterpene
8	unknown III	unknown
9	unknown sesquiterpene	mono-oxygenated sesquiterpene
10	drimenol	bicyclic sesquiterpene alcohol
11	polygodial	bicyclic sesquiterpene dialdehyde
12	unknown diterpene	diterpene

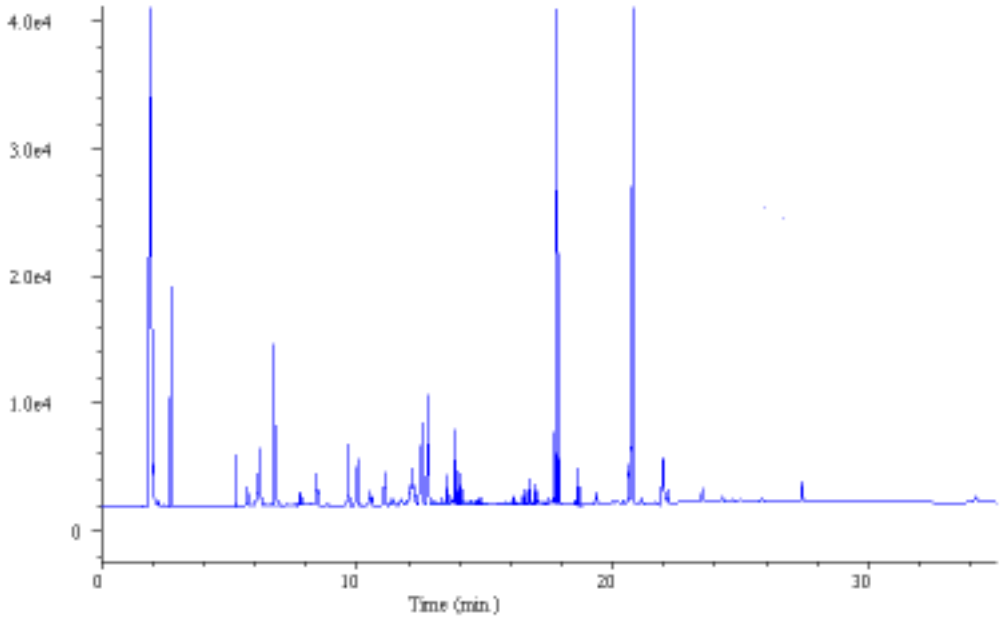


Figure 4.18 Gas chromatogram for NW Red soil containing 0.5%v/v *T. lanceolata* essential oil at time 0.

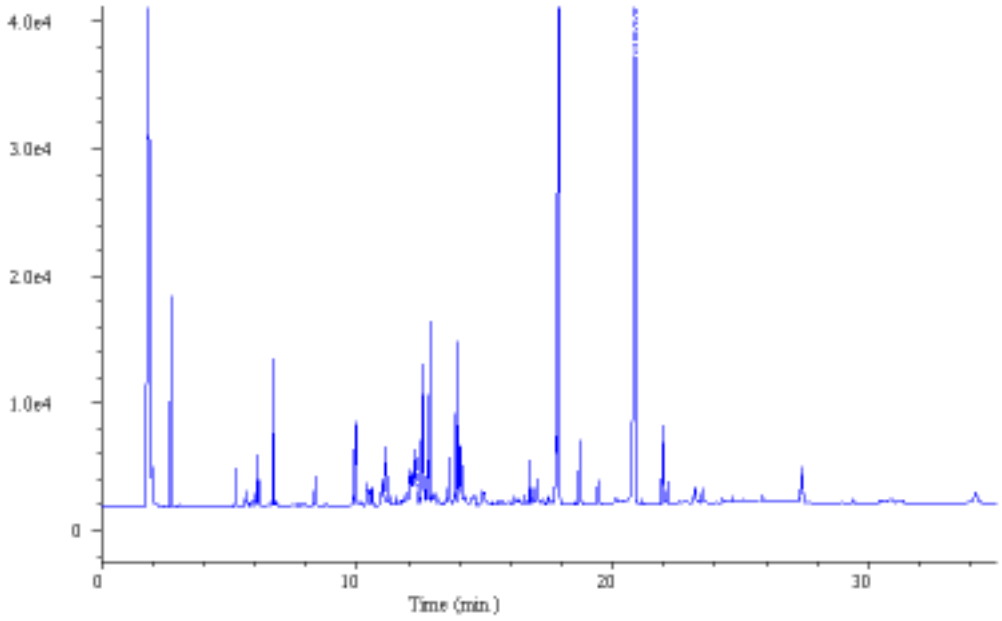


Figure 4.19 Gas chromatogram for volatilisation control containing 0.5%v/v *T. lanceolata* essential oil at time 0.

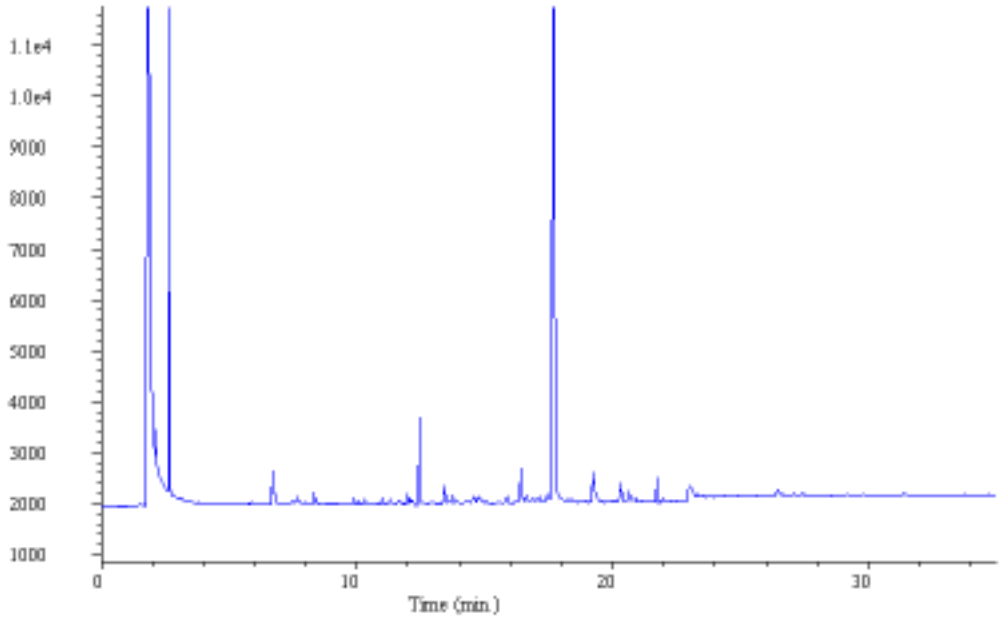


Figure 4.20 Gas chromatogram for NW Red soil containing 0.5%v/v *T. lanceolata* essential oil after eight weeks incubation at 25^0C.

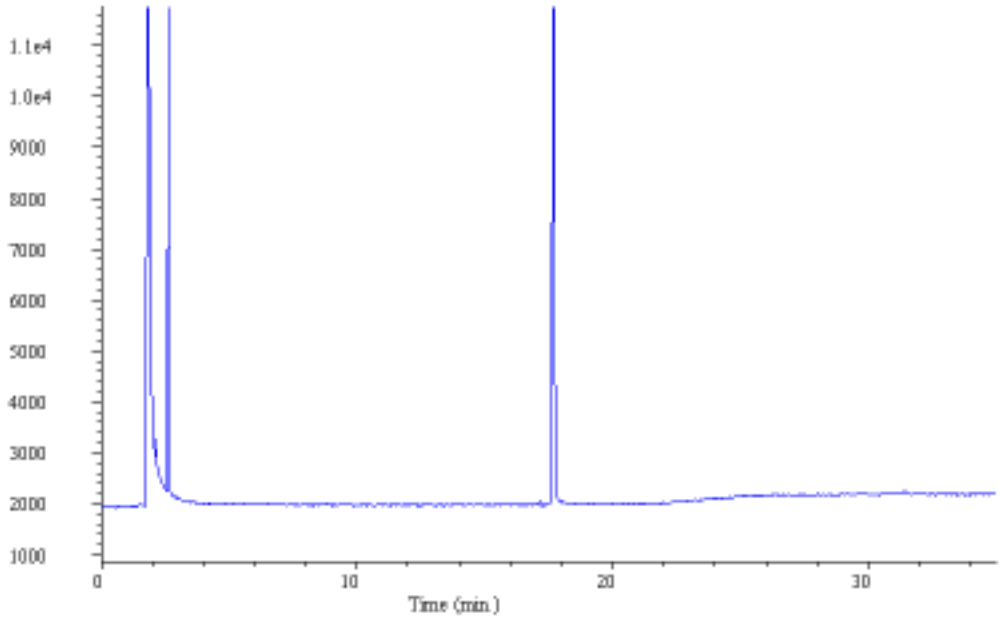


Figure 4.21 Gas chromatogram for volatilisation control containing 0.5%v/v *T. lanceolata* essential oil after eight weeks incubation at 25^0C.

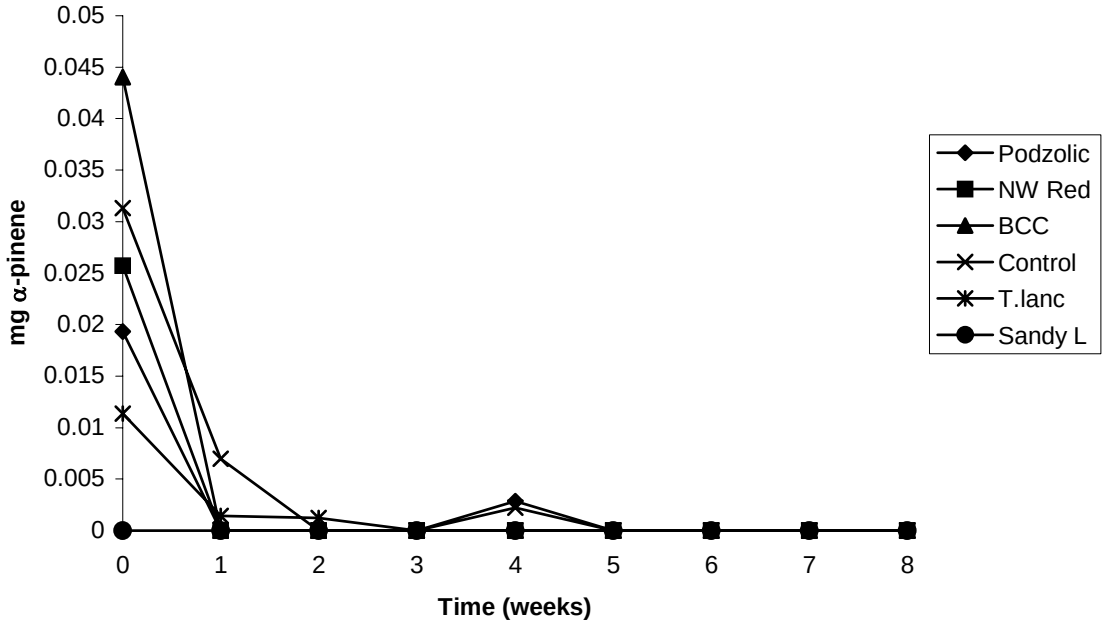


Figure 4.22 Changes in levels of α-pinene and in soil microcosms over time relative to control.

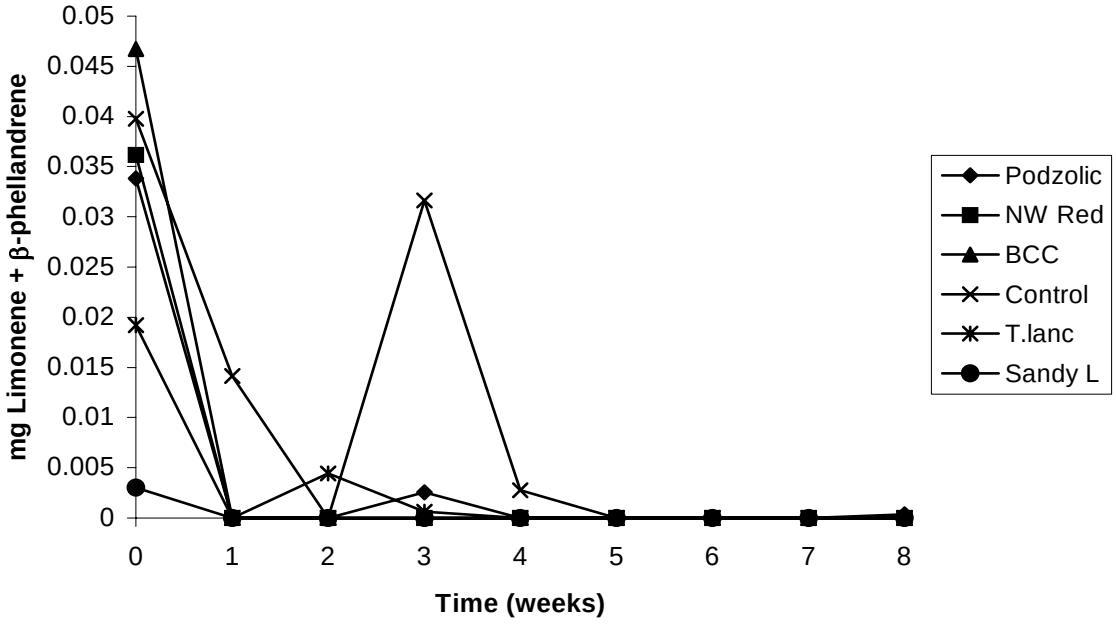


Figure 4.23 Changes in levels of β-phellandrene + limonene in soil microcosms over time relative to control.

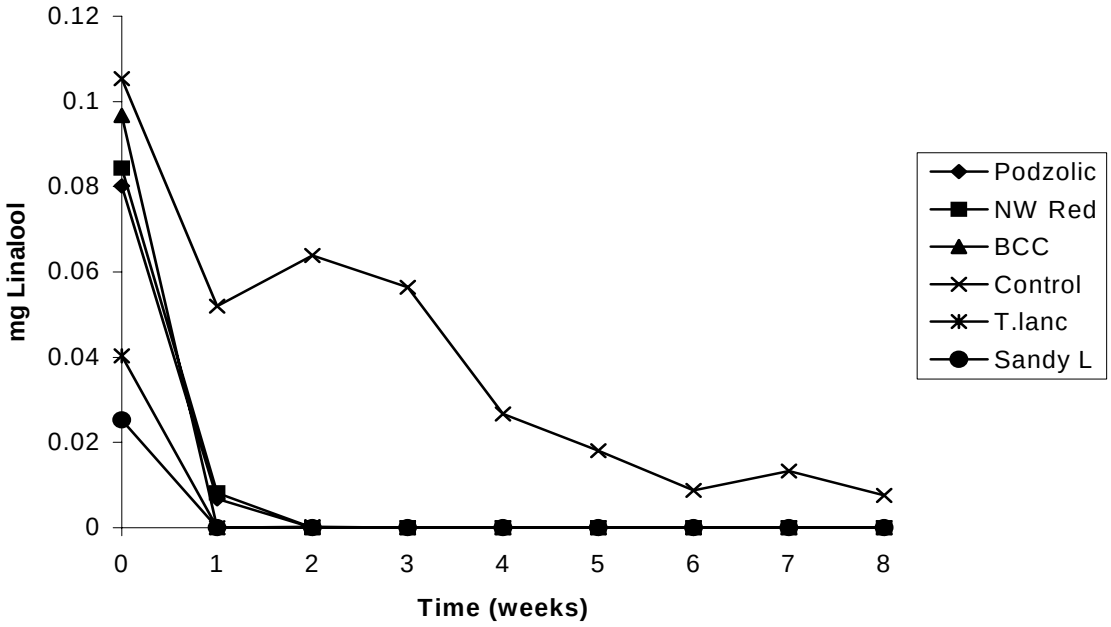


Figure 4.24 Changes in levels of linalool in soil microcosms over time relative to control.

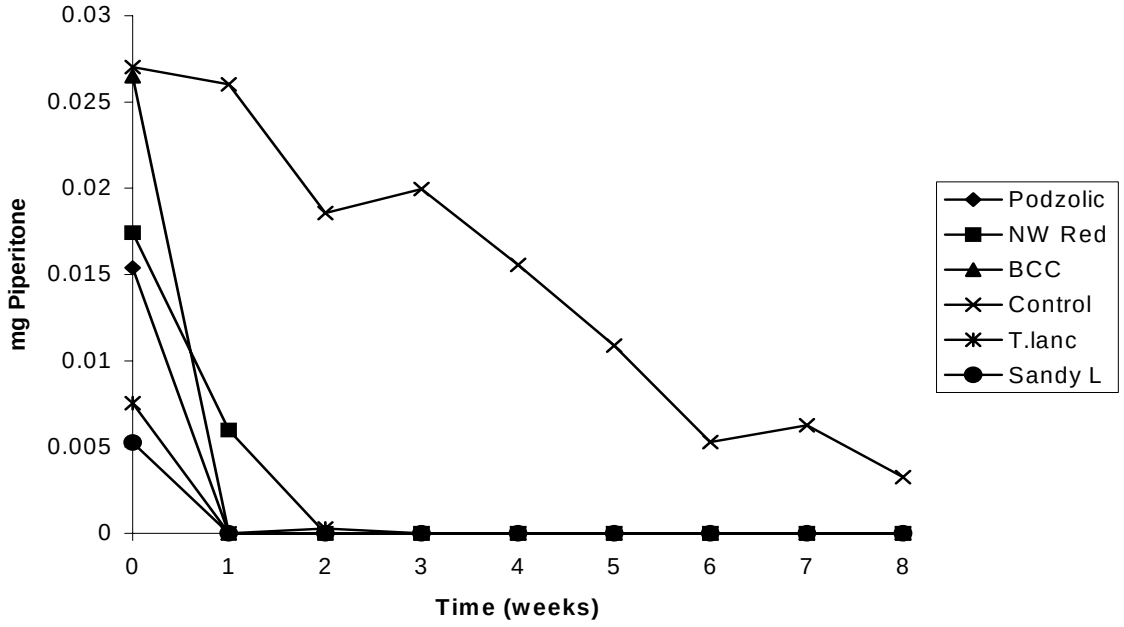


Figure 4.25 Changes in levels of piperitone in soil microcosms over time relative to control.

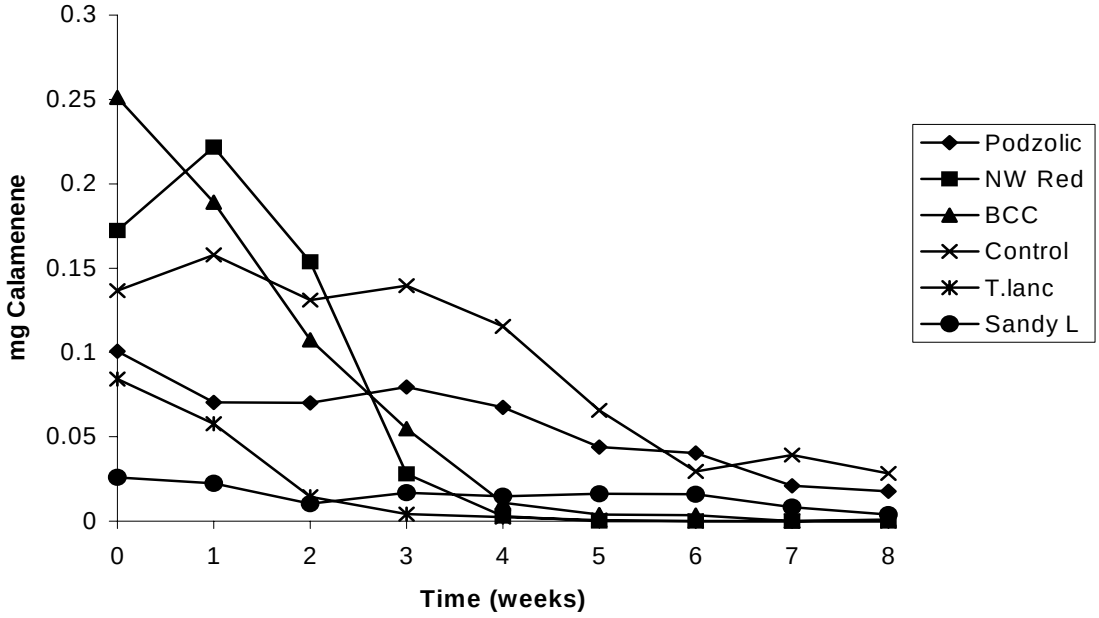


Figure 4.26 Changes in levels of α-cubebene in soil microcosms over time relative to control.

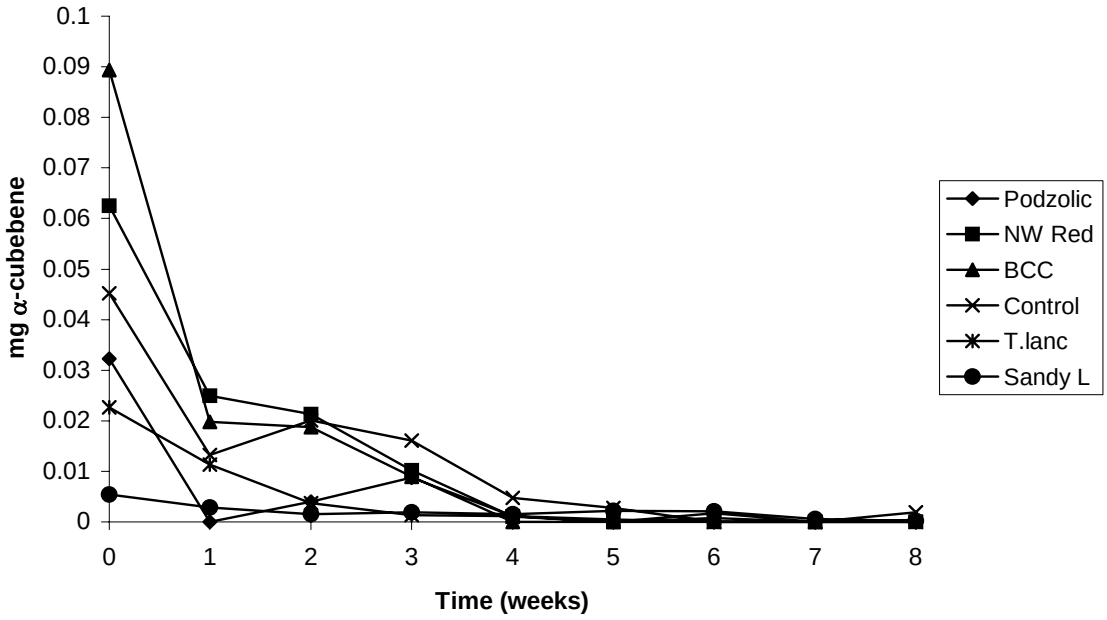


Figure 4.27 Changes in levels of calamenene in soil microcosms over time relative to control.

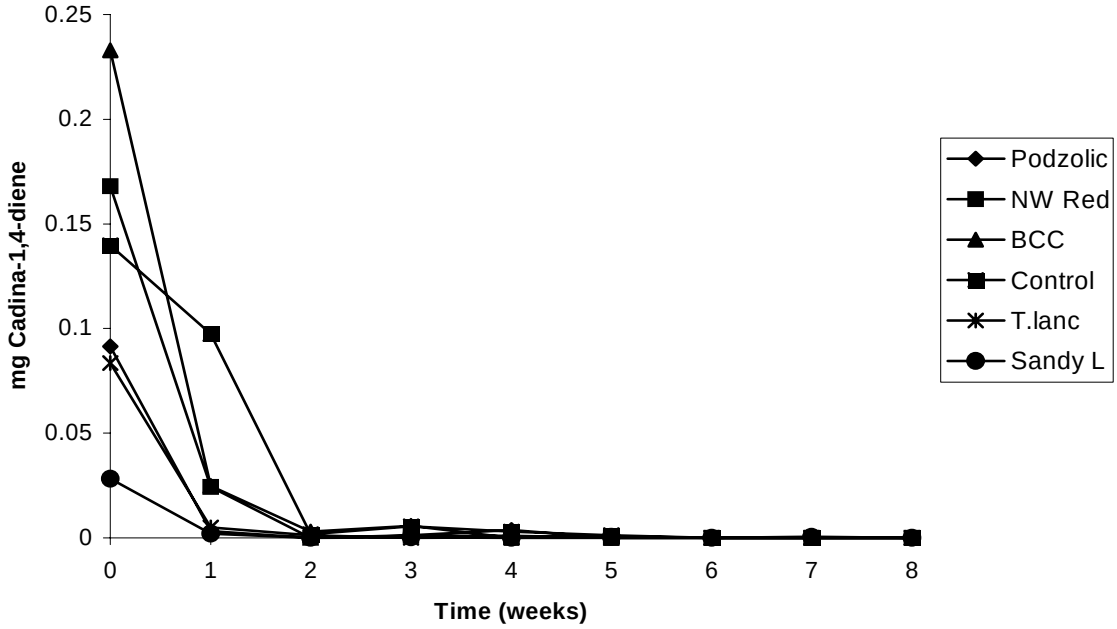


Figure 4.28 Changes in levels of cadina-1,4-diene in soil microcosms over time relative to control.

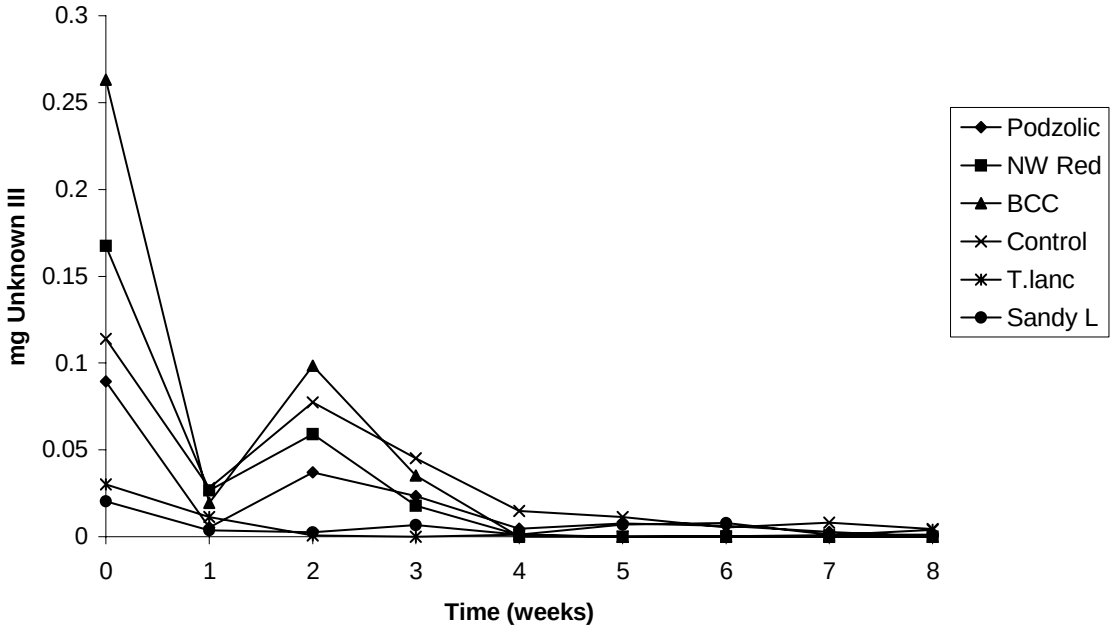


Figure 4.29 Changes in levels of unknown compound III in soil microcosms over time relative to control.

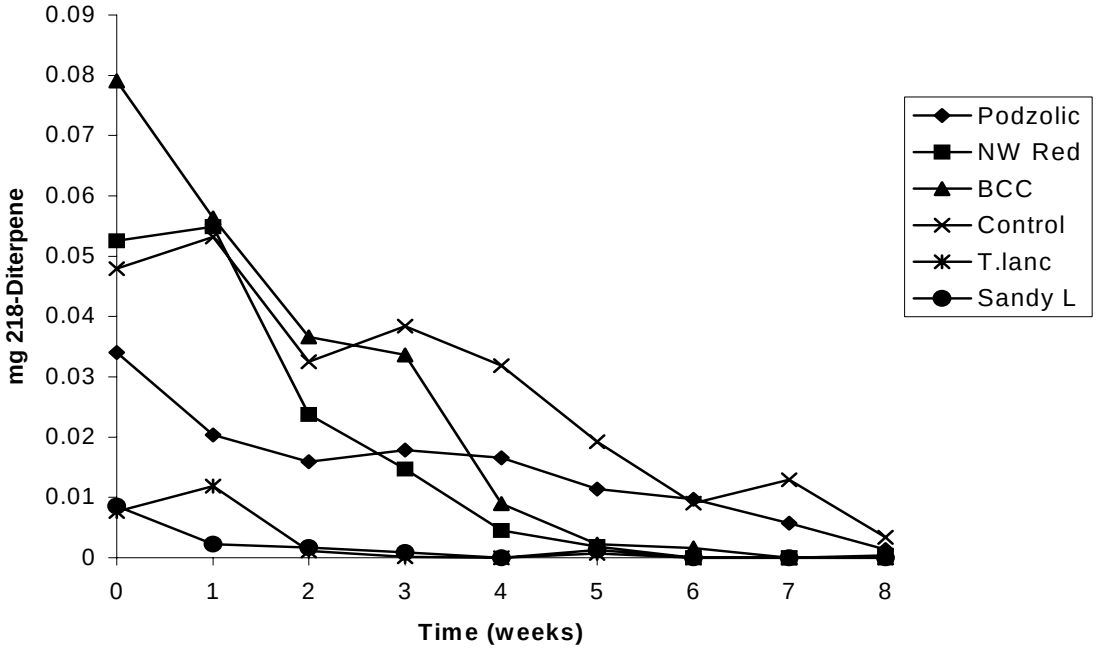


Figure 4.30 Changes in levels of 218-diterpene in soil microcosms over time relative to control.

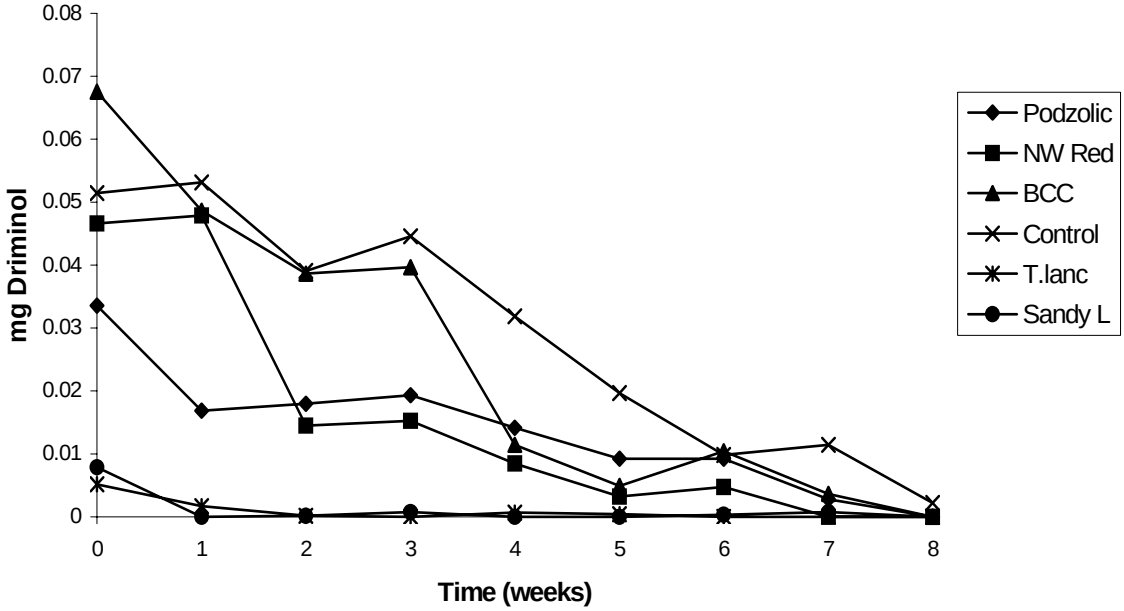


Figure 4.31 Changes in levels of drimenol in soil microcosms over time relative to control.

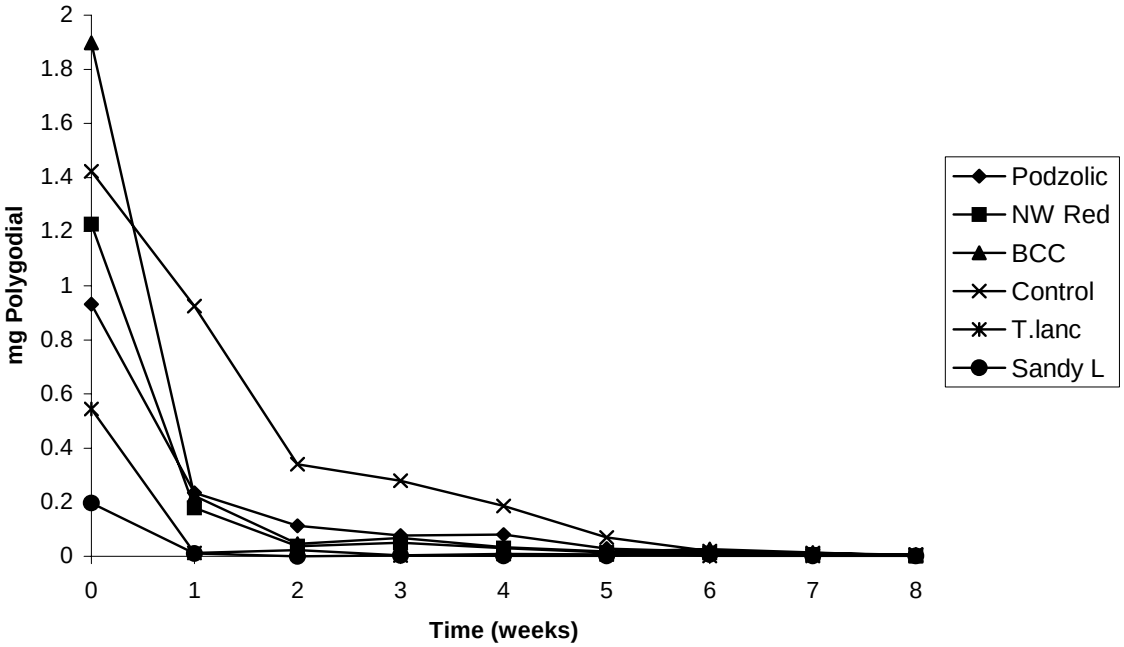


Figure 4.32 Changes in levels of polygodial in soil microcosms over time relative to control.

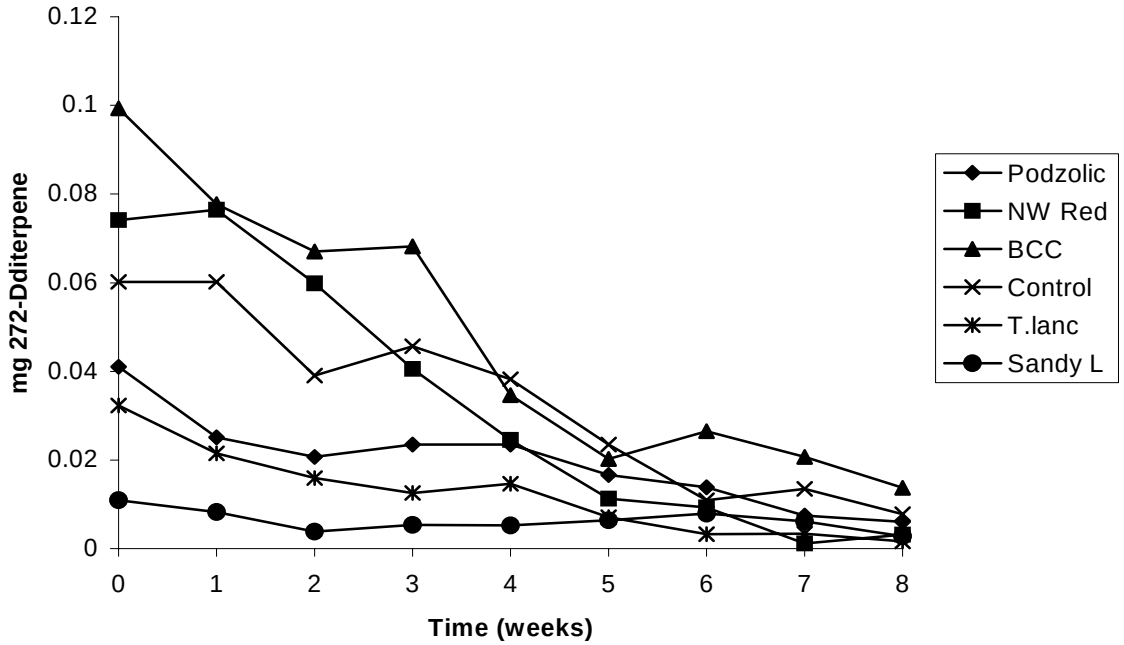


Figure 4.33 Changes in levels of 272-diterpene in soil microcosms over time relative to control.

4.4 Commercial Extract

4.4.1 Determination of Safrole Levels in Commercial *T. lanceolata* Blend

The statistical analysis and standard curve are shown Table 4.6 and Figure 4.34 respectively. The average level of safrole in the current commercial blend of *T lanceolata* extract was 4.7ppm. This represents a safe level, considering that the product is used at low concentrations for most applications. These applications also represent a very low proportion of average daily food consumption.

Table 4.6 Statistical analysis of results of safrole determination in commercial *Tasmannia lanceolata* extract

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.99596394
R Square	0.99194416
Adjusted R Square	0.98925888
Standard Error	0.0009461
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.0003307	0.000331	369.401	0.00030762
Residual	3	2.685E-06	8.95E-07		
Total	4	0.0003333			

	Coefficients	*Std Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.00044281	0.0005941	0.745357	0.51014	-0.00144	0.002333	-0.00144	0.0023
ppm Safrole	0.11132489	0.0057922	19.2198	0.00031	0.092891	0.129758	0.092891	0.1298

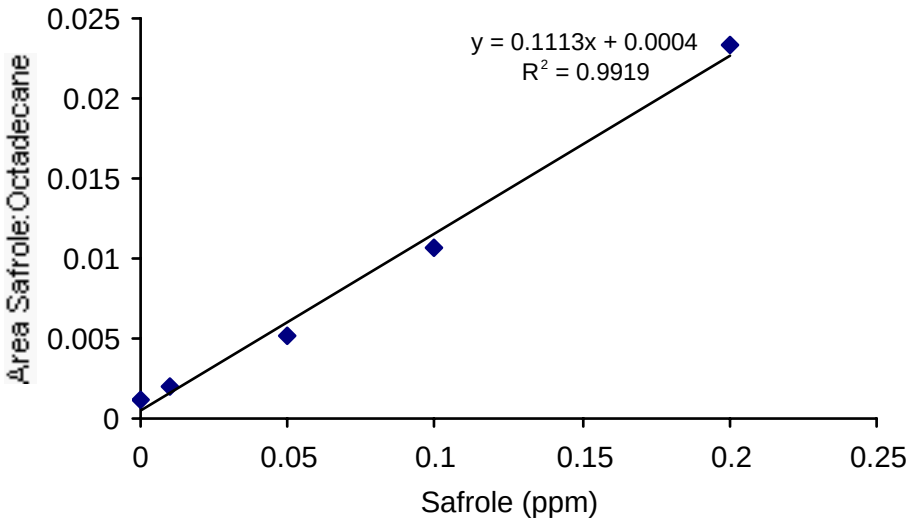


Figure 4.34 Safrole standard curve

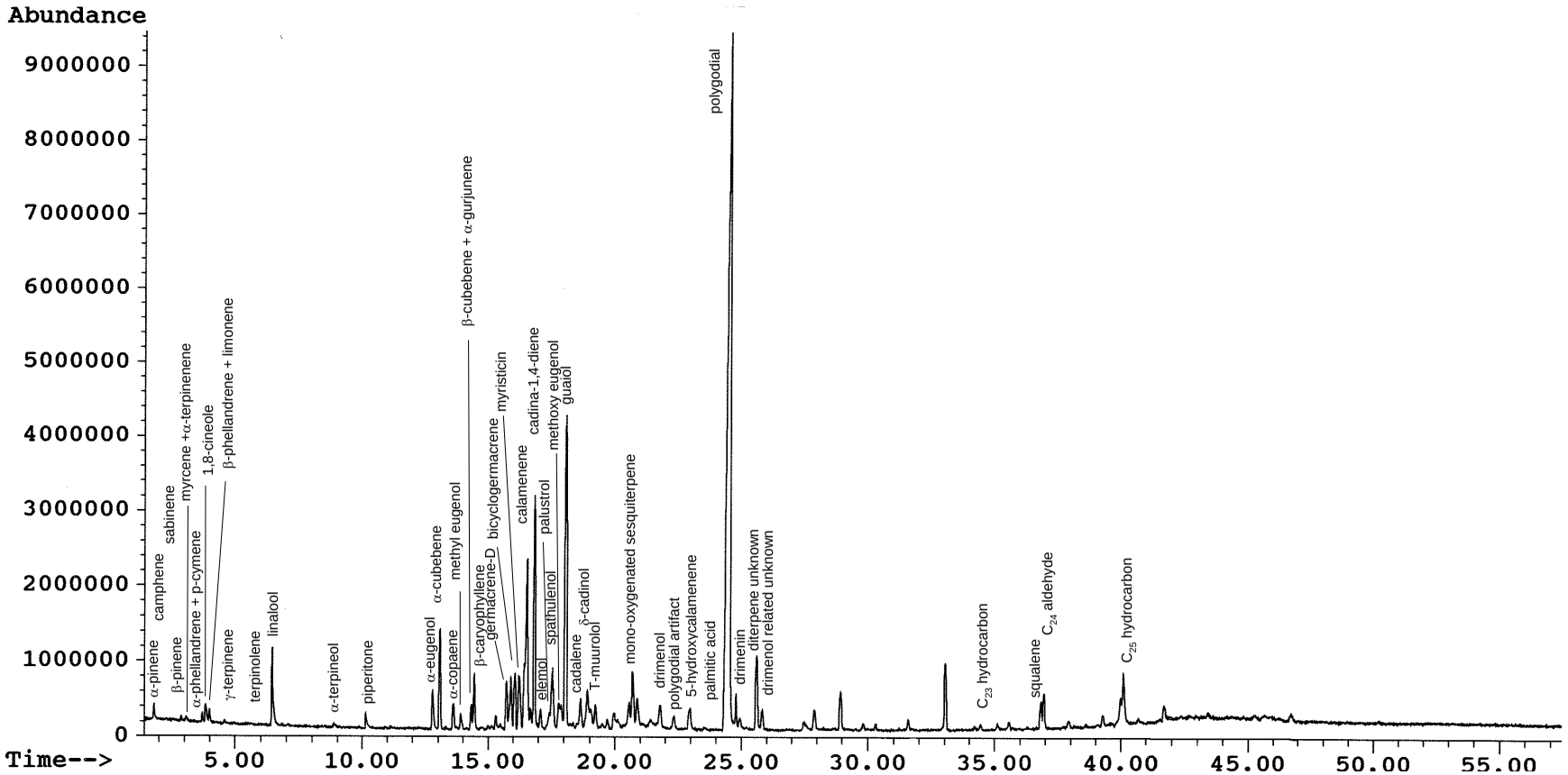
4.4.2 Commercial Blend Analysis

The chromatogram reproduced in Figure 4.35 represents the commercial blend currently produced by Essential Oils of Tasmania. The list of compounds is shown in Table 4.7.

Table 4.7 Components of commercial *Tasmannia lanceolata* extract

COMPONENT	% PEAK AREA
α-pinene	0.86
camphene	0.02
sabinene	0.03
β-pinene	0.38
myrcene + α-terpinene	0.10
α-phellandrene +p-cymene	0.26
1,8-cineole	0.77
β-phellandrene + limonene	0.03
γ-terpinene	0.02
terpinolene	0.03
linalool	1.81
α-terpineol	0.06
piperitone	0.55
α-eugenol	0.95
α-cubebene	0.88
methyl eugenol	0.24
α-copaene	0.48
β-cubebene	0.15
α-gurjunene	0.04
β-caryophyllene	0.87
germacrene-D	0.33
bicyclogermacrene	1.15
myristicin	1.00
calamenene	3.42
cadina-1,4-diene	1.58
elemol	0.39
palustrol	0.38
spathulenol	1.94
guaiol	4.46
cadalene	0.44
d-cadinol	0.40
T-muurolol	0.39
drimenol	1.91
polygodial artifact	2.38
5-hydroxycalamenene	1.47
palmitic acid	0.08
polygodial	36.74
drimenin	0.45
diterpene unknown	1.18
drimenol related unknown	0.51
C23 hydrocarbon	0.14
n pentacosane	1.54
squalene	0.12
tetracosanal	0.45
C27 hydrocarbon	0.34
hexacosanal	2.71
C29 hydrocarbon + linolenic acid	1.76
octacosanal	0.86
TOTAL	77.06

Figure 4.35 Gas Chromatogram of *Tasmannia lanceolata*
Commercial Blend Extract showing Identified Components



5. Discussion

5.1 Clone collection

The maintenance of the collection of *Tasmannia lanceolata* clones at the Horticultural Research Centre entailed routine inspection of the area to locate plants that were not thriving or that had been attacked by rabbits or other predators. These were replaced whenever possible. As a consequence of this operation, several clones can be described as having a low field survival rate. For instance, BR1 did not survive, even after several replacements. Such types were eventually replaced in the overall design, with substitutes. These alternative plants were selected on the basis of vigour and extract character.

In all, there are 63 clones within the replicated area and a further 14 that are present as border plants, but that may be used as alternatives in the event of a search for specific extract compositions or genetic material.

5.2 Population Variation

The population of *Tasmannia lanceolata* of which the 18ha sampling site described by this report was part, extended over some 300Ha of undulating terrain, previously cleared of native myrtle (*Nothofagus cunninghamii*) forest, and since left to regenerate, more or less undisturbed.

Particular care was taken during sampling to ensure leaf material chosen was formed during the preceding summer, and was reasonably free of evidence of predation or disease. On the other hand, no attempt was made to estimate the age or potential leaf yield of sampled individuals, since trees were chosen only on the basis of their proximity to the grid point.

The use of the GPS system in this survey enabled random choice of sample trees in a relatively uniform distribution across the site, notwithstanding the (in places) rugged terrain and thick vegetation. Furthermore, point coordinates, together with visual markers will allow easy relocation of individual trees should repeat sampling be required.

The assay used lends itself to rapid and economical screening of the natural population. Collected samples can be air-dried in 48hrs, and leaf samples for extraction can be prepared at the approximate rate of 15 per hour.

Since the results provide only extract compositions expressed as a percentage of dry weight of leaf, it is not possible to propose that a commercial harvest gathered from the sample trees would result in an average composition approximating the means reported here, since the quantity of dry leaf available ranges between a few hundred grams for the smallest trees, to many tens of kilograms in the case of the largest trees.

Nevertheless, the trial provides the basis for three important lines of investigation.

1) Management of extract composition from wild-harvested leaf

The large number of samples gathered from randomly sampled trees at this site provides a sound estimate of the variability of the natural population, and (for this site), an estimate of the mean and median levels for the three parameters reported.

We can derive confidence intervals from these statistics which can be used to establish sampling protocols for other, extensive natural populations of the species.

Hence from a transects of local populations of *Tasmannia lanceolata* containing 30 randomly chosen individuals we can propose with 99% certainty that the mean % polygodial determined for the sample is that of the population +/- 0.436, and so on.

(A one-tailed test might be more appropriate for safrole, for which the main concern is that the levels found in the extract be *less than* some pre-determined level).

Determination of appropriate threshold levels for the extract parameters, based on commercial, toxicological or technical requirements will enable estimates of the potential of other sites to deliver commercial volumes of extract to specification.

While the data presented here apply only to the three parameters: percent volatiles, polygodial and safrole, the statistics could easily be derived for other components of the extract detected in this analysis.

2) Selection of suitable genetic material for propagation

The individuals identified in this population which are low in safrole and relatively high in polygodial (Tables 4.2 and 4.3) represent a useful, if limited selection from the available wild type material, suitable for inclusion in the existing database and clonal collection.

Significantly, the data collected shows the nature of the distribution pattern for leaf extracts of the species, and indicates the scope for careful selection in favour of one or more extract parameters from the natural population. However, it is most important to recognise the need for reappraisal of the 'preferred' types with regard to the form and vigour of the plant *in situ*, since even casual observation shows a wide range of bush size, leaf and shoot vigour, and other evidence of phenotypic variation in many individuals.

The additional analysis of the low safrole samples showed that the population distribution is skewed. This may be an indication that there are only one or two genes controlling the safrole level in this species, and that the controlling alleles are dominants.

3) Investigation of factors affecting extract composition

A previous survey of *Tasmannia lanceolata* leaf extracts in which whole extract was prepared by filtration and removal of the solvent by evaporation, showed a wide range of polygodial concentrations in the leaf (Dragar, 1998). Small numbers of plants at each of twelve locations, (altitudes between 0 and 800m asl), around Tasmania, produced polygodial yields between 0.11 and 2.9% of dry leaf, the highest result arising from material collected at 300m above sea level, on the west coast of Tasmania. No attempt was made to discern trends in yield with altitude or any other locality parameter with the small number of individuals sampled.

The results of the present trial indicate that a reliable estimate of population means for extract constituents may be obtained with relatively modest sample sizes of 20 individuals. Consequently comparing local populations for extract composition, and testing correlations with environmental parameters such as altitude, aspect, radiation levels or soil nutrient levels should be relatively straightforward.

5.3 Product Registration

A pre-submission review was undertaken by the Flavour and Extract Manufacturers' Association of the United States (FEMA) staff. Their comments did not represent the opinion of the Expert Panel and were made following examination of data on chemically related substances and past actions taken by the Panel on structurally related substances used as flavour ingredients;

i. The Panel has adopted a screening paradigm to evaluate the safety of naturals based on the identity, structure, and relative amount of each constituent in the natural. The identity and %composition of 94.8% of the *Tasmannia* extract was provided. Thirty five (35) constituents and 4 unknowns have been identified. A gas chromatogram with

labelled peaks, and data accounting for the relative amount of each chemically-identified constituent in the natural complex must be provided. Data should be presented to demonstrate the variation in composition based on the different sources of mountain pepper extract. The mass, NMR and IR spectra of polygodial should be included in the application.

ii. The annual volume of use of mountain pepper extract in Japan is anticipated to be 250 kg (550lb). The anticipated volume in the United States must be provided. A figure for anticipated use in Europe should also be provided, if available. It is requested that the use of mountain pepper extract be approved in 4 food categories: snack foods (0.5 ppm), non-alcoholic beverages (0.5 ppm), hard candy (6 ppm usual; 10 ppm max), and chewing gum (50 ppm). If the maximum levels are not provided, usual levels are assumed to be the maximum.

iii. The history of use of mountain pepper extract has been well documented. Copies of relevant articles should be provided in the application. Documentation of regulatory approval in Japan should also be provided.

iv. Information on the metabolism and toxicology of mountain pepper extract as well as major constituents should be included in the application. Data on major constituents such as polygodial are valuable in the absence of extensive toxicity on mountain pepper extract itself. Metabolic and toxicology data on substances structurally related to major constituents are also informative. Its is emphasised that the chemical structure of the constituent polygodial is unique for a flavouring substance. The FEMA Expert Panel is aware that 1,4-dials are biologically reactive substances. Other 1,4-dials, such as *cis*-2-butene-1,4-dial are potent liver toxicants (Chen *et al*,1995; Chen *et al*, 1997). During consideration of other unique substances, the Panel requested toxicity studies be performed to document safety under conditions of use. Therefore, there is a high probability that additional studies may be requested on the major constituent polygodial.

Mr Tim Adams, a technical representative from FEMA, suggested that the application be submitted to a 1999 meeting. Additional information was required on the variation due to sources of plant material and the metabolism and toxicity with reference to toxicology of polygodial (the main constituent of the extract). The first of these issues has been addressed, as described in this report.

Toxicological testing of the extract of *Tasmannia lanceolata* has been indicated as a matter of course for any substance to be used as a flavouring or flavour enhancer. In addition, the presence of a 1,4-dial group in polygodial is further cause to perform toxicological studies, since this group has proved to be a liver toxicant in other instances.

A mouse toxicity test performed by Kyoto P. University showed that toxicity has been detected with 30µg/ml and strong toxicity was found at 100µg/ml.

The Japanese company Lotte & Toyotama has registered polygodial and polygodial plant extracts as food additives in the United States. The patent number is US 5,523,105 JP 93-293114 (931124).

This company has employed the extract of *T. lanceolata* in chewing gum and toothpaste preparations. Typical usage rates as a flavour enhancer with mint, peppermint, spearmint, herbmint, wasabi and lemon essence are shown in Table 5.1

Table 5.1: Typical usage rates of *Tasmannia lanceolata* in various products

	Flavour	**Rate In Flavour**	**Rate In Product**
Chewing Gum	Mint Flavour A	0.60%	0.0005%
	Peppermint Flavour B	0.60%	0.0005%
	Spearmint Flavour C	0.60%	0.0005%
Candy	Spearmint Flavour C	0.20%	0.00006^
	Herb Mint Flavour	0.20%	0.001%
Seasoning for Snack	Wasabi Powder	0.25%	0.00005%
Beverage	Lemon Essence	0.02%	0.00002%

Polygodial has been (as is being) investigated by various workers, including the Japanese company 'Takasogo International Corporation'. The inhibition of microorganisms for cosmetics and flavours has been researcher by Dr Tsukasa. Nagashima, the Chemist and Manager of the Fragrance Division with Takasogo. The work on *T. lanceolata* extract by Ms S. Thomas, presented in this report, complements the findings already available.

Polygodial:[1R-(1α,4aβ.8aα)]-1,4,4a,5,6,7,8,8a-Octahydro-5,5,8a-trimethyl-1,2-naphthalenedicarboxaldehyde, is the main constituent of the extract. It has well recognised anti-feedant properties and occurs naturally in the (-)- form. In the pure state it forms colourless crystals with the structural formula shown in Figure 5.1 below.

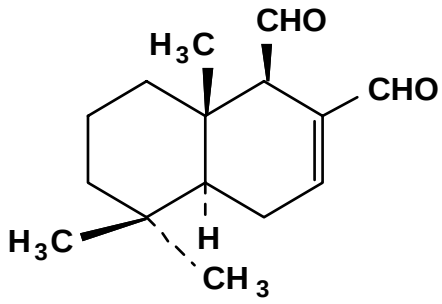


Figure 5.1 Structural formula for polygodial

The issue of the presence of safrole in the extract had to be addressed when the International Organisation of the Flavour Industry (IOFI) placed a limit of 1mg/kg in foods and beverages. From the present report, it is clear that there is potential to select clonal material that has very low or no safrole, or that breeding may be undertaken which will result in varieties that exhibit low safrole levels coupled with other desireable characteristics. In addition, the reports from Japan on usage levels suggest that at the envisage rates of application, the level of safrole in the final product is well below the 1ppm limit, even with a high safrole extract.

Traditional consumption of at least one of the species containing polygodial as a flavouring relish (*Polygonum hydropiper*) and more recently, over twenty years of use in Australia of *Tasmannia* spp.as novel foodstuffs (Cribb and Cribb, 1974; Low, 1988 and Cherikoff, 1989) would seem to offer anecdotal evidence for the safety of small quantities of the compound for human consumption.

A product sheet has been developed by Essential Oils of Tasmania, in conjunction with the University. This, together with the material safety data sheet, is presented in Appendix 3.

The extract has a Chemical Abstract Service number: CAS No. 183815-52-3.

Other avenues towards registration of the product have been explored. These include possible European registration through a laboratory in the United Kingdom, with the European list of notified chemical substances (EINECS).

FEMA has begun approval of applications for mixtures (naturals) in April 1998. All previous listings for GRAS (list of generally recommended as safe food additives) were for single substances. Upon completion of the toxicological studies, the application will be re-submitted to FEMA.

5.4 Environmental Breakdown

5.4.1 Biodegradation of the Essential Oil of *T. lanceolata* by Pure Culture

These assays were carried out prior to identification of the isolates. Results were subsequently combined to give a mean result for isolates which were subsequently found to be identical. Representative peaks of oil components were chosen that could clearly be distinguished on the initial chromatograms and which had a known identity. It was clear that for the cyclic monoterpenes α-pinene and β-phellandrene the rate of loss could be attributed entirely to volatilisation. To distinguish degradation from volatilisation, samples would need to be assessed on a more frequent basis within the first week of incubation. Airtight-sealed flasks would have also been advantageous. The sesquiterpenes, polygodial, cadina-1,4-diene and α-cubebene showed a clear decrease in amount present in the first three weeks of incubation, with little loss to volatilisation. These results were supported by the fact that sesquiterpenes are generally less volatile than monoterpenes. Clearly the structure of the compounds will influence both degradation and volatilisation rates. All isolates showed the same general pattern of degradation. The complexity of the oil makes determination of the components targeted difficult, as isolates may use similar compounds. While there is little information regarding the biodegradation of essential oils, there has been considerable work on the utilisation of some of the essential oil constituents by pure cultures. This is particularly so for monoterpenes such as α-pinene (Gibbon & Pirt, 1971; Griffiths *et al.*, 1987; Tudroszen *et al.*, 1977), linalool (Madyastha *et al.*, 1977) and 1,8-cineole (Trudgill, 1990; Trudgill, 1994; Williams *et al.*, 1989).

5.4.2 Soil Microcosms

The rate of degradation of a chemical in the environment is dependent on the physical, chemical and microbial characteristics of that environment (Heitkamp *et al.*, 1987). The complexity of essential oils adds another factor to their rate of degradation as some components are likely to be readily degraded and others may be persistent. There is also the problem of toxicity of some compounds inhibiting the microbiota in the soil environment.

The use of microcosms allowed the small-scale study of the biodegradation of the essential oil in the soil environment. In all of the five non-sterile soil types, the degradation (combined with volatilisation) of the essential oil appeared to occur rapidly, as the majority of components were no longer detectable by GC analysis after eight weeks. This indicates the presence of active degrading microbial populations in these soils despite the lack of pre-exposure to *T. lanceolata* essential oil. The microbiota may be exposed to similar chemicals through the decay of plant matter and there is also the possibility of co-metabolism of compounds. However, volatilisation would also play an important role in the removal of the essential oil from the soil environment, especially for monoterpenes such as α-pinene. As with the pure culture study, air-tight, sealed containers would have been more appropriate for this experiment. Another possible extension of the experiment would be headspace analysis to determine those compounds that have volatilised or the production of radioactive carbon-14 labeled carbon dioxide from the degradation of labelled components.

As with the bacterial cultures, these assays also encountered the problem of the complexity of the essential oil. The same choice of components was made for this experiment as for the pure culture study. It is clear that for the monoterpenes such as α-pinene, β-phellandrene and limonene, volatilisation was significant in the initial week of incubation as was apparent for the pure culture assay. Analysis of variance showed no significant variation from the volatilisation control for any soil types for α-pinene. However, for β-phellandrene and limonene there was a significant difference between the volatilisation control and most of the other soils after one week, suggesting biodegradation of the components in the first week. No conclusions could be drawn for the sandy loam and *T. lanceolata* soils which contained low initial levels of oil.

The clearest indication of biodegradation was seen for linalool and piperitone with a sharp decrease in amounts present to the point where both were undetectable after two weeks in all soils irrespective of the type. The volatilisation control was significantly different ($p<0.05$) to all other soil types with slow volatilisation occurring over the eight-week period. The polygodial curve showed a rapid decrease in the first week for all soils relative to the volatilisation control, indicating microbial utilisation, with all soils being significantly different to the volatilisation control for the first four weeks. Sesquiterpenes possess five more carbon atoms than monoterpenes and are generally less volatile than monoterpenes. However, the reverse was seen in this study, with volatilisation of the sequiterpenes polygodial, cadina-1,4-diene and α-cubebene occurring much more rapidly than the monoterpenes linalool and piperitone. Linalool is an acyclic monoterpene alcohol, piperitone is a cyclic monoterpene ketone and polygodial a sesquiterpenoid dialdehyde. The presence of oxygen-containing side-chains may influence the degradability of these compounds as such groups are absent in α-pinene, β-phellandrene and limonene. This contention is partially supported by the pure culture study where there was a clear decrease in content in the first two weeks with little volatilisation.

There was little evidence to suggest degradation of α-cubebene as analysis showed rapid volatilisation in the first week with no significant difference in loss-rates between the volatilisation control and soil samples after this time. There may be have been some degradation of calamenene in BCC, NW Red and Tlanc soils, however this was not as clear cut as for piperitone and linalool. Analysis showed unknown compound III levels to be clearly lower in the NW Red and Tlanc soils after week two with no difference between the two types, suggesting a degrading population common to both. There appeared to be little evidence for the degradation of driminol or 272-diterpene which volatilised slowly over the eight-week incubation period. For 218-terpene in podzolic soil, there was evidence of degradation occurring in the first three weeks. It is possible that this compound is co-metabolised with another compound, such as β-phellandrene or limonene, neither of which was detectable after the first few weeks.

Degradation and volatilisation were often rapid in the soil microcosms which contrasts to the pure culture study with relatively little decrease over four weeks. This may suggest that soil provides better conditions for microbial utilisation or perhaps a microbial consortium is required for degradation. Other factors to consider are the greater surface area to volume ratio found in the soil microcosms allowing a much larger area for attack. Volatilisation may also be influenced by the matrix, therefore comparison of a component on its own, to within the complex *T. lanceolata* extract may not be very straightforward. Overall there was virtual loss of all oil components examined within an eight-week period under constant environmental conditions except in volatilisation controls. The same may not, however happen in the soil environment where sub-optimal temperatures and water contents will affect the rates of volatilisation and degradation. Therefore, this study could be extended to examine the effect of such parameters on the degradation rates in soil.

Statistical analyses of the data for all components revealed these to be significantly lower in the sandy loam and Tlanc soils at time zero for most compounds ($p<0.05$). This may be due

to poor dispersion of the oil through these soils or different levels of sorption of the oil to soil particles. The low levels in these soils to begin with made distinguishing between degradation and volatilisation in these soils difficult, limiting any conclusions.

The incorporation of a surfactant into the system allowed an even dispersion of the viscous oil through the soil and subsequently exposure to a greater surface area to volume ratio. However, it does introduce another variable into the system that may influence the physiology of the degrading population as well as the rate of degradation. Volkering *et al.* (1998) suggest that addition of a surfactant in soil remediation can stimulate growth by the provision of a cosubstrate, or inhibit growth due to toxicity. Clearly, a surfactant needs to have low toxicity to warrant its use in remediation, otherwise it will only add to the site contamination. The formation of microemulsions of non-aqueous phase liquids can lead to an increase in surface area, thereby aiding mass transport (Volkering *et al.*, 1998). Surfactants may assist the dispersion of hydrophobic organic compounds owing to the formation of micelles, thereby enhancing the rate of uptake by microbiota. The reverse can also be true as micelles may decrease the bioavailability of some insoluble compounds. There is also the possibility of cometabolism of surfactant and hydrophobic organic compounds providing enhancement of degradation rate.

5.5 Commercial Extract

5.5.1 Commercial Blend

The commercial blend at present, as created by Essential Oils of Tasmania Pty. Ltd. has low safrole levels, and complies with the organoleptic requirements imposed by the Japanese client that currently employs this product. It is the result of combining extracts derived from two wild stands of *Tasmannia lanceolata*. Namely, 'Read' and 'Farquhar'. The possibility of creating other blends could be valuable, in the event that specific client requirements need to be met. Consequently, the product that is registered with FEMA as GRAS may alter slightly from time to time, but would fall within a specified range of values for major components and physical characteristics.

5.5.2 Chemical Analysis

The chemical analysis of the commercial blend has resulted in the identification of over 85% of the extracts' composition. The absolute resolution of all of the constituents is possibly not required for registration purposes, since the major components are known. In some instances, over 94% of the extract can be identified, depending on the particular clonal extract in question. Future work may produce a complete identification, though resolution of the very minor peaks would be time consuming and expensive, which may not prove cost effective.

6. Implication

The implications of successfully registering *Tasmannia lanceolata* extract with FEMA as GRAS are significant. In particular, from a commercial point of view, the market then becomes wholly accessible. At present there are very firm limits to where and how the extract can be used. With registration, users in many flavour applications will have access to this novel preparation. A broad range of applications includes:

- Cheeses
- Liqueurs
- Bakery products
- Meat marinades
- Sauces
- Cooking oils
- Chocolate and candy
- Condiments

Since the extract has such a spicy character, and given that 30% of the fragrance industry is based on spicy odours, this is another potential new sales avenue.

Polygodial has also been shown to be an effective anti-fouling agent in the control of marine barnacles. The Mitsubishi Co. is cooperating in extensive research in this area.

The extract, or its major constituent, polygodial, may be used industrially, as an insecticide, due to its proven anti-feedant qualities. An Australian company, which has an extract from *Tasmannia stipitata*, is seeking registration of its polygodial extraction process. This product is envisaged as an insecticide, particularly in the organic food industry.

Due to the anti-microbial properties, the extract would also find application in minimally processed crops.

7. Recommendations

The progress towards registration of *Tasmannia lanceolata* extract has been a slow and deliberate process. Most of the requirements for registration with FEMA on the GRAS list have been fulfilled. However, a substantial body of work is yet to be carried out on the toxicology of the extract. Negotiations are being undertaken between those flavour companies that have expressed an interest in assisting with funding for the registration, and toxicological laboratories that are able to carry out the necessary procedures.

Clones for commercial plantations would be desirable from the point of view of maintaining strict quality controls, and this work has shown that the variability within populations is such that many acceptable selections could be made. It has also highlighted the fact that the current commercial blend, derived from a wild population, has an acceptable level of safrole for present applications.

Following the FEMA pre-submission appraisal, it is recommended that the extract be submitted in the next round, provided that further toxicological data is available.

To achieve and maintain quality standards, the transect method of sampling of wild populations would be used.

Our work with end users should continue, in order to apply the latest anti-microbial findings.

Due to its large potential in market sectors other than the flavouring application, research should also continue in these areas.

8. Appendices

Appendix 1: Summary of Composition Data for 307 Samples

Tree	% Volatiles	% Safrole	% P'	% Polygodial	Total Polygodial
MEAN	*4.01*	*0.0022*	*0.20*	*1.84*	*2.04*
Variance	*1.37*	*0.000010*	*0.02*	*0.77*	*0.91*
Std Dev.	*1.17*	*0.0032*	*0.13*	*0.88*	*0.95*
Median	*3.87*	*0.0013*	*0.17*	*1.82*	*2.08*
A1	2.53	0.0026	0.03	1.48	1.51
A2	4.98	0.0180	0.03	1.17	1.21
A3	2.66	0.0019	0.02	0.97	0.99
A4	6.70	0.0000	0.15	4.52	4.68
A5	5.54	0.0039	0.01	0.29	0.30
A6	3.79	0.0028	0.02	0.79	0.82
A7	5.65	0.0013	0.08	2.29	2.37
A8	3.66	0.0108	0.07	2.17	2.24
A9	5.10	0.0048	0.11	3.24	3.35
A10	2.79	0.0003	0.06	1.76	1.82
A11	5.53	0.0018	0.12	3.34	3.46
A12	3.13	0.0012	0.08	1.88	1.96
A16	3.45	0.0017	0.08	2.14	2.22
A17	2.94	0.0021	0.02	0.56	0.58
A19	3.25	0.0034	0.06	1.60	1.66
A20	2.51	0.0012	0.03	0.98	1.01
A21	6.35	0.0000	0.05	1.29	1.33
B1	3.74	0.0018	0.09	2.14	2.22
B2	3.68	0.0035	0.09	2.28	2.37
B4	3.76	0.0000	0.04	1.17	1.21
B5	5.54	0.0033	0.11	2.82	2.93
B6	5.05	0.0047	0.11	2.73	2.84
B7	3.37	0.0000	0.08	2.20	2.28
B9	4.04	0.0010	0.02	0.58	0.60
B10	4.90	0.0010	0.12	2.98	3.10
B11	6.11	0.0015	0.02	0.31	0.33
B13	5.31	0.0007	0.12	3.07	3.19
B15	5.29	0.0006	0.15	3.60	3.75
B16	2.80	0.0048	0.03	0.79	0.82
B19	3.49	0.0000	0.09	2.10	2.19
B20	3.62	0.0024	0.10	2.31	2.42
B21	1.63	0.0000	0.04	0.95	0.99
C1	4.61	0.0040	0.08	2.05	2.13
C2	5.84	0.0106	0.14	3.19	3.33
C3	3.62	0.0015	0.02	0.45	0.47
C5	2.59	0.0048	0.07	1.44	1.51
C6	4.79	0.0019	0.05	1.06	1.11
C7	3.47	0.0045	0.09	1.83	1.91
C9	2.72	0.0024	0.08	1.65	1.73
C10	3.91	0.0015	0.12	2.52	2.64
C11	6.24	0.0026	0.16	3.60	3.77
C12	2.41	0.0000	0.04	0.83	0.87
C14	2.90	0.0030	0.02	0.45	0.47
C15	4.42	0.0013	0.04	0.76	0.79
C16	3.11	0.0000	0.08	1.61	1.69
C17	4.62	0.0051	0.17	3.03	3.20
C18	3.43	0.0007	0.12	2.05	2.17

Tree	% Volatiles	% Safrole	% P'	% Polygodial	Total Polygodial
C19	4.18	0.0000	0.18	2.94	3.13
C20	2.57	0.0000	0.09	1.66	1.75
C21	2.18	0.0062	0.07	1.28	1.35
D1	2.17	0.0000	0.08	1.29	1.37
D2	3.03	0.0000	0.11	1.98	2.09
D3	2.99	0.0011	0.11	1.80	1.90
D4	2.45	0.0000	0.10	1.42	1.51
D5	3.81	0.0000	0.08	1.43	1.50
D6	3.34	0.0000	0.13	1.95	2.08
D7	3.58	0.0000	0.12	2.15	2.27
D10	4.19	0.0051	0.04	0.83	0.88
D12	2.81	0.0023	0.11	2.12	2.23
D13	4.56	0.0000	0.15	2.79	2.95
D14	5.60	0.0032	0.21	3.61	3.82
D15	3.87	0.0019	0.08	1.42	1.50
D16	3.59	0.0030	0.14	2.29	2.44
D17	5.65	0.0028	0.22	3.57	3.79
D18	3.50	0.0000	0.05	0.74	0.80
D20	3.86	0.0013	0.14	2.37	2.51
D21	3.09	0.0020	0.10	1.59	1.69
E1	2.01	0.0005	0.07	1.07	1.14
E2	4.88	0.0000	0.16	2.09	2.25
E3	2.69	0.0014	0.03	0.58	0.62
E4	3.24	0.0055	0.15	2.11	2.26
E6	4.61	0.0026	0.16	3.02	3.19
E7	4.40	0.0048	0.10	1.72	1.82
E8	4.97	0.0000	0.19	3.22	3.41
E9	3.37	0.0000	0.14	2.10	2.24
E12	4.08	0.0000	0.17	2.68	2.85
E13	4.36	0.0011	0.19	2.70	2.89
E14	3.70	0.0037	0.07	1.04	1.10
E15	3.87	0.0000	0.15	2.50	2.65
E16	3.83	0.0012	0.06	1.15	1.22
E17	3.46	0.0013	0.11	1.87	1.98
E18	6.06	0.0034	0.11	1.64	1.75
E19	3.83	0.0030	0.15	2.42	2.58
E20	4.86	0.0133	0.20	3.27	3.47
E21	3.33	0.0115	0.14	2.15	2.28
F1	4.97	0.0052	0.15	2.83	2.98
F2	2.69	0.0050	0.03	0.42	0.45
F3	4.66	0.0005	0.09	1.46	1.55
F4	4.69	0.0031	0.02	0.41	0.43
F5	4.34	0.0000	0.13	2.90	3.03
F6	5.34	0.0035	0.08	1.65	1.73
F7	3.80	0.0023	0.07	1.42	1.49
F8	4.24	0.0021	0.15	2.62	2.77
F9	4.23	0.0023	0.13	2.70	2.83
F10	4.60	0.0008	0.05	0.96	1.01
F11	4.73	0.0043	0.16	3.09	3.25
F12	5.72	0.0097	0.21	3.59	3.80
F13	3.56	0.0039	0.17	2.20	2.37
F14	4.72	0.0018	0.21	3.15	3.36
F15	4.30	0.0000	0.10	1.45	1.55
F16	3.12	0.0028	0.13	1.74	1.87
F17	4.13	0.0021	0.21	2.52	2.73
F18	4.67	0.0016	0.16	2.25	2.41
F19	5.22	0.0017	0.24	3.37	3.60

Tree	% Volatiles	% Safrole	% P'	% Polygodial	Total Polygodial
F20	1.94	0.0033	0.03	0.42	0.45
F21	6.55	0.0008	0.33	4.18	4.51
G1	3.59	0.0019	0.13	2.15	2.28
G2	3.40	0.0018	0.09	1.60	1.69
G3	3.01	0.0000	0.11	1.92	2.03
G4	3.97	0.0014	0.03	0.35	0.38
G5	3.87	0.0008	0.14	2.34	2.48
G6	2.92	0.0064	0.06	1.09	1.15
G7	4.14	0.0029	0.18	2.50	2.68
G8	4.64	0.0035	0.18	2.60	2.78
G9	4.31	0.0006	0.09	1.02	1.10
G10	8.04	0.0008	0.19	2.92	3.11
G11	2.21	0.0058	0.09	1.14	1.22
G12	6.79	0.0000	0.29	4.68	4.97
G13	3.80	0.0011	0.19	2.25	2.44
G14	5.54	0.0038	0.29	3.60	3.90
G15	5.27	0.0019	0.12	1.72	1.84
G16	3.96	0.0000	0.17	2.26	2.43
G17	2.45	0.0017	0.06	1.29	1.36
G18	5.35	0.0000	0.18	3.00	3.18
G19	3.57	0.0009	0.04	0.61	0.65
G20	4.54	0.0000	0.25	2.77	3.02
G21	4.73	0.0073	0.25	2.97	3.22
H1	3.57	0.0047	0.07	0.88	0.95
H2	4.36	0.0023	0.11	1.36	1.47
H3	4.42	0.0041	0.24	2.58	2.83
H4	3.34	0.0030	0.15	1.68	1.83
H5	2.41	0.0034	0.13	1.40	1.53
H6	3.50	0.0015	0.18	1.94	2.12
H7	4.13	0.0039	0.25	2.57	2.82
H8	4.72	0.0000	0.12	1.22	1.34
H9	3.17	0.0029	0.15	1.70	1.85
H10	4.50	0.0006	0.20	2.11	2.31
H11	3.90	0.0000	0.23	2.35	2.58
H12	5.77	0.0007	0.13	1.30	1.43
H13	4.30	0.0000	0.14	1.52	1.65
H14	4.61	0.0009	0.32	2.95	3.27
H15	4.67	0.0000	0.26	2.57	2.83
H16	4.32	0.0000	0.29	2.49	2.77
H17	4.57	0.0000	0.26	2.72	2.98
H18	3.86	0.0065	0.08	1.04	1.12
H19	4.86	0.0156	0.25	3.10	3.35
H20	4.05	0.0000	0.22	2.56	2.79
H21	4.14	0.0000	0.24	2.51	2.75
I1	3.69	0.0000	0.18	1.97	2.16
I2	3.56	0.0018	0.19	2.14	2.33
I3	3.57	0.0009	0.16	1.81	1.97
I4	3.12	0.0000	0.15	1.80	1.96
I5	6.09	0.0044	0.05	0.51	0.56
I6	5.14	0.0000	0.14	1.77	1.92
I7	2.01	0.0009	0.12	0.88	1.01
I8	2.63	0.0000	0.16	1.34	1.50
I9	4.86	0.0000	0.39	3.05	3.45
I10	3.82	0.0000	0.14	1.20	1.34
I11	4.12	0.0013	0.33	2.51	2.84
I12	3.48	0.0026	0.27	1.85	2.12
I13	3.65	0.0010	0.06	0.46	0.52

Tree	% Volatiles	% Safrole	% P'	% Polygodial	Total Polygodial
I14	2.06	0.0000	0.05	0.36	0.41
I15	3.99	0.0012	0.31	2.36	2.67
I16	5.33	0.0030	0.40	3.03	3.43
I17	6.91	0.0040	0.59	4.10	4.69
I18	3.45	0.0000	0.26	1.71	1.97
I19	3.99	0.0015	0.30	2.09	2.39
I20	3.07	0.0017	0.24	1.72	1.96
I21	3.38	0.0021	0.24	1.57	1.81
J1	4.67	0.0000	0.37	2.64	3.02
J2	4.78	0.0081	0.09	0.75	0.84
J3	3.90	0.0000	0.08	0.58	0.66
J4	3.18	0.0018	0.08	0.61	0.69
J5	4.21	0.0000	0.33	2.04	2.37
J6	1.96	0.0000	0.03	0.18	0.21
J7	5.60	0.0006	0.06	0.40	0.45
J8	5.34	0.0000	0.41	3.08	3.49
J9	2.99	0.0038	0.23	1.59	1.82
J10	3.54	0.0000	0.30	1.83	2.13
J11	3.74	0.0013	0.28	1.80	2.08
J12	3.75	0.0000	0.27	1.92	2.20
J13	4.10	0.0000	0.12	0.83	0.95
J14	3.63	0.0020	0.30	1.98	2.28
J15	5.17	0.0017	0.45	3.02	3.47
J16	3.37	0.0014	0.32	1.85	2.16
J17	4.87	0.0000	0.09	0.62	0.71
J18	3.49	0.0012	0.14	0.88	1.02
J19	2.53	0.0007	0.22	1.23	1.45
J20	3.17	0.0006	0.29	1.67	1.96
J21	2.73	0.0030	0.22	1.47	1.69
K1	3.07	0.0009	0.13	0.84	0.96
K2	1.91	0.0000	0.09	0.48	0.57
K3	4.37	0.0000	0.14	0.85	0.98
K4	3.00	0.0026	0.27	1.66	1.93
K5	3.99	0.0000	0.24	1.39	1.63
K6	3.58	0.0000	0.31	1.75	2.06
K7	3.62	0.0000	0.11	0.68	0.79
K8	2.09	0.0006	0.04	0.27	0.31
K9	3.35	0.0035	0.28	1.83	2.11
K10	6.06	0.0010	0.23	1.69	1.92
K11	2.56	0.0000	0.19	1.01	1.20
K12	2.72	0.0170	0.08	0.44	0.52
K13	3.95	0.0042	0.29	1.92	2.22
K14	3.46	0.0009	0.24	2.01	2.25
K15	2.85	0.0000	0.26	1.35	1.60
K16	2.36	0.0000	0.19	1.15	1.34
K17	3.02	0.0027	0.19	1.14	1.33
K18	5.45	0.0016	0.47	3.25	3.72
K19	4.21	0.0026	0.30	1.80	2.10
K20	10.54	0.0052	0.49	3.01	3.50
K21	4.11	0.0000	0.32	1.90	2.21
L1	3.60	0.0028	0.32	1.97	2.29
L2	4.97	0.0013	0.10	0.63	0.73
L3	4.93	0.0007	0.48	2.64	3.12
L4	3.44	0.0011	0.35	1.98	2.33
L5	2.88	0.0014	0.08	0.47	0.55
L6	4.98	0.0000	0.23	1.58	1.81
L7	5.13	0.0030	0.37	2.85	3.22

Tree	% Volatiles	% Safrole	% P'	% Polygodial	Total Polygodial
L8	4.87	0.0016	0.33	2.54	2.88
L9	4.86	0.0005	0.38	2.85	3.23
L10	2.96	0.0029	0.28	1.52	1.80
L11	3.22	0.0010	0.34	1.82	2.16
L12	3.85	0.0000	0.03	0.16	0.18
L13	3.14	0.0000	0.24	1.41	1.65
L14	2.85	0.0000	0.27	1.41	1.68
L15	4.71	0.0018	0.24	1.41	1.65
L16	3.37	0.0014	0.31	1.61	1.91
L17	2.95	0.0012	0.27	1.47	1.74
L18	5.15	0.0000	0.39	2.30	2.70
L19	3.03	0.0000	0.15	0.87	1.02
L20	2.58	0.0008	0.09	0.52	0.61
L21	1.24	0.0012	0.32	1.72	2.04
M1	1.72	0.0019	0.11	0.53	0.64
M2	5.09	0.0000	0.23	1.21	1.43
M3	4.25	0.0020	0.22	1.31	1.53
M4	1.40	0.0030	0.02	0.09	0.11
M5	3.44	0.0014	0.33	1.74	2.08
M6	4.21	0.0053	0.41	2.21	2.62
M7	3.64	0.0000	0.34	1.83	2.17
M8	4.29	0.0011	0.11	0.65	0.75
M9	3.15	0.0022	0.23	1.17	1.40
M10	3.67	0.0000	0.36	2.00	2.37
M11	4.30	0.0000	0.33	1.92	2.24
M12	3.24	0.0000	0.32	1.71	2.03
M13	4.43	0.0098	0.41	2.23	2.63
M14	5.21	0.0009	0.18	1.11	1.30
M15	3.63	0.0000	0.30	1.92	2.23
M16	8.19	0.0000	0.17	1.26	1.43
M17	3.82	0.0006	0.32	1.87	2.19
M18	6.31	0.0138	0.59	3.53	4.13
M19	3.70	0.0020	0.32	1.92	2.24
M21	4.46	0.0000	0.33	1.77	2.10
N1	6.25	0.001	0.56	3.73	4.30
N2	4.48	0.003	0.30	2.16	2.45
N3	3.76	0.009	0.18	1.34	1.52
N4	4.89	0.005	0.20	1.29	1.49
N5	3.96	0.000	0.33	1.83	2.16
N6	4.95	0.003	0.21	1.30	1.51
N7	4.06	0.000	0.34	2.19	2.53
N8	5.22	0.005	0.48	3.07	3.55
N9	5.34	0.015	0.37	2.23	2.60
N10	3.89	0.001	0.35	2.00	2.35
N11	4.90	0.002	0.53	2.71	3.24
N12	2.60	0.009	0.21	1.12	1.33
N13	4.10	0.008	0.43	2.46	2.89
N14	3.78	0.001	0.35	2.02	2.37
N15	3.88	0.000	0.44	2.10	2.54
N16	4.25	0.002	0.32	1.96	2.28
N17	3.85	0.003	0.32	2.26	2.58
N18	4.97	0.000	0.50	2.85	3.34
N19	4.37	0.004	0.26	1.48	1.74
N20	3.08	0.002	0.30	1.52	1.82
N21	2.81	0.002	0.25	1.61	1.86
O1	3.40	0.001	0.29	1.85	2.13
O2	2.97	0.000	0.17	0.83	1.00

Tree	% Volatiles	% Safrole	% P'	% Polygodial	Total Polygodial
O3	3.36	0.005	0.25	1.63	1.88
O4	4.29	0.002	0.41	2.38	2.80
O5	3.95	0.001	0.34	2.21	2.55
O6	5.20	0.000	0.22	1.40	1.62
O7	5.61	0.000	0.50	2.92	3.42
O8	4.34	0.000	0.17	0.81	0.98
O9	1.98	0.003	0.15	0.68	0.83
O10	5.89	0.018	0.54	3.72	4.26
O11	3.64	0.008	0.15	0.84	0.99
O12	3.99	0.000	0.32	1.72	2.03
O13	3.64	0.000	0.15	0.80	0.95
O14	1.79	0.000	0.03	0.21	0.24
O15	5.22	0.000	0.32	1.94	2.25
O16	2.55	0.002	0.18	1.07	1.25
O17	4.63	0.001	0.46	2.79	3.24
O18	4.91	0.021	0.50	2.54	3.04
O19	5.12	0.002	0.24	1.26	1.50
O20	4.97	0.001	0.47	2.82	3.29
O21	5.29	0.001	0.51	2.73	3.25
P10	3.71	0.000	0.39	1.97	2.36
P11	3.24	0.000	0.31	1.92	2.23
P12	3.20	0.005	0.28	1.78	2.07
P13	4.52	0.005	0.38	2.32	2.70
P14	2.29	0.000	0.20	1.34	1.54
P15	2.96	0.000	0.23	1.64	1.87
P16	3.66	0.003	0.35	2.17	2.52
P17	2.45	0.001	0.09	0.58	0.67
P18	4.66	0.001	0.19	1.21	1.40
P19	3.81	0.001	0.31	1.94	2.25
P20	4.05	0.002	0.34	2.31	2.65
P21	3.81	0.000	0.31	2.15	2.47

Appendix 2 Results of GC-SIM analysis of low safrole samples

Sample No	mg volatiles in vial	area safrole (SIM)	conc safrole in vial ug/ml	total safrole from leaf (ug)	% safrole in leaf
2	2.00	1232698	6.501	32.862	0.01619
4	2.82	4009	0.018	0.088	0.00004
7	2.58	79144	0.415	1.847	0.00091
11	2.16	83166	0.436	2.278	0.00112
21	2.87	31481	0.163	0.736	0.00036
25	1.50	27032	0.140	0.712	0.00035
28	1.19	2607	0.011	0.062	0.00003
31	1.82	17831	0.091	0.496	0.00020
34	1.81	28255	0.146	0.877	0.00043
36	1.83	23897	0.123	0.727	0.00036
40	1.26	30986	0.161	0.926	0.00044
42	0.60	56025	0.293	1.619	0.00080
52	0.88	30235	0.157	1.489	0.00070
54	0.90	1254	0.004	0.020	0.00001
58	1.10	1892	0.007	0.042	0.00002
60	1.19	26399	0.136	0.822	0.00039
61	1.74	29800	0.154	0.771	0.00037
62	0.96	16986	0.087	0.483	0.00023
64	0.91	4227	0.019	0.095	0.00005
65	1.29	10169	0.051	0.252	0.00012
67	0.78	10744	0.054	0.351	0.00017
68	1.59	8836	0.044	0.221	0.00010
69	1.41	1167	0.003	0.016	0.00001
70	1.41	1245	0.004	0.020	0.00001
76	1.61	9751	0.049	0.284	0.00014
81	1.46	891	0.002	0.009	0.00000
83	1.70	26312	0.136	0.644	0.00031
86	1.40	1014	0.002	0.018	0.00001
92*	1.75	4644	0.022	--	-
93	1.51	16547	0.084	0.388	0.00019
96	1.79	8141	0.040	0.196	0.00009
97	1.81	24407	0.126	0.635	0.00030
99	1.63	4181	0.019	0.098	0.00005
110	1.66	1877	0.007	0.037	0.00002
119	1.76	38128	0.198	1.072	0.00053
120	1.82	815	0.001	0.007	0.00000
124	2.09	61578	0.322	1.639	0.00080
126	2.78	43479	0.226	1.113	0.00053
131	1.63	31483	0.163	0.829	0.00039
136	3.52	39333	0.205	0.966	0.00047
138	2.86	2163	0.008	0.042	0.00002
139	1.67	42722	0.222	1.040	0.00051
142	1.50	1652	0.006	0.033	0.00002
144	1.98	17009	0.087	0.499	0.00023
146	2.02	14784	0.075	0.353	0.00017

Sample No	mg volatiles in vial	area safrole (SIM)	conc safrole in vial ug/ml	total safrole from leaf (ug)	% safrole in leaf
155	1.86	780	0.001	0.006	0.00000
157	1.67	26308	0.136	0.746	0.00037
158	1.64	2300	0.009	0.047	0.00002
160	1.49	16547	0.084	0.490	0.00024
161	1.68	57904	0.303	1.688	0.00083
162	1.76	2375	0.010	0.053	0.00003
163	1.71	1399	0.004	0.023	0.00001
164	1.78	3888	0.018	0.093	0.00005
167	1.70	26597	0.137	0.672	0.00033
168	1.88	13706	0.069	0.327	0.00015
169	1.62	2649	0.011	0.052	0.00003
172	1.47	8355	0.041	0.185	0.00009
176	1.20	21839	0.112	0.517	0.00025
177	2.01	15186	0.077	0.397	0.00019
178	1.64	30063	0.156	0.756	0.00036
183	1.64	58767	0.307	1.528	0.00075
186	1.18	6455	0.031	0.186	0.00009
191	1.80	1505	0.005	0.027	0.00001
193	1.49	20925	0.107	0.580	0.00028
195	1.59	2259	0.009	0.049	0.00002
198	2.18	2366	0.010	0.048	0.00002
200	1.48	1911	0.007	0.035	0.00002
202	1.67	1709	0.006	0.028	0.00001
203	1.51	1323	0.004	0.023	0.00001
205	2.20	10881	0.054	0.265	0.00013
207	2.19	13460	0.068	0.308	0.00015
213	0.87	21191	0.109	0.503	0.00024
214	2.11	61556	0.322	1.400	0.00067
216	1.63	25366	0.131	0.669	0.00032
217	1.46	2157	0.008	0.042	0.00002
218	1.54	9154	0.045	0.227	0.00011
222	1.07	1548	0.005	0.027	0.00001
226	1.14	773	0.001	0.006	0.00000
227	0.92	16045	0.082	0.433	0.00021
229	2.28	82053	0.430	2.093	0.00103
232	1.59	13350	0.068	0.363	0.00017
235	1.71	25737	0.133	0.798	0.00038
241	1.85	17922	0.092	0.500	0.00024
244	1.21	589	0.000	0.001	0.00000
245	1.08	881	0.002	0.010	0.00001
246	1.11	1425	0.005	0.024	0.00001
250	2.09	14299	0.073	0.361	0.00018
251	1.22	207	-0.002	-0.010	-0.00000
255	1.92	20004	0.103	0.557	0.00027
260	1.38	685	0.001	0.004	0.00000
263	1.50	1418	0.005	0.023	0.00001

Sample No	mg volatiles in vial	area safrole (SIM)	conc safrole in vial ug/ml	total safrole from leaf (ug)	% safrole in leaf
264	1.87	16268	0.083	0.394	0.00019
265	1.27	125701	0.660	3.556	0.00168
268	1.37	3228	0.014	0.077	0.00004
269	3.21	128	-0.002	-0.012	-0.00001
273	1.46	103078	0.541	2.927	0.00139
274	1.82	37089	0.193	0.970	0.00047
275	2.20	50719	0.265	1.545	0.00075
279	1.41	3303	0.015	0.085	0.00004
281	1.51	3529	0.016	0.086	0.00004
288	1.46	106840	0.561	3.007	0.00145
289	1.37	2167	0.009	0.050	0.00002
292	1.70	3274	0.014	0.087	0.00004
301	1.33	34464	0.179	1.445	0.00070
302	1.46	1880	0.007	0.055	0.00003
303	1.49	16441	0.084	0.502	0.00024
307	1.66	13554	0.069	0.344	0.00016
308	1.18	3620	0.016	0.103	0.00005
309	0.60	2147	0.008	0.051	0.00003
310	1.86	10091	0.050	0.290	0.00014
312	1.99	474776	2.502	11.982	0.00582
313	1.74	1138302	6.003	34.154	0.01694
315	1.76	36493	0.190	1.119	0.00054
316	1.87	99875	0.524	3.055	0.00148
318	1.16	1057	0.003	0.015	0.00001
321	0.85	1061	0.003	0.015	0.00001
322	1.09	2560	0.011	0.060	0.00003
328	1.46	6416	0.031	0.169	0.00008

Appendix 3 Product Specification

PRODUCT DESCRIPTION: TASMANIAN MOUNTAIN PEPPER EXTRACT

Source: Leaf material of the Tasmannia lanceolata bush (commonly known as Mountain Pepper)

Extraction Procedure: Non chlorinated solvent extraction.

Storage Conditions: Very stable when stored in closed light proof container below 10¡C

Physical Data:

Polygodial content (by weight): 20 - 30%
Colour (Wilson Horticultural Colour Chart): Citron green/twice grade, code #000763
Specific gravity (25¡C): 0.98
Flash Point (Pensky-martens closed cup): 52¡C
Optical Rotation(20¡C):
Refractive Index (20¡C): 1.64
Freezing Point:
Solubility: Insoluble in water.
Soluble in ethanol.

FEMA No.: NA

CAS No.: 183815-52-3

Declaration: We hereby confirm this product is 100% natural without any additions of either artificial or nature identical substances.

Date: 15 January,1997

MATERIAL SAFETY DATASHEET

IDENTIFICATION

1. Label Name: Tasmanian Mountain Pepper Extract

2. Trade Name:

3. FEMA Number:

4. CAS Number: 183815-52-3

FIRE EXPLOSION AND REACFFVITY

1. Flash Point: (Pensky-martens closed cup) 52¡C

2. Dot Hazard Classification:

3. Extinguishing Media: Fog, Foam, C02, Dry Chemicals

4. Special firefighting procedures:

5. Unusual fire and explosion hazards:

6. Hazardous combustion or decomposition products: None

7. Stability: Stable

8. Conditions to avoid: N/A

9. Materials to avoid: May react with some plastics & rubber

10. Hazardous polymerization products: None

PHYSICAL DATA

1. Odour, appearance and physical state:
Dark citron green, which has a distinctive and exotic aroma, possessing fresh, spicy top notes overlying a peppery background.

2. Boiling Point:

3. Melting Point:

4. Specific gravity: 0.98

5. Vapour pressure:

6. Vapour density:

7. Solubility (in water): Non-soluble

PROTECTION INFORMATION

1. Respiratory:

No special requirements. Use of face mask with solvent/organic vapour cartridge advisable if exposure causes irritation.

2. Ventilation: Ventilate

3. Eyes: Chemical splash goggles advisable

4. Skin: Oil/solvent resistant gloves

OCCUPATIONAL EXPOSURE LIMIT

1. Threshold limit value: Unknown

2. OSHA Permissible Exposure Limit (PEL):

3. Has the substance ever been listed as a carcinogen or potential carcinogen in the "Annual Report on Carcinogens" published by N.T.P., by The International Agency for Research on Cancer or by OSHA?: No

HEALTH HAZARD INFORMATION

1. Health Hazard Determination:

EMERGENCY AND FIRST AID PROCEDURES

1. Inhalation exposure:
Move to well ventilated area.

2. Eye Contact:
Flush with water for 15 mins. If irritation persists seek medical advice.

3. Skin Contact:
Wash with mild soap and warm water.

4. Other:

SPILL, LEAK AND DISPOSAL PROCEDURES

1. Precautions if material is spilled or released:
Ventilate area & mop up excess. Wash with detergent & water.

2. Waste disposal methods:
Incinerate.

HANDLING AND STORAGE PROCEDURES

Store in full sealed containers, keep cool and protect from light to preserve quality.

Chemical and Common Names
Not Applicable

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