Identification of *Leptospermum scoparium* essential oils and their genetic control

by

Ravinka Dimal Kannangara

Submitted in partial fulfilment of the requirements for the degree of Master of Applied Science (Agricultural Science)

University of Tasmania, Hobart

November 2020

Author's Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma and, to the best of my knowledge, contains no copy or paraphrase or material published or written by any other person, except where due reference is made in the text of this thesis.

Ravinka Dimal Kannangara

University of Tasmania, Hobart

13th November 2020

Acknowledgements

Firstly, I would like to thank my supervisor, Dr. Sandra Garland, for her unwavering support, guidance and encouragement throughout this extensive thesis project. Even though the project included a steep learning curve from every aspect, she allowed me to work independently, which boosted my self-confidence, and steered me in the right direction to access help whenever needed.

I would like to extend my appreciation to Dr. Anthony O'Grady from CSIRO, for his efforts in setting up the trial, sharing relevant data, and aiding me with the field sample collection. A sincere appreciation goes to Dr. René Vaillancourt, for his invaluable advice and contributions to the project. I must also thank Dr. Ross Corkrey, who helped with the statistical aspects of the project. I would also like to thank Dr. David Nichols from central science laboratory (CSL), University of Tasmania for his technical expertise on GC-MS. My gratitude is extended to Dr. Liz Barbour and her team from the Collaborative Research Centre for Honey Bee Products (CRCHBP) for initiating research into the Australian honey industry and for the financial support and permissions to use the plants for the study.

I would like to thank Caroline Claye for her continual support and technical assistance in the laboratory. I would also like to thank Dr. Shane Powel, Dr. Saideepa Kumar, Sharee McCammon and Adam Smolenski who have accommodated my requests during the project. A helping hand was extended by Christopher Wellington, Nabeela Dar and Chanjoo Park, and their feedbacks and involvement throughout the project were immensely appreciated. I would like to thank all TIA staff and students working in the perennial horticulture laboratories who helped me bring this project to success.

Finally, I would like to thank my family and friends. It has been a long journey with a lot of ups and downs and I could not have done it without their support. I’m truly grateful to my parents, without their unswerving support and care, I would not have made this far.

Table of Contents

Abstract

Leptospermum scoparium J.R. Forst. & G. Forst (Myrtaceae), is commonly known as manuka, a name often associated with New Zealand flora. From an established trial site in Southern Tasmania, ethanol based leaf extracts from 172 individual plants collected from 39 provenances around Tasmania, were analysed using GC FID. As many as 70 different oil components were detected, of which 17 peaks were identified and quantified; α-pinene, β-pinene, β-myrcene, p-cymene, 1,8-cineole, γ-terpinene, linalool, terpinen-4-ol, α-terpineol, β-caryophyllene, humulene, nerolidol, caryophyllene oxide, globulol, γ-eudesmol, cubenol, α & β-eudesmol. Despite there being signs of a peak eluting in the same region as leptospermone, no triketones were detected in Tasmanian samples. Ethanol extracts produced high levels of nerolidol (0 - 3.6 mg/g DW) and eudesmols (0 – 10.7 mg/g DW). K-means clustering and principal component analysis identified three clusters within Tasmania and recursive partitioning identified components that best predict Tasmanian chemotypes. α & β-Eudesmol and α-terpineol were used to define the three chemically distinctive clusters, confirming chemotypes exist within Tasmania. Despite being able to define clusters within the state, the geo-coordinates of the clusters did not correspond to specific geographically distinct regions.

Key words: *Leptospermum scoparium*, leptospermone, nerolidol, α-terpineol, α & β-eudesmol

CHAPTER 1: Project Rationale

Essential oils are concentrated natural plant products containing a mixture of volatile aromatic compounds. These compounds are mainly extracted by steam distillation and are known to exhibit various beneficial bioactive properties (Adorjan & Buchbauer 2010).

Leptospermum scoparium, commonly identified as manuka, is considered to be the most important and widespread native plant species in New Zealand (Stephens, Molan & Clarkson, 2005). During the past decade, essential oils distilled from *L. scoparium* leaves have been commercially exploited in New Zealand (Christoph, KubecZka & Stahl-Biskup, 1999; Maddocks-Jennings et al. 2005). Comprehensive studies have been conducted on New Zealand *L. scoparium* populations which have aided in developing different chemotypes that correlate with favourable oil characteristics (Porter & Wilkins 1998; Douglas et al. 2004; Maddocks-Jennings et al. 2005). Studies have shown that essential oils derived from New Zealand *L. scoparium* populations are claimed to be a superior product compared to the Australian populations due to the presence of elevated levels of triketones and sesquiterpene hydrocarbons, which confer health benefits (Perry et al. 1997; Douglas et al. 2004). A study conducted by Brophy et al. (2000) found both monoterpenes and sesquiterpenes, but no triketones, in the essential oils produced within a *L. scoparium* population in eastern Australia. In contrast, Perry et al. (1997) reported the presence of triketones; flavesone, leptospermone and isoleptospermone within the Australian *L. scoparium* population at levels similar to those in Northern New Zealand *L. scoparium* populations. Furthermore, the paper also stated that the Tasmanian population of *L. scoparium* were morphologically different from other Australian populations and also from New Zealand *L. scoparium* populations.

These studies have revealed that there is scope to identify genotypes within Australia that produce elevated levels of triketones and sesquiterpenes. Thus, this thesis examines oil components from *L. scoparium* populations across Tasmania with a view to identifying chemotypes that are on par or better than New Zealand *L. scoparium* populations.

CHAPTER 2: Literature Review

2.1 Taxonomy

Leptospermum scoparium J.R. Forst. & G. Forst, commonly known as manuka, belongs to the family Myrtaceae. This family contains more than 3800 species of which 83 species are of the *Leptospermum* genus (Stephens, Molan & Clarkson, 2005). The Myrtaceae family is distinguished by cohesion of several features: flowers containing half inferior, to inferior ovary, usually several stamens, internal phloem, entire leaves containing oil glands, and vestured pits on the xylem vessels (Wilson et al. 2001; Stephens, Molan & Clarkson, 2005). Leaf anatomy analysis of 40 different *Leptospermum* species have shown that *L. scoparium* contains a xeromorphic structure which is typical of the genus (Johnson 1980), while the wood anatomy (Johnson 1984; Patel, Manaaki & Research, 1994) and pollen morphology (McIntyre 1963) supports the genus classification.

2.2 Morphology

L. scoparium is a fast growing small tree or shrub with a height ranging from 1 m to about 7 m (Bond, Dickinson & Mark, 2004). The bark is firm and close with juvenile stems bearing a silky pubescence which soon becoming glabrous. Leaves appear to be highly variable in both shape and size, from lanceolate to broadly elliptical, incurved margins, coriaceous appearance with sharply pointed, distinct ends. Flowers are white or red or rarely pink, appearing on axillary or occasionally, terminal positions on branchlets. Overall control of inflorescence is influenced by day length and temperature. During winter, bud development is restricted due to cold temperatures but as the temperature gets warmer with long days, flowering is initiated and, the blossoms appear between the months of October to February (Thompson 1989; Stephens, Molan & Clarkson, 2005).

2.3 Distribution

L. scoparium is widely spread throughout New Zealand and is now recognized to be the only indigenous species of *Leptospermum* in that country out of the 87 wild species of *Leptospermum* found worldwide (Maddocks-Jennings et al. 2005; Dawson 2012). However, some controversy exists that even this species is not endemic to New Zealand, as it also naturally found in south-

eastern mainland Australia and is reported to sprout across most of its range within Tasmania, particularly in heath vegetation (Thompson 1989; Bond, Dickinson & Mark, 2004). Similarly, in New Zealand, the species is able to thrive over a wide range of geographical areas and has highly variable physiology and morphology (Perry et al. 1997; Bond, Dickinson & Mark, 2004).

Burrell (1965) reported that all New Zealand populations of *L. scoparium* are readily killed by fire though Harris (2002) reported varying levels of serotiny in plants grown from seeds collected from different provenances across New Zealand. Thompson (1989) suggested that the serotinous trait evolved either in situ or spread to New Zealand from Australia as it is a common trait found in Australian flora. *L. scoparium* populations found in Victoria, Australia and in Tasmania were found to be strongly serotinous as they has been observed to withstand fires due to the presence of lignotubers (Bond, Dickinson & Mark, 2004), especially in the endemic Tasmanian *L. scoparium* var. *eximium* species. This is a feature not found in New Zealand's *L. scoparium* populations (Stephens, Molan & Clarkson, 2005). Tasmanian manuka and New Zealand manuka were reported to be different in terms of their colour and form, along with differences in leaf thickness and shape (Bond, Dickinson & Mark, 2004).

2.4 Essential oils

As with many of the species from the Myrtaceae family, the essential oil production in *L. scoparium* occurs within the schizogenous cavities (oil sacs) that are found on the underside of the leaf surfaces and the seed capsules (Retamales 2007). Commercially it is extracted by steam distillation of harvested leaves and terminal branches.

Over the last decade, essential oils distilled from *L. scoparium* leaves have been commercially exploited in New Zealand with a range of oils originating from different provenances, often with varying properties (Porter et al. 1998; Christoph, KubecZka & Stahl-Biskup, 1999). The essential oil compositions are variable between populations from different provenances, and this is reflected in the varying degree of biological activity of the oil (Porter et al. 1998). To aid in the identification of a distinctive population, chemotaxonomic analysis of *L. scoparium* essential oils is suggested as it reveals a distinctive chemical composition for a plant species (Perry et al. 1997).

Analysis of *L. scoparium* honey have revealed that its biological activity is due to different constituents to those which confer bioactivity in *L. scoparium* essential oils (Tan et al. 1988). The bioactivity of manuka honey is attributed to the presence of methylglyoxal (MGO) (Alvarez-Suarez et al. 2014) whereas the triketones; leptospermone, isoleptospermone and flavesone contribute to bioactivity in the leaf essential oils (Douglas et al. 2004).

2.4.1 Chemistry

Essential oils are made up of terpenoid compounds which are identified and quantified via gas chromatography–mass spectrometry (GC-MS) (Chizzola 2013; Dhifi et al. 2016). Some of the major constituents which make up the *L. scoparium* essential oils are triketones, sesquiterpene hydrocarbons and monoterpene hydrocarbons (Christoph, KubecZka & Stahl-Biskup, 1999; Maddocks-Jennings et al. 2005).

Monoterpenes are made up of two isoprene units (C_{10}) (Banthorpe, Charlwood & Francis, 1972). The basic structure is a pinane skeleton bearing a bicyclic structure and the major species include α-pinene and β-pinene (Banthorpe, Charlwood & Francis, 1972). Sesquiterpenes are made up of three isoprene units (C_{15}) and, similar to monoterpenes, two species of sesquiterpenes are found; sesquiterpene hydrocarbons and oxygenated sesquiterpenes (Cordell 1976). Many sesquiterpene hydrocarbons are present in *L. scoparium* essential oils.

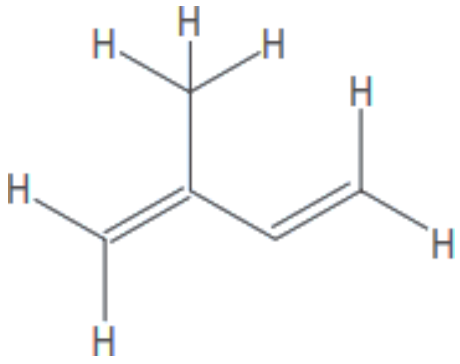
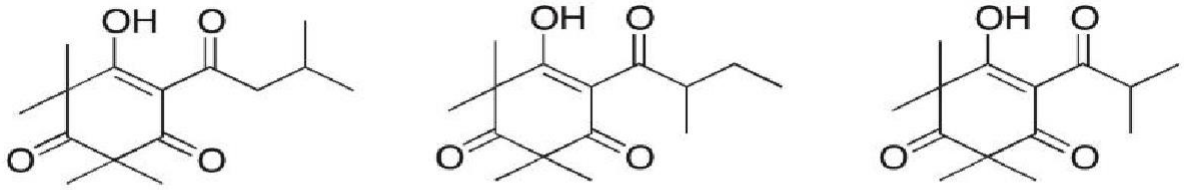


Figure 1: The chemical structure of an isoprene unit (Aldred, Buck & Vall, 2009).

2.4.2 Composition specific to *L. scoparium* essential oils

A chemical class in New Zealand *L. scoparium* oil is the triketones, which are characterized by cyclic polyketones, subsequently called β-triketones. They contain an acyl side, and four methyl substituents attached on to a six membered ring. The three main β- triketones that have been identified in *L. scoparium* essential oils are flavesone, isoleptospermone and leptospermone (Figure 2). In solution, all three components have keto-enol tautomerism, with the enol form dominating (Van Klink et al. 1999).



Leptospermone $C_{15}H_{22}O_4$ | Isoleptospermone $C_{15}H_{22}O_4$ | Flavesone $C_{14}H_{20}O_4$

Figure 2: Three main β-triketones found in *L. scoparium* essential oils (Manuka Beta Oil β-Triketones n.d.).

The GC and GC-MS analysis of *L. scoparium* essential oils typically produces a pattern of around 100 different components. Comprehensive studies by Brophy et al. (2000) and Christoph (2001) listed several monoterpene and sesquiterpene compounds that were analysed by GC fitted with a column containing a polar stationary phase (Table 1), however, in both studies, triketones were not detected in the samples.

Table 1: Compounds detected in the essential oils of *L. scoparium* based on Brophy et al. (2000) (A) and Christoph (2001) (B).

No.	Compound	RI	
		A	B
1	α-Pinene	1020	1021
2	α-Thujene	ND	1025
3	β-Pinene	1105	1109

4	Sabinene	ND	1121
5	Myrcene	1156	1164
6	α-Terpinene	1174	1184
7	Limonene	1200	1201
8	1,8-Cineole	1206	1209
9	(Z)-β-ocimene	1237	ND
10	γ-Terpinene	1244	1251
11	(E)-β-Ocimene	1254	1255
12	*p*-Cymene	1264	1275
13	Terpinolene	1283	1287
14	α-Cubebene	ND	1460
15	α-Ylangene	ND	1482
16	α-Copaene	ND	1494
17	α-Gurjunene	ND	1529
18	Linalol	1545	1548
19	β-Elemene	1585	1592
20	β-Caryophyllene	1594	1600
21	Terpinen-4-ol	1603	1603
22	Aromadendrene	1610	1608
23	α-Bulnesene	1613	ND
24	Cadina-3,5-diene	ND	1635
25	*allo*-Aromadendrene	1642	1644
26	Humulene	1667	1670
27	Selina-4,11-dien-14-al	ND	1676

28	γ-Muurolene	ND	1689
29	Viridiflorene	1695	1694
30	α-Terpineol	1700	1698
31	Germacrene-D	ND	1709
32	α-Muurolene	ND	1716
33	β-Selinene	1720	1724
34	α-Selinene	1725	1728
35	Geranial	1728	ND
36	Bicyclogermacrene	1732	1732
37	δ-cadinene	1758	1756
38	Cadina-1,4-diene	ND	1782
39	Benzyl valerate	ND	1822
40	(-)-*trans*-Calamenene	ND	1835
41	Geraniol	1836	ND
42	Palustrol	1934	1938
43	Caryophyllene oxide	1983	1981
44	Ledol	ND	2026
45	(E)-Nerolidol	2049	2042
46	Globulol	2086	2065
47	Viridiflorol	2091	2085
48	Spathulenol	2136	2121
49	γ-Eudesmol	2176	2176
50	α-Eudesmol	2229	2220
51	β-Eudesmol	2238	2230

2.5 Chemotypes

The literature revealed that there is a large variation in the essential oil composition found between *L. scoparium* populations. This has allowed researchers to categorise them into different chemotypes (Porter & Wilkins 1998; Christoph, KubecZka & Stahl-Biskup, 1999; Douglas et al. 2004).

A study conducted by Perry et al. (1997) stated that plants from distinct populations in New Zealand, grown from seed, but cultivated on a single site, had different chemical profiles and showed that East Cape population contained the highest level of triketones (>30%). In contrast, populations from the rest of the country reported contrastingly low triketone levels (1-2.4%). Some of the lowest levels of triketones were identified in North Cape populations which had high levels of monoterpene hydrocarbons such as α-pinene (45–47%) and β-pinene (7–10%) whereas the populations from further south contained a complex mixture of sesquiterpenes and oxygenated sesquiterpenes. The paper further stated that chemotypes reported in *L. scoparium* populations were matched to the morphological types to a certain degree and further sampling would allow for the identification of distinct oil compositions between populations and within geographic boundaries. Porter & Wilkins (1998) provided supportive evidence to those results obtained by Perry et al. (1997). Four different geographical sites have been identified based on distinguishing oil components; oils distilled from wild East Cape population contained high levels of triketones, Nelson and Kaiteriteri areas were rich in eudesmol and linalool, Woodstock and Canterbury areas rich in pinene whilst a population that was deficient in the triketone, eudesmol and linalool was spread across the rest of New Zealand.

A comprehensive field study conducted by Douglas et al. (2004) analysed essential oils from 261 *L. scoparium* plants across 87 locations throughout New Zealand. Oils collected from the plants found in the East Cape reported high triketone levels (>20%). These results are in accordance with the results obtained by Perry et al. (1997). Ten distinct chemotypes were identified and are characterised by the following hydrocarbons;

1. α-pinene
2. sesquiterpene/myrcene
3. caryophyllene/humulene

4. sesquiterpene 33
5. geranyl acetate
6. γ-ylangene/α-copaene
7. sesquiterpene plus East Cape triketone chemotype
8. methyl-cinnamate/sesquiterpene
9. linalool
10. elemene/selinene

The use of chemotypes can be confounded by variation in component profiles across different seasons. Douglas et al. (2004) investigated the seasonal variation in East Cape triketone chemotype by studying the oil composition from 36 individual plants and reported that the mean values of triketones were highest in late spring reaching about 35% and gradually decreasing to about 25% in autumn.

A chemotaxonomic study on plants that were collected and raised from seeds of a single wild population in Nelson, New Zealand, revealed that there were no differences between plants based on oil composition, but reported variation in the oil components between young (1–2 years) and mature plants (Porter et al. 1998). The paper further stated that the total monoterpene level gradually decreased from 45.6% in midsummer to 38.9% in autumn, indicating essential oil with elevated compositions are to be obtained during the main spring to summer periods of leaf growth. A similar study revealed that *L. scoparium* oil compositions were largely under the influence of genetics, as the plants that were raised from seeds in a single study site, produced similar oil compositions to those of plants found at the seed source site (Perry et al. 1997). Furthermore, the paper stated that phenotypic variability can be a response mechanism to changes between and within seasons, and to climatic variability. Results of Perry et al. (1997), Porter et al. (1998) and Douglas et al. (2004) provide conclusive evidence that there is an effect of seasonal variation on oil composition. Thus, the time of sampling could have a confounding effect when defining each chemotype and should be considered when selecting for improved chemotypes.

Most of the chemotaxonomic analysis of *L. scoparium* essential oils has been undertaken for New Zealand populations. As stated, this has revealed a pattern of about 100 different constituents, with 50% of them contributing 95% of the volume (Christoph, KubecZka & Stahl-Biskup, 1999;

Maddocks-Jennings et al. 2005). Christoph, KubecZka & Stahl-Biskup (1999) studied 16 different commercial samples of *L. scoparium* essential oils from New Zealand and reported that most contained around 35 sesquiterpene hydrocarbons (60-70% of the oils) with cadina-1,4-diene (mean 4.7%), α-copaene (5.3%), cadina-3,5-diene (6.3%), δ-cadinene (6.3%) and (−)-trans-calamenene (12.5%) making up the majority of the oils. The triketones (leptospermone, isoleptospermone and flavesone) made up to around 20% (mean 22.1%), while monoterpene hydrocarbons were found to be in a range between 2.2-14.1% (mean 4.5%). It was found that four of the sixteen samples were distinct, having much lower quantities of the triketones (mean 14%) and relatively higher quantities of α-pinene (8-11%).

Australian population includes several endemic varieties that are reported to also have high monoterpene content (Perry et al. 1997; Porter & Wilkins 1998; Christoph, KubecZka & Stahl-Biskup, 1999; Douglas et al. 2004). These were found to be similar to *L. scoparium* population found in northern New Zealand (Perry et al. 1997). An extensive study was conducted by Brophy et al. (2000) to identify the essential oils derived from the genus *Leptospermum* in eastern Australia. Two *L. scoparium* varieties were analysed and the major monoterpenes and sesquiterpenes identified (Table 2).

Table 2: Comparison of essential oils from two plant varieties from the genus Leptospermum in eastern Australia (Brophy et al. 2000).

	***Leptospermum* species**	
	***L. scoparium* var. *scoparium* (%)**	***L. scoparium* var. *eximium* (%)**
α-Pinene	0.7–13	2–25
1,8-Cineole	2–10	
Linalool	0.4–5	
Terpinen-4-ol	0.8–2	
α-Terpineol	1–5	
β-Caryophyllene	2–15, the majority being <5	
Humulene	0.4–7, the majority being <4	
Globulol	2–6	5–8
Viridiflorol	3–8	4–11

Spathulenol	2–4	
α-, β- and γ-Eudesmol	12–27 in total	2–38 in total

The β-triketones, flavesone and leptospermone were previously found in var. *eximium* in a study conducted by Perry et al. (1997) but were not detected by Brophy et al. (2000). As previously noted, the Tasmanian population of *L. scoparium* were different from both mainland Australian populations and from the New Zealand *L. scoparium* populations (Perry et al. 1997). This is supported by Bond, Dickinson & Mark (2004) who reported that East Australian and Tasmanian populations of *L. scoparium* differed from each other and from the taxon in New Zealand. This has serious trans-Tasman implications. *L. scoparium* is also known colloquially as Manuka, and this label has been heavily promoted by New Zealand, not only in relation to its essential oils, but more famously, for its honey which is produced from the nectar of the *L. scoparium* flowers. New Zealand has invested heavily in the R&D of these products and has undertaken significant genetic selection and characterisation of wild stock to produce oils and honey with high levels of bioactivity.

With New Zealand having invested resources in R&D and promotion, there is an ongoing trademark dispute between Australia and New Zealand (Beavis 2018; Frykberg 2019) with regards to the use of generic name of "manuka" (Lloyd 2017), and in early 2017, the Ministry for Primary Industries in New Zealand introduced a scientific regulatory definition for exported manuka honey based on pollen DNA marker analysis and chemical fingerprinting (McDonald et al. 2018). On the other hand, very little work has been done to investigate Australian *L. scoparium* populations, particularly those endemic to Tasmania. This suggest that further investigation is needed. This chemotypic study may demonstrate a close relationship between Tasmanian and New Zealand *L. scoparium* populations and challenge New Zealands' exclusive claim to manuka essential oil and honey.

2.6 Genetic influence on phenotype

There have been several studies that have examined the variable phenotypic characteristics expressed by New Zealand *L. scoparium* populations. An extensive field study conducted by Ronghua, Mark & Wilson (1984) analysed the leaf variation in 182 herbarium plant specimens

that were representing most of its natural habitat. This revealed that leaf morphology (length: width ratio and leaf length) is significantly correlated with latitude, altitude, annual and winter temperatures and distance from the coast. Furthermore, the paper stated that a seedling population raised from a uniform environment expressed different leaf morphologies, and age at first onset of flowering, thus suggesting a significant genetic influence.

A recent study was conducted to assess mechanisms and patterns of *L. scoparium* shrub encroachment across three geological areas within Grampians National Park, south-eastern Australia and it was stated that there was no significant variation identified in relative growth of seedling across the areas (Price & Morgan 2006). The paper further stated that growth, germination and survival in the field were not influenced by seed source. However, variation in biomass and shoot length measured when seeds were grown under uniform conditions suggests possible genetic variation. These results agree with that of Ronghua, Mark & Wilson (1984).

2.7 Extraction of essential oils

Essential oils and chemical components can be extracted from various plant materials by different methods. The form and state of the material, along with the time required to extract oil components from the material are factors that are needed to consider when determining the most appropriate method (Cassel et al. 2009; Tongnuanchan & Benjakul 2014). The use of unsuitable extraction methods can alter the chemical composition of the essential oil, leading to a loss in natural character and bioactivity.

2.7.1 Steam distillation

Steam distillation is the most common extraction methods used for extracting essential oils from plants (Reverchon & Senatore 1992; Chemat & Boutekedjiret 2015). Fundamentally, the steam is usually generated from an external boiler and is directed through the plant material which is placed on a perforated grid. The water vapour penetrates the vegetative mass and the heat applied acts as a lysis mechanism to break down the cellular structures thus aiding the release of essential oils from the plant material. The emerging mixture of oil and vaporised water is passed through a condensing vessel, where the steam is condensed. The mixture of essential oil and condensed water is collected into a separation vessel the oil and water partition occurs, primarily due to the density

difference between essential oil and water at ambient temperature (Chemat & Boutekedjiret 2015; Butnariu & Sarac 2018).

Use of water as an extraction solvent has several advantages; it is inexpensive, non-toxic, non-flammable and environmentally friendly (Filly et al. 2016). At elevated temperatures and with prolonged extraction periods, solvent-matrix interaction is increased, but this can lead to the loss of volatile compounds and result in the chemical modification of the essential oil components (Chemat & Boutekedjiret 2015; Filly et al. 2016). Most literature studies have reported an extraction period of 2 h for the production of essential oils from *L. scoparium* foliage by steam distillation (Perry et al. 1997; Porter & Wilkins 1998; Douglas et al. 2004). However, the use of steam distillation to extract oils may not be feasible in the present study primarily due to the restricted time availability to complete the project and the low biomass of the young plants within the trial.

2.7.2 Solvent extraction

Baker, Lowe & Southwell (2000) stated that the use of solvent extraction is a possible alternative method for extracting oils from plant leaves. Organic solvents are often used to extract oils from delicate flower materials where the volatile oil compounds are likely to degrade rapidly if exposed to the heat of steam distillation. The most commonly used solvents include hexane, acetone, petroleum ether, ethanol or methanol (Tongnuanchan & Benjakul 2014; Chemat & Boutekedjiret 2015; Butnariu & Sarac 2018). The solvents are removed from the oil by evaporation but, residues are often retained due to incomplete removal (Tongnuanchan & Benjakul 2014). However, studies have reported that in some cases, solvent extraction is preferred over steam distillation as the end product composition is not dependent on component volatility. In addition, extract composition is not changed as a result of extended thermal reactions (Boland et al. 1982; Weston 1984). Solvent extraction for the purposes of analyses, can be used for smaller sample size and can be used to extract multiple samples simultaneously (Baker, Lowe & Southwell, 2000), thus allowing for more efficient throughput of plant samples.

With view to establishing an efficient analytical methodology, Baker, Lowe & Southwell (2000) quantitatively compared the major oil components recovered from tea tree (*Melaleuca alternifolia*) foliage using a 2 h steam distillation method and an ethanol based solvent extraction. The paper

concluded that ethanol extraction produced 14% to 16% higher mg/g of oil yield, compared to the oil recovery from a six hour steam distillation of leaves the same trees. Furthermore, the total monoterpenes and sesquiterpenes made up a greater proportion of the extract from ethanol extracts compared to steam distillation. Southwell & Stiff (1989) conducted a similar study into the variation in monoterpenes from steam distillation oil compared to that of ethanol extraction of *M. alternifolia* leaves. The paper reported that analysis of the solvent extract of a single mature leaf produced almost identical GC chromatograms to that of steam distilled oils when comparatively equal concentrations of each extract were injected. However, analysis of flush growth (leaves near the apex) reported much higher levels of thujanes (sabinene, *trans*-sabinene hydrate and *cis*-sabinene hydrate) with concentration of 52.2% in the solvent extract compared to only 0.5% in steam distilled oils, while the opposite was reported for the terpinols and terpinenes which made up 74.2% of the steam distilled oils and only 16.2% of the ethanol extract.

2.8 Identification of terpenoid compounds

It is widely accepted, that essential oils are mainly made up of terpenes, which are the most structurally varied group of natural plant products (Zellner et al. 2008). The separation and identification of terpenoid compounds in plant essential oils relies heavily on GC (Davies 1990).

2.8.1 Kovats indices

The mass spectra of many terpenes are often identical due to the similarities in structures or due to rearrangements of isomers during fragmentation and ionization in the mass spectrometer (Jennings & Shibamoto 1980). As a result, peak assignment can be difficult therefore, retention characteristics during GC are often needed to complement the mass spectral data. Calculation of Kovats retention indices provide an effective tool as it allows retention time of an unknown analyte to be expressed relative to the retention times of known standards such that the parameter is independent of the system on which the data was acquired. These independent constants describing its' elution behaviour (Wilson, Barnes & Boswell, 2014). The use of GC-MS structural information combined with retention indices is widely accepted and used to confirm the identity of compounds (Zellner et al. 2008).

Retention indices (RI) are determined using a homologous series of straight chained paraffins as reference peaks. Under isothermal GC conditions, retention times increase exponentially, therefore a semilogarithmic relationship exists between the adjusted retention of the *n*-paraffins and their carbon numbers (Figure 3). As a result each unknown analyte is referenced between the two *n*-paraffins that bracket its retention time (Zellner et al. 2008).

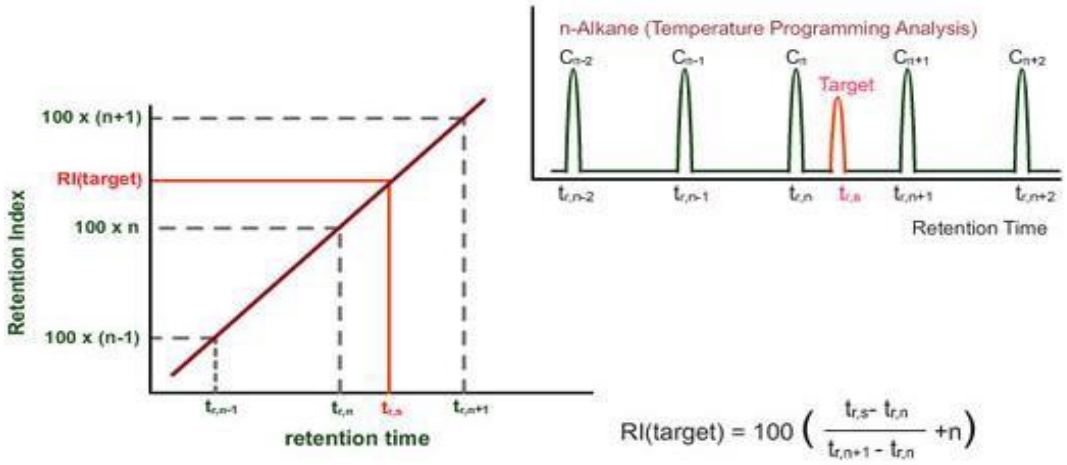


Figure 3: Scheme of the calculation of isothermal retention indices (Shimadzu n.d.).

2.8.2 Single ion monitoring

Single ion monitoring (SIM) in MS allows qualitative determination of a certain specific compound within the total complexity of the ion trace of the analyte (Kitson, Larsen & McEwen, 1996). As only specific ions, unique to the fragmentation pattern of the target analyte are monitored within a retention time, high specificity and high sensitivity are achieved, especially when the constituents are present in low concentrations (Kitson, Larsen & McEwen, 1996; Xiao, Zhou & Ressom, 2012). Although SIM can detect very low concentrations of a constituent within an analyte, this detection level is highly correlated to the compounds of interest and the interferences that are generated (Kitson, Larsen & McEwen 1996).

2.9 Project context and aims

This project seeks to investigate the prevailing paradigm that Australian *L. scoparium* populations have low levels of triketones and sesquiterpenes. It seeks to establish the differences in the essential oils derived from Tasmanian populations as compared to those of the Australian mainland and to the essential oils produced by New Zealand populations.

Both genotype and environment influence the essential oil production. As a result there is a large variation in the essential oil composition found between wild *L. scoparium* populations. It can be difficult to distinguish the effect of genotype on the oil composition within their natural habitats. Therefore, this study provides a unique opportunity to investigate essential oils produced by genetically diverse Tasmanian *L. scoparium* plants established and grown under similar conditions. Specifically, it aims to:

1. Determine the variation in oil composition between families of *L. scoparium* collected from a range of provenances across Tasmania and grown under similar conditions in a trial plot.
2. Investigate if identified chemotypes can be used to differentiate closely related varieties of *L. scoparium* across Tasmania.

CHAPTER 3: Materials and Methods

3.1 Study of provenances and seed collection

Seeds had been collected from wild species of *L. scoparium* found within 39 provenances around Tasmania (Figure 4). At each provenance, five trees (families) had been randomly selected and from each family, 5-10 g of wood seeds pods had been collected. Each family within a provenance had been selected from plants at least two tree heights apart where possible, but the distances between each family were highly variable due to the number of plants available and the ability to safely access the plants.

Seeds had been collected in 2016 and a specific geotag number was allocated to each provenance based on their collection date (Table 3).

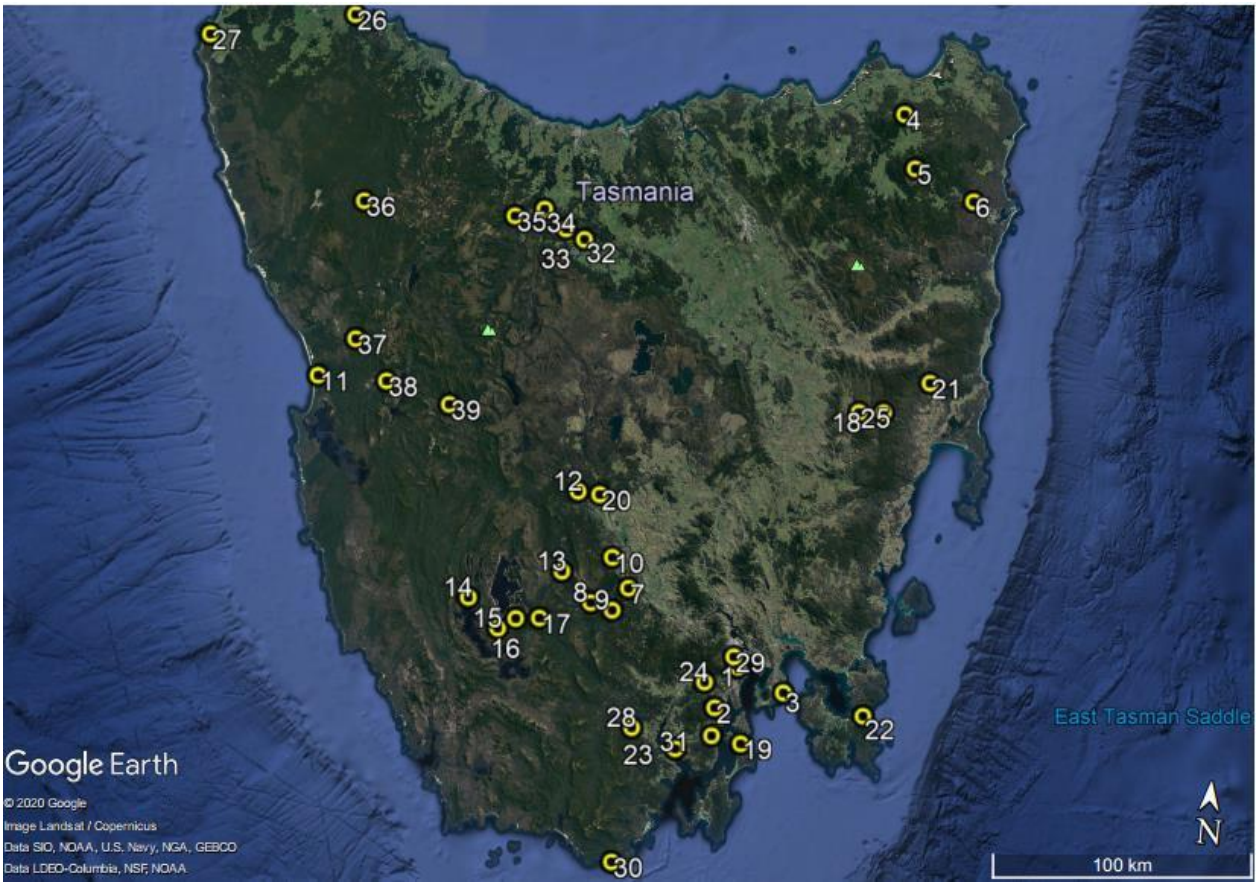


Figure 4: Distribution of the 39 provenances across Tasmania.

Table 3: Summary of the seed collection data used in this study.

Geotag	Provenance	State Region	Latitude	Longitude	Elevation	Collection Date
1	Mount Nelson	SE	-42.913	147.316	245	15-Jan-16
2	Margate	SE	-43.057	147.228	229	17-Jan-16
3	North Clifton	SE	-42.983	147.537	8	07-Feb-16
4	Cameron	NE	-41.003	147.804	151	13-Feb-16
5	Moorina	NE	-41.182	47.876	141	13-Feb-16
6	Goshen	NE	-41.270	148.154	82	13-Feb-16
7	Westerway	SE	-42.685	146.779	110	06-Mar-16
8	Junee Rd	SE	-42.746	146.613	302	06-Mar-16
9	Florentine	SE	-42.764	146.715	274	14-Mar-16
10	Ellendale	SE	-42.587	146.692	336	14-Mar-16
11	Henty Dunes	SW	-42.066	145.282	21	27-Mar-16
12	Wayatinah	SE	-42.378	146.508	247	28-Mar-16
13	Eleven Rd	SE	-42.650	146.468	421	28-Mar-16
14	Lake Pedder	SW	-42.767	146.052	323	28-Mar-16
15	Sentinel Range	SW	-42.861	146.199	346	28-Mar-16
16	The Wedge	SW	-42.823	146.278	406	28-Mar-16
17	Frodshams Pass	SW	-42.814	146.385	506	28-Mar-16
18	Meetus Falls Rd	NE	-42.010	147.853	685	16-Apr-16
19	Murrayfield	SE	-43.170	147.365	21	22-Apr-16
20	Catagunya Rd	SW	-42.382	146.608	527	30-Apr-16

21	Old Coach Rd	NE	-41.894	148.048	335	06-May-16
22	Eaglehawk Neck	SE	-43.033	147.914	399	15-May-16
23	Lymington Rd	SE	-43.208	147.067	52	20-May-16
24	Longley	SE	-42.974	147.169	363	12-Jun-16
25	Lake Leake Rd	SE	-42.019	147.741	610	12-Jun-16
26	Peggys Beach	NW	-40.842	145.315	26	09-Jul-16
27	West Point Rd, Arthur River	SW	-40.942	144.670	51	10-Jul-16
28	Geeveston	SE	-43.152	146.856	295	17-Jul-16
29	Knocklofty	SE	-42.880	147.292	313	23-Jul-16
30	South East Cape	SE	-43.611	146.824	37	06-Aug-16
31	Pullens Rd, Woodbridge	SE	-43.152	147.228	57	02-Sep-16
32	Alum Cliffs	NW	-41.534	146.430	389	24-Sep-16
33	Gog Forest	NW	-41.505	146.343	345	24-Sep-16
34	Claude Rd	NW	-41.446	146.239	263	24-Sep-16
35	Wilmot Power Station	NW	-41.477	146.109	468	24-Sep-16
36	Waratah	NW	-41.469	145.426	660	24-Sep-16
37	Mount Dundas	SW	-41.934	145.440	220	25-Sep-16
38	Gormanston	SW	-42.066	145.595	309	25-Sep-16
39	World Heritage Apiary Site	SW	-42.127	145.889	410	25-Sep-16

3.2 Seedling establishment

Seed capsules from each family that had been stored in brown paper bags were allowed to dry slowly until the seeds were relased. Seeds were stored in 5 ml glass vials until ready to be planted.

Seeds had been germinated over the 2016/2017 summer in a glasshouse at CSIRO Land and Water at Sandy bay in Hobart, Tasmania. Seeds from the species are quite small, therefore mutiple seeds

belonging to each family had been raised in a locally sourced seed raising mix within individual forestry tubes. Later, established seedlings had been thinned until only one seedling remained within each tube (O'Grady & Worledge 2020).

3.3 Experimental design

A trial plantation of *L. scoparium* was established using the seedlings at Pittwater Road in Southern Tasmania, using a randomized complete block design with five blocks as replicates of 195 families from 39 provenances. Within each block, plants had been established in a 3 x 3 m grid spacing and irrigated using an inline dripper. Weed control had been maintained using a combination of chemical control and manual weeding (O'Grady & Worledge 2020).

Climate at the site is classified as temperate oceanic and the site lies in a fairly dry zone, with an annual rainfall of approximately 500 mm and a mean pan evaporation of about 1300 mm per year. Mean minimum daily temperatures range from 4.2°C to 12.2°C between July and January, while the mean maximum daily temperatures range from 22.7°C to 12.5°C between January to July (Bureau of Meteorology 2020). Soils had a texture contrasting profile, an aeolian derived sandy top soil over a deep heavy clay subsoil (O'Grady, Worledge & Battaglia, 2005).

3.4 Plant collection

On a single collection day, laterals were manually harvested from each plant in block one representing each of the five different families from each provenance (a total of 172 plants). Branches of similar age and aspect were sampled to obtain a total leaf weight of 10-15 g. Sampling of smaller plants with low biomass were avoided (plant height below 40 cm). Sampled material was transferred to a zip lock bag and placed in an esky containing ice for transport. The samples were stored in a walk in freezer at -20°C until processing.

3.5 Sample preparation and solvent extraction

Samples were processed in the same randomized order as found in the field trial. Leaves and fine tips were stripped off and mixed thoroughly (Figure 5A). A sub-sample of approximately 1-3 g of wet weight (determined by the total sample weight) were weighed into separate brown paper bags to determine the dry weight. These bags were placed in a drying oven for a period of 3 days at 60

°C, after which the dry weight of the samples was measured. The remaining leaves were placed into a stainless steel mortar and ground under liquid nitrogen using a motar and pestle. Leaves were ground up into a moderately coarse power (Figure 5B). A sub-sample of 3 g of this powder was transferred to a centrifuge tube into which 6 mL of analytical grade ethanol solution was added. The samples were agitated using a tube rotator for a period of 24 hours and 30 µL of the internal standard (Carvone, conc.: 2.4509 g in 100 ml of ethanol) was added into the tube. Following a final vortex for 2 minutes, the samples were centrifuged at 4000 rpm for 5 minutes and the 1 mL of the supernatant was transferred into a gas chromatography (GC) sample vial for the analysis of oil components.

Figure 5: Stripped leaves (A) and ground up leaf sample (B).

3.6 Gas chromatography analysis

Essential oil components were analysed using Agilent 8890 GC system (Santa Clara, California, United States) equipped with a flame ionization detector (FID). The column (Agilent J&W HP-5MS UI) consisted of (5%-phenyl)-methylpolysiloxane as the stationary phase with column dimensions; 30 m x 320 µm and film thickness 0.25 µm. Injection volume was 1 µL (split ratio of 5:1) with injector at 250°C. Oven temperature was increased from 60°C to 210°C at 6°C/min and to 280°C at 25°C/min. Nitrogen carrier gas was used in the column with a flow rate maintained at 4 mL/ min and the detector was set at 300°C. The total analysis time was 38 minutes.

GC-MS data were collected on a Varian CP-3800 GC (Palo Alto, California, United States) coupled to a Bruker 300-MS triple quadrupole MS (Billerica, Massachusetts, United States). Agilent J&W DB-5 column was used with column dimensions; 30 m x 250 µm and film thickness 0.25 µm. A split ratio of 3:1 for 1 min, then 20:1 was used with injector at 220°C. The oven temperature program was set to increase from 60°C to 240°C at 3°C/min. An ionization energy of 70 eV and detector voltage operated in extended dynamic range was used for the analysis. Helium carrier gas was used in the column with a flow rate maintained at 1.3 mL/ min.

3.7 Peak identification

Individual peaks were identified by calculating their Kovats indices and comparing them to published Kovats indices established under similar conditions and on similar, non-polar GC columns. GC-MS spectral data were related to those published in the MS database of National Institute of Standards and Technology (NIST).

Kovats index equation:

$$RI_i = 100\left[n + \frac{t_i - t_n}{t_{n+1} - t_n}\right]$$

RI_i = Kovats retention index of peak *i* (target peak)

n = carbon number of *n*-alkane peak heading peak *i*

t_i = retention time of compound *I* (minutes)

t_{n+1} = retention time of heading n-alkane (minutes)

t_n = retention time of trailing n-alkane (minutes)

3.8 Peak quantification

The quantification of individual peaks was done using carvone as an internal standard and peak areas measured by normalization of GC-FID data. Mass of the target compound was represented against its dry sample weight.

Conversion of peak area into weight of the target compound:

$$\text{weight}_{target} = [\frac{weight_{IS}}{area_{IS}}]\text{area}_{target}$$

weight_{target} = weight of the target compound (mg)

weight_{IS} = weight of the internal standard added (mg) - constant

area_{IS} = area of the internal standard

area_{target} = area of the target peak

3.9 Statistical analysis

Statistical analysis of the experimental results was completed using R statistical software (version 3.6). The matrix of chemical abundances was analysed using *k*-means cluster analysis. The number of clusters was set to four following examination of the within groups sums of squares. Principle components analysis (PCA) was used to generate an ordination and to display the four groups from the k-means clustering analysis.

Analysis of similarity was conducted to determine if the four groups differed significantly. Recursive partitioning analysis was also conducted to identify possible oil constituents that could be used to predict the memberships of the four groups. A p value of < 0.05 was considered as the level of significance using the software.

CHAPTER 4: Results

4.1 Analytical method optimisation

Dry weight analysis revealed that the moisture content of the samples was between 48% - 88%, indicating higher water concentration available within the leaves. Hexane was initially considered as a solvent to extract chemicals from vegetative material as it is non-polar and will solubilise chemicals of similar polarities, such as monoterpenes and sesquiterpenes. However, due to its non-polar nature, a polar/non-polar interface can form between hexane and water such that the solvent cannot penetrate the aqueous cell. Thus, ethanol was chosen as the solvent as it has sufficient polarity to be miscible with the cell contents yet still solubilise organic compounds.

Octadecane ($C_{18}H_{38}$) is a straight chain alkane which is popular as an analytical internal standard during GC analysis. However, it's only partially soluble in ethanol (Seyer, Patterson & Keays, 1944) and there was a possibility that it would come out of solution and crystallize during refrigerated storage. This could result in inconsistent and inaccurate measurements of the internal standard. Carvone was selected as the internal standard as it has not been reported to be present in *L. scoparium* and was found to have a GC retention time of 8.45 mins under the temperature gradient adopted in this study which didn't overlay with peaks of interest in *L. scoparium* samples.

Initial extractions were run on an Agilent J&W HP-1 column and revealed poor chromatography of peaks within 0 – 12 mins of the analysis. This led to the installation of a new column: Agilent J&W HP-5ms Ultra Inert with has very low bleed characteristics.

4.2 Peak identification

Both GC and GC-MS data provide useful data for the identification of the oil constituents. The GC analysis of all the 172 Tasmanian *L. scoparium* samples revealed a pattern of about 70 different oil components (Figure 6A). Monoterpenes have low molecular weights compared to sesquiterpenes; therefore, they elute faster, thus their retention times within the GC column in less. From figure 6A, elution of strong peaks of monoterpenes were observed between 5 – 20 mins in the Tasmanian *L. scoparium* samples whilst the New Zealand *L. scoparium* oil sample reported either low or no peaks within this time (Figure 6B). However, after 20 mins, densely packed

sesquiterpene peaks were observed eluting between 20 – 32 mins in New Zealand *L. scoparium* oil sample whilst dispersed peaks were observed in Tasmanian *L. scoparium* samples within the same time frame.

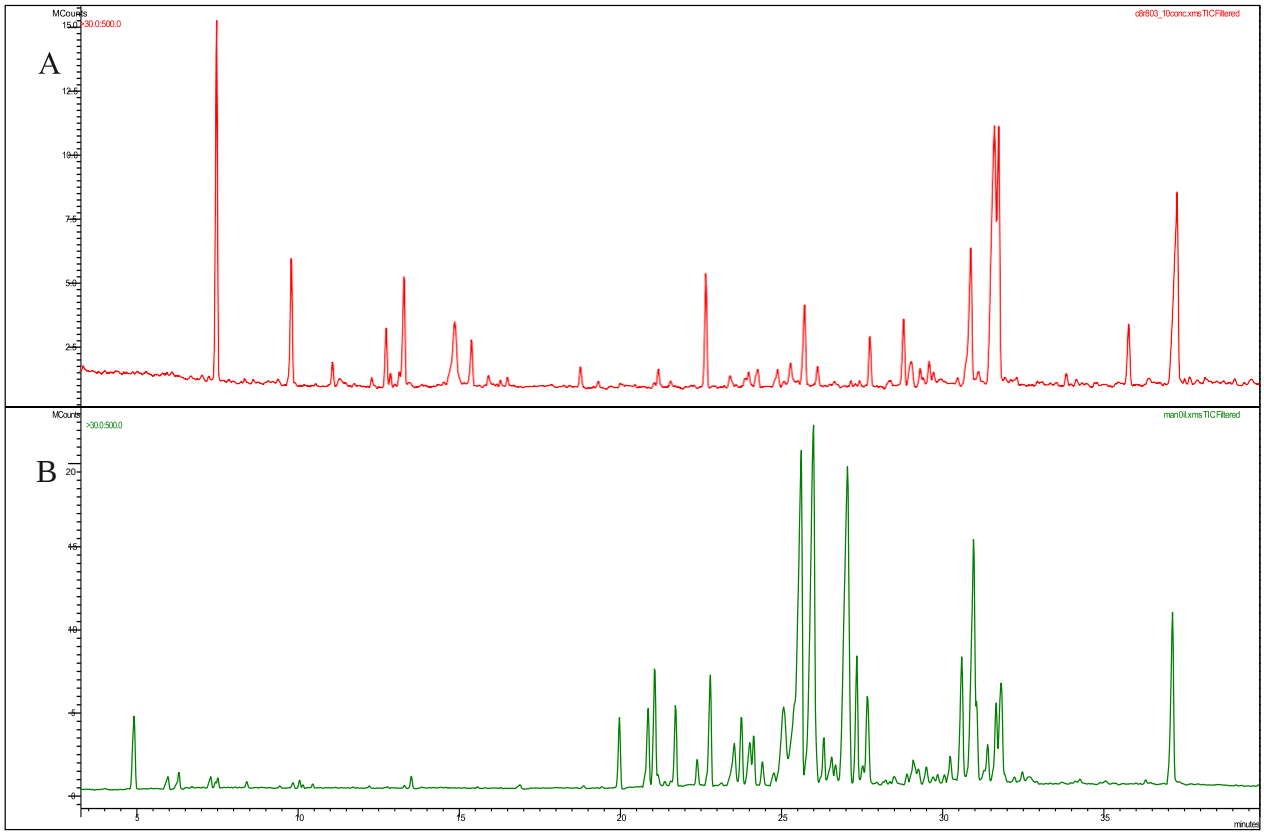


Figure 6: GC-MS chromatograms of oil constituents of a Tasmanian *L. scoparium* leaf extract (graph A) and a New Zealand *L. scoparium* oil sample (graph B) (see appendix 1 for enlarged graphs).

By way of example, a chemical with a mass spectrum shown in figure 7A, was identified as 1,8-Cineole by a NIST database search (Figure 7B). It eluted with a retention time of 7.529 mins between internal standards of C_{10} and C_{11} which had retention times of 6.570 mins and 9.909 mins respectively. Its' Kovats index can be calculated by using the equation introduced earlier and this value can be compared to that published data in the NIST MS database for identification.

For example: 1,8-Cineole; Retention time: target peak - 7.529 mins, C_{10} – 6.570 mins, C_{11} – 9.909 mins

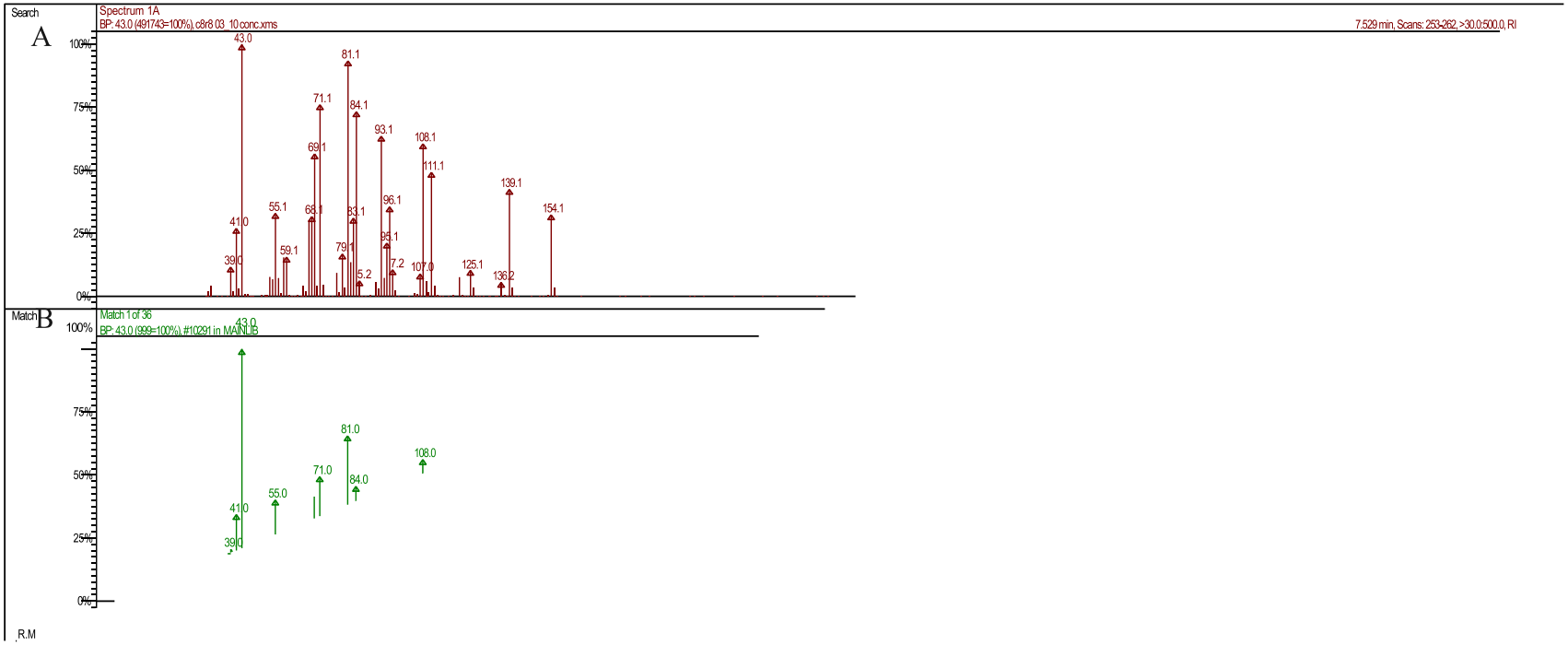


Figure 7: Mass spectrum of 1,8-Cineole as extracted from *L. scoparium* in this study (graph A) compared against the published NIST MS spectral database for identification of the constituent (graph B).

For verification, Kovats index was calculated:

$$RI_i = 100\,[10 + \frac{7.529 - 6.570}{9.909 - 6.570}] \qquad RI_i = 1029$$

4.2.1 Table of peaks identified

Nineteen peaks corresponding to oil constituents and the internal standard were monitored throughout the 172 *L. scoparium* leaf extracts, as these were consistently present at relatively high concentrations in most of the analysed samples across the provenances.

Table 4: Verification of the identified compounds based on their calculated KI.

Retention time-MS (mins)	Compound name	Calculated KI	NIST published KI	Reference
4.871	α-Pinene	943	943.5	El-Massry, El-Ghorab & Farouk, 2002
5.723	β-Pinene	965	965	Senatore & De Feo 1999
5.900	β-Myrcene	972	972	Storer, Elmore & Van Emden, 1993
7.254	p-Cymene	1020	1020	Georgiou et al. 2010
7.529	1,8-Cineole	1029	1029	Bos et al. 2007
8.200	γ-Terpinene	1049	1049	Agnihotri et al. 2004
9.798	Linalool	1097	1097.6	Kant et al. 2004
12.743	Terpinen-4-ol	1172	1172	Georgiou et al. 2010
13.296	α-Terpineol	1186	1186	Wu et al. 2007
15.393	Carvone (Int. Std.)	1237	1237	Hamm et al. 2003
23.409	β-Caryophyllene	1432	1432	Ali et al. 2008
24.000	Humulene	1447	1447	Bos et al. 2007
27.451	Nerolidol	1535	1535	Bin Jantan et al. 2003
29.005	Caryophyllene Oxide	1576	1576	Morteza-Semnani & Saeedi 2003
29.310	Globulol	1584	1584	Lucero, Estell & Fredrickson, 2003
29.500	Unknown 1	1589		
29.800	Unknown 2	1596		
30.800	γ-Eudesmol	1624	1624	Sylvestre et al. 2007
31.083	Cubenol	1632	1632	González et al. 2004
31.541	α & β-Eudesmol	1643	1643	Srivastava et al. 2006

4.2.2 Investigation on the presence of leptospermone in Tasmanian *L. scoparium*

As discussed earlier, the New Zealand essential oil is valued for its high concentration of triketones, especially leptospermone and isoleptospermone. It was important to identify if these chemicals were present in the Tasmanian populations included in this study. It was not within the scope of this study to analyse every extract by GC-MS, as this would not only be expensive, it would also not provide quantitative data. MS relies on the fragmentation and ionisation of fragments of the parent molecule as chemicals elute from the gas chromatogram and enter the ionisation chamber of the MS. In the first chromatograms of the ethanol extracts of Tasmanian *L. scoparium* run by GC-MS was for the purpose of structural identification and establishing Kovats indices to confirm the peak identification, the ketones were not identified. However, some of the GC FID chromatograms showed a strong peak in the region in which leptospermone and isoleptospermone elute. For this reason, one of these extracts were re-analysed by GC-MS.

Both leptospermone and isoleptospermone have a molecular weight of 266.33 and these parent molecules fragment under MS conditions into ions with *m/z* values of 196, 251, and 266 (Muturi et al. 2020). Manuka oil that had been distilled from *L. scoparium* plants sourced from New Zealand, which had previously been shown to contain triketones, was analysed alongside the ethanol extract of Tasmanian *L. scoparium* leaf extracts. Two strong peaks were confirmed to be leptospermone and isoleptospermone in the Manuka oil, based on their mass spectra and Kovats indices (Figure 8B). The ions for m/z 196, 251 and 266, characteristic of the mass spectra of these two analytes, were extracted from the total ion current (TIC) and are shown in graph C and D for the ethanol extract of the Tasmanian plant and for the Manuka oil sample respectively. Despite there being signs of a peak eluting in the same region in graph A, it can be seen that the diagnostic ions for the two triketones are not present at any appreciable levels in graph C of Figure 8 and the peaks that did elute in the same region as that of leptospermone and isoleptospermone were not of sufficient intensity and did not align with those detected in Manuka oil.

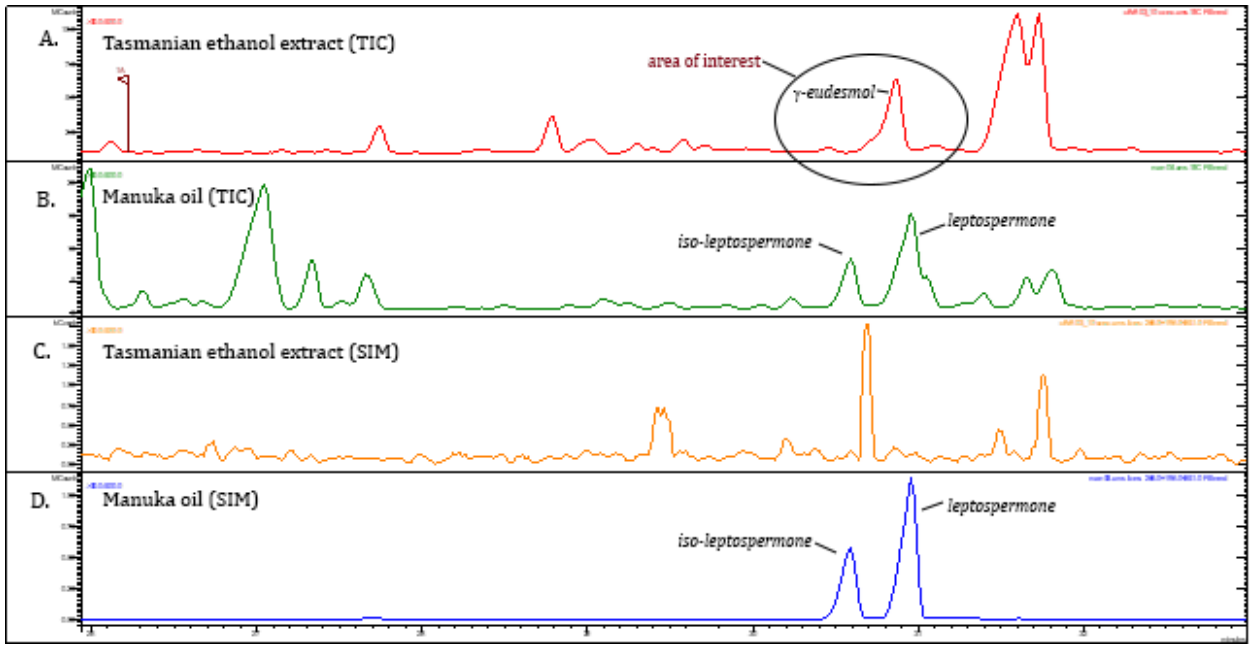


Figure 8: Ion trace of the area of interest in Tasmanian *L. scoparium* leaf extract (graph A) and its SIM peaks for leptospermone and isoleptospermone (graph C), and ion trace of the area of interest in New Zealand *L. scoparium* oil sample (graph B) and its SIM peaks for leptospermone and isoleptospermone (graph D).

4.3 Identification of possible chemotypes across Tasmania

The recoveries for the plants grown in the trial plot calculated from the total peak areas and assuming a 1:1 response to the area of the internal standard, ranged from 0.8 – 41.3 mg/g DW.

The GC dataset (Table 5) showed a wide variation in the concentration of the 19 oil constituents across the 172 samples and within provenances.

Table 5: Range of components detected across Provenances (mg/g DW).

Provenance	Code	α-pinene	β-pinene	β-myrcene	p-cymene	1,8-cineole	γ-terpinene	terpinolene	terpinen-4-ol	α-terpineol	α-caryophyllene	β-caryophyllene	α-humulene	caryophyllene oxide	unknown sesquiterpene alcohol	unknown sesquiterpene alcohol	unknown sesquiterpene alcohol	g-eudesmol	α & β-eudesmol	Total
Alum cliffs	1	0.6-3.5	0.1-0.4	0.1-0.4	0-0.1	.1-7.0	0-0.2	0.3-0.7	0.1-0.8	0.1-1.5	0.2-1.9	0.1-0.2	0.4-3.6	0-0.5	0-0.1	0.5-1.2	0-0.3	0.4-1.5	0.2-0.6	1.7-5.1
WH Apiary site	2	1.1-5.4	0.1-0.4	0.1-0.4	0-0.3	1.3-6.8	0-0.2	0.3-2.4	0.2-0.7	0.3-1.4	0.7-2.5	0-1.0	0.1-1.1	0-0.5	0-1.5	0.2-1.4	0-0.2	0.1-2.8	0-0.5	2.2-10.7
Cameron	3	0.1-1.0	0-0.1	0-0.2	0.1-0.3	0.4-0.7	0-0.3	0.1-0.7	0.1-0.2	0.1-0.2	0.1-0.5	tr	0-0.4	bdl	bdl	0-0.2	0-0.1	bdl	bdl	0-0.4
Catagunya	4	0..7-2	0-0.1	0-0.2	0.1-0.2	1.0-2.4	bdl	0.3-1.5	0.1-0.3	0.2-0.6	0.2-0.8	0-0.3	0.1-0.7	0-0.1	bdl	0-0.5	0-0.4	0-0.6	bdl	0.7-2.0
Court Rd	5	1.1-3.1	0.1-0.4	0.1-0.4	0-0.2	1.2-2.6	0-0.2	0.3-1.3	0.1-0.5	0.2-1.1	0.6-1.5	0-1.2	0.5-2.6	0-0.7	0.1-0.2	0.2-1.0	0-0.3	0.1-1.4	0.2-0.4	0.7-4.2
Ellendale	6	0.2-1.9	0-0.1	0-0.2	0-0.3	0.2-3.5	tr	tr	0.1-0.4	0.1-0.9	0.1-1.0	0-0.1	0-0.2	bdl	0-0.2	0-0.2	bdl	bdl	bdl	0-1.6
Eagle Hawk Nck	7	0.4-1.5	0-1.2	bdl	0-0.1	0.9-1.5	tr	0.3-0.5	0.1-0.2	0-0.2	0.2-2.0	0.1-0.2	0.2-0.9	0.0	0-0.2	0-0.3	0-0.1	0-2.9	0-0.4	0-10
Eleven Rd	8	0.2-1.1	0-0.1	0-0.1	0-0.1	0.5-1.9	bdl	0-0.2	0.1-0.3	0.2-0.4	0.2-0.8	0-0.6	0-0.2	0-0.8	0-0.1	0-0.4	bdl	0.1-0.6	bdl	0.6-1.8
Florentine	9	0-1.9	0-0.1	bdl	0-0.3	0.7-1.0	tr	0-0.2	0.1-0.1	0.2-0.3	0.3-2.3	0-1.4	0.1-0.4	0-0.3	0-0.1	0-0.6	0-0.2	0.2-0.8	bdl	1.3-2.5
Frodshams Pass	10	0.5-1.7	0.1-0.1	0.1-0.2	0-0.2	0-3.9	tr	0-0.8	0-0.5	0.1-0.8	0.3-1.5	0.2-1.2	0-0.2	0-0.7	bdl	bdl	bdl	0.2-0.9	bdl	1.2-3.3
Grog Forest	11	1.2-2.9	0.1-0.3	0.2-0.4	0.1-0.7	2.3-4.1	0-0.3	0.2-1.2	0.3-0.5	0.4-1.0	0.9-3.4	0.1-0.2	1.1-4.4	0-0.6	0-0.2	0.4-0.9	0-0.4	0.2-0.7	0-0.5	1.3-2.8
Gormanston	12	0.5-2.4	0.1-0.2	0-0.2	0.1-0.4	1.6-2.6	0-0.1	0.5-0.8	0-0.3	0.3-0.6	0.2-0.9	0.1-0.5	0-0.9	0-0.3	0-0.2	0-0.6	0-0.2	0.7-1.9	0.2-0.2	2.5-8.1
Goshern	13	0.2-0.7	0-0.1	0-0.2	0-0.1	0.2-1.6	0-0.3	0-0.6	0-0.2	0-0.3	0.2-0.4	tr	0-0.3	0.2-0.2	0-0.1	0.00	bdl	0-0.1	tr	0-0.6
Geeveston	14	0.5-1.7	0-0.2	0-0.2	0.1-0.6	0.1-4.2	0-0.1	0-0.8	0.2-0.4	0.2-0.7	0.4-1.9	0.2-1.0	0-0.3	0-0.1	0-0.1	0.00	tr	0-0.8	0-0.2	1.0-2.7
Henty Dunes	15	0-0.8	bdl	bdl	bdl	0-0.7	tr	0-1.3	tr	0-0.2	0.6-2.3	0.1-1.1	0-0.1	0-0.2	0-0.1	0.30	0-0.25	0-1.3	0-0.2	0-5.5
Longley	16	0.3-0.8	0-0.1	0-0.1	0.1-0.1	0.6-1.3	tr	0-0.7	0.1-0.2	0.1-0.3	tr	bdl	0-0.2	0-0.4	tr	0.23	0-0.1	0-0.3	0-0.2	0-1.0
Lake Leake Rd	17	0.3-0.6	0-0.1	0-0.1	0-0.1	0.5-0.8	bdl	0-1.0	0-0.1	0.1-0.3	0-0.3	0-0.2	0-0.1	0-0.5	bdl	0.00	bdl	bdl	bdl	0-0.7
Lymington Rd	18	0.4-0.7	0-0.1	0-0.2	0-0.1	0.2-1.8	tr	bdl	0-0.2	0.1-0.5	0.2-1.0	0-0.2	0-0.5	0-0.9	bdl	0.45	0-0.1	0-0.4	bdl	0-0.9
Lake Pedder	19	1.0-2.0	0.1-0.2	0-0.3	0.1-0.2	1.8-2.7	tr	0.3-0.7	0.2-0.3	0.5-0.6	0.4-1.4	0.1-0.8	0.1-0.5	0-2.2	0-0.2	0.00	0-0.1	0-1.8	0-0.3	tr-7.5
Junee Rd	20	0.6-2.7	0.1-0.4	0.1-0.3	0.1-1.5	0-6.7	bdl	0-1.4	0.1-0.8	0.1-1.4	0.2-1.0	0-0.6	0-0.3	bdl	0-0.1	1.65	tr	0.2-0.7	0-0.3	1.3-3.6
Knocklofty	21	0.1-3.4	0.1-0.3	0-0.3	0.1-0.2	1.1-5.4	0-0.1	0-0.9	0.1-0.5	0.3-1.0	0.3-1.8	0.1-0.3	0.1-1.3	0.1-0.4	bdl	0.00	0-0.2	0-0.3	0.3-0.5	0.5-1.5
M t Dundas	22	1.0-3.2	0.1-0.3	0.2-0.2	0.1-0.1	2.2-3.2	bdl	0.4-0.9	0.2-0.3	0.6-0.6	0.9-2.3	0.1-0.9	0.2-0.3	0-0.6	-0.70	0.00	0-0.1	2-2.0	0.2-0.4	1.9-8.0
M eetus Falls Rd	23	0.4-0.9	0-0.1	0-0.1	0.00	0-1.3	bdl	0-0.1	0-0.2	0-0.3	0-0.5	bdl	0-0.2	bdl	bdl	0.00	0-0.1	0-0.5	0.1-0.3	0.7-1.0
M urray Field	24	0.4-2.0	0-0.1	bdl	0-0.1	0-1.5	tr	0-0.23	0-0.2	0.1-0.3	0-2.4	0-0.3	0-0.8	bdl	bdl	0.22	0-0.1	0-0.3	0-0.3	0.2-1.4
M argate	25	1.0-1.7	0-0.2	0-0.3	0-0.2	0-3.2	0-0.3	0-0.6	0.2-0.3	0.4-0.6	0.3-1.7	0-0.2	0-0.8	0-0.4	bdl	0.23	0-0.2	0-0.3	0-0.4	0-1.2
M t Nelson	26	0.5-1.3	0-0.1	bdl	0-0.2	0.6-2.5	tr	0-1.0	0.1-0.3	0.1-0.5	0.2-1.1	0-0.2	0.1-0.3	0-0.6	0-0.1	0.00	tr	0-0.2	0-0.3	0.4=0.9
M oorina	27	0-0.6	0-0.1	bdl	0-0.1	0.3-1.9	tr	0-0.3	tr	0-0.4	1-1.0	bdl	0-0.3	0-0.1	bdl	0.00	tr	0-0.2	0-0.5	0-0.9
N Clifton	28	0.3-1.9	0-0.2	0-0.2	bdl	0.4-2.2	bdl	0-1.3	0-0.3	0-0.6	0-1.9	0-0.1	0-2.7	0-0.4	bdl	0.67	0-0.2	0-0.9	0-0.5	0-2.6
Old Coach Rd	29	0.4-0.7	0-0.1	bdl	0-0.2	0-1.1	tr	0-0.9	0.1-0.2	0.1-0.3	0.3-0.5	bdl	0-0.1	bdl	bdl	0.13	tr	tr	0.00	0.00
Peggys Bch	30	0.5-1.3	1-0.1	0-0.3	0-0.2	0.6-3.3	0-0.2	0.2-0.6	0.1-0.2	0.1-0.7	0.6-1.6	0-0.3	0.1-0.4	0-0.6	0-0.5	0.25	0-0.1	0-1.0	0-0.2	0.3-3.3
Pullens Rd WB	31	0.2-0.7	0-0.1	0-0.1	tr	0-0.6	bdl	0.3-1.0	0-0.1	0-0.2	0.3-0.8	0-0.3	0-0.2	0-0.4	tr	0.31	0-0.1	0-0.5	0-0.4	0-1.2
SE Cape	32	0.1-0.8	0-0.1	bdl	0-0.2	0-1.3	bdl	0.4-0.8	0.1-0.2	0-0.3	0.1-0.9	0-0.7	0.1-0.2	0-0.6	bdl	0-0.2	bdl	0-0.7	0-0.3	0-1.6
Sentinel Rg	33	0-1.1	0-0.2	0-0.3	0-0.3	0-6.2	bdl	0-1.3	0-0.7	0-1.3	0.1-1.3	0.1-0.7	0-0.1	0-0.3	0-0.1	0-0.5	tr	0-1.5	0-0.4	0.6-6.1
Wayatinah	34	0.2-1.1	0-0.1	0-0.1	0-0.1	0.5-2.3	bdl	0.2-0.7	0-0.2	0.1-0.5	0.1-0.3	0-0.3	0-0.2	0-0.4	bdl	0-0.2	0-0.1	0-0.6	0-0.3	0-1.9
The Wedge	35	0.2-2.8	0-0.2	0-0.2	0-0.2	0-3.8	bdl	0.2-1.0	0-0.3	0-0.8	0.2-1.2	0.1-0.8	0-0.1	0-2.0	0-0.3	0-0.2	tr	0-2.0	0-0.5	2.3-7.4
Wilmont Power	36	0.5-3.7	0.1-0.5	0.2-0.8	0-0.7	2.5-13.3	0.1-0.6	0.3-2.2	0.3-1.5	0.5-3.0	0.7-2.8	0.1-3.7	0.5-2.9	0.2-0.7	0-1.2	0.3-1.3	0-0.1	0.5-2.5	0.1-0.4	2.3-9.2
W Point Rd AR	37	0.3-1.5	0-0.1	0-0.2	tr	0.3-2.2	0-0.1	0-0.3	0-0.3	0-0.5	0.4-2.8	0.1-0.3	0.1-0.4	0-0.3	bdl	0-0.1	tr	0-0.2	bdl	0-0.9
Waratah	38	0.6-2.0	0.1-0.2	0-0.2	0.1-0.5	0.7-3.3	0.1-0.2	0.2-0.4	0.1-0.3	0.2-0.7	0-1.1	0-0.1	0-1.5	0-0.4	0-0.4	0-0.8	0.1-0.2	0.7-1.0	0.1-0.3	2.6-4.2
Westeway	39	0.2-1.7	0-0.1	bdl	0-0.1	0.7-2.3	bdl	0-0.5	0.1-0.2	0.1-0.4	0.2-0.3	0.1-0.2	0.1-0.2	bdl	tr	0-0.4	tr	0-0.5	0.1-0.2	0.7-1.9

N.B. bdl: below detection level & tr: trace levels

After examination of the within-groups sums of squares (variation of individual scores around each group mean) four groups were selected for the *k*-means analysis as clustering beyond fourth have little value which is indicated by the gradient of the slope as it starts to flatten out (Figure 9).

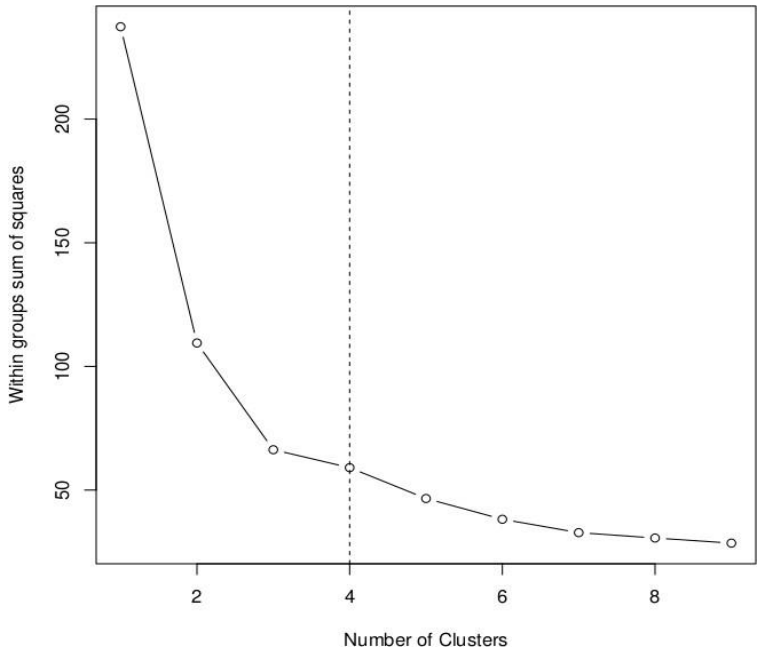


Figure 9: The within groups sums of squares from the k-means cluster analysis. Four groups were selected as shown by the vertical line.

A principle components analysis was used to generate an ordination (Figure 10). This is a two dimensional plot that is used to reduce complex multidimensional data such as provenances and chemical concentrations into smaller groups with similar characteristics. This analysis explained 79% of the variation within the data by the 1st dimension (stronger separation of provenances along x-axis) compared to the 2nd dimension (y-axis) and resulted in the four groups being visually distinct, although some were more diffuse than others.

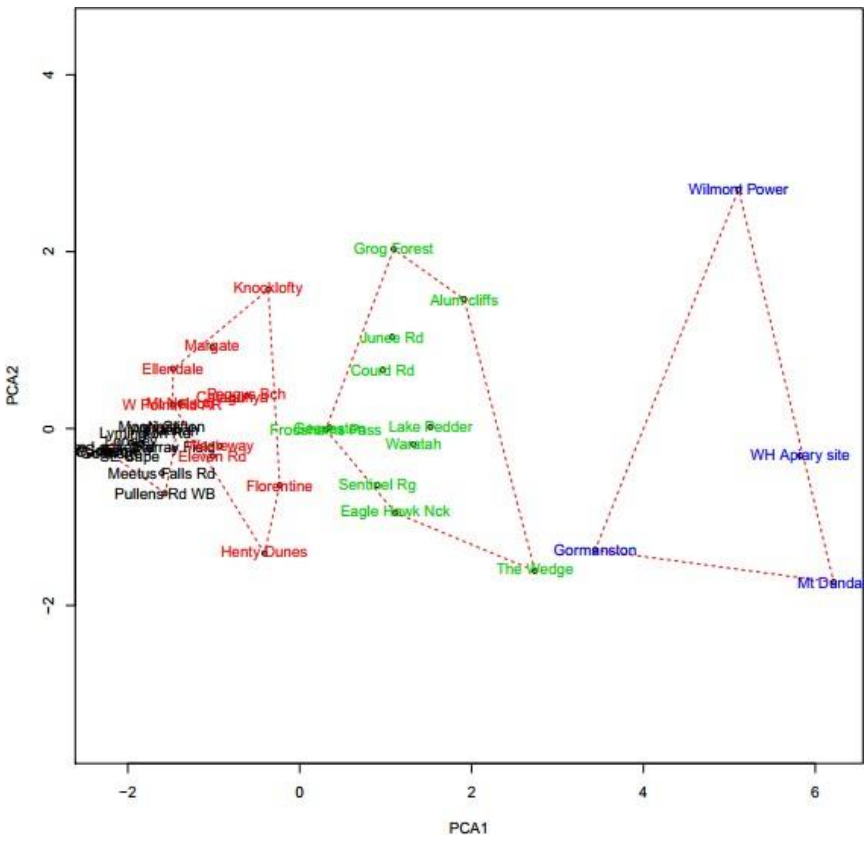


Figure 10: PCA ordination showing the four groups identified in the k-means analysis. The four groups are indicated by the dotted lines and by colour.

Analysis of similarity (ANOSIM) was then conducted to compare the four groups. This showed that the four groups differed significantly (ANOSIM statistic r = 0.703, *P* = 0.001). The results of the ANOSIM are represented graphically in Figure 11. The plot indicated that one group was quite compact (i.e. members within the group are self-similar), whereas members in group two and three were more diffuse, and the members within group four were very dispersed (although it only consisted of four sites).

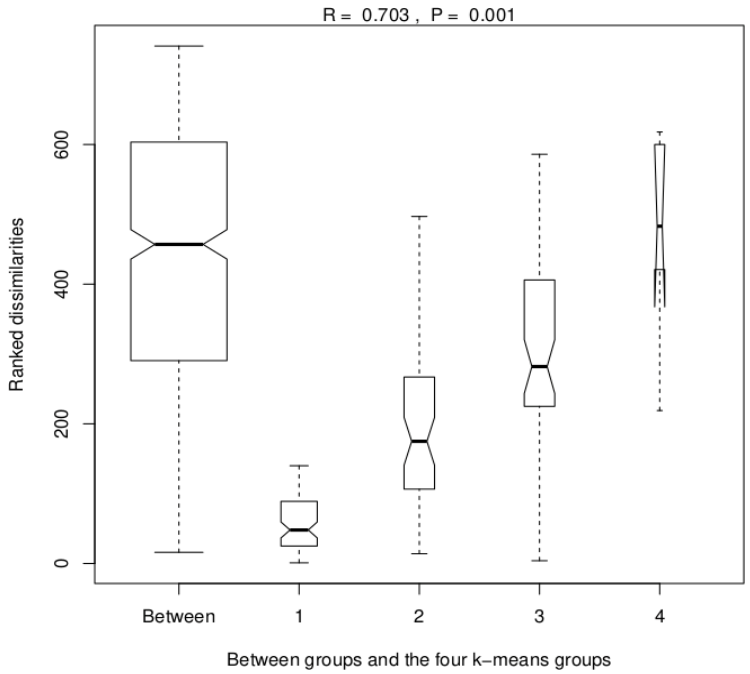


Figure 11: Boxplots of the ANOSIM variation within the k-means groups and between them.

Recursive partitioning analysis was conducted on the four groups to determine which oil constituents could be used to best predict membership of the groups. This identified two important predictors, viz, the levels of α & β-eudesmol and α-terpineol. These two predictors by themselves could account for 82% of the variation in the analyte data. The results of the recursive partitioning analysis are shown in Figure 12. Using these two predictors, three main clusters were apparent, which are described below.

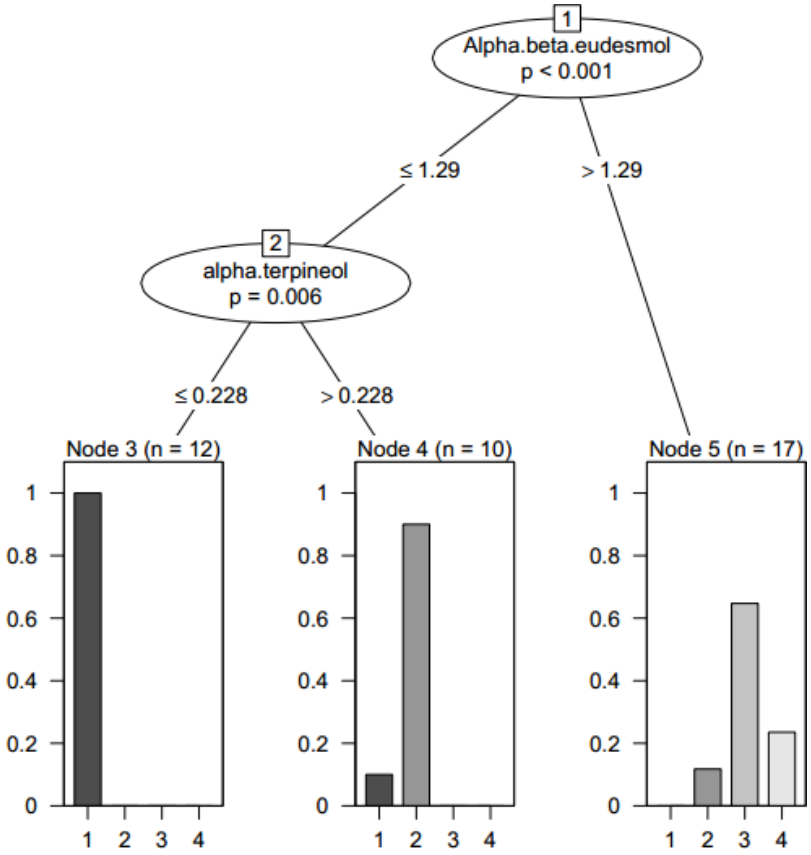


Figure 32: Conditional inference tree from the recursive partitioning. At each node, a cut point is chosen for a predictor to maximally separate the four groups. The terminal nodes are represented using bar charts each of which shows the proportion of data in each *k*-means group. The analysis resulted in two predictors being selected. The four k-means groups were reallocated into 3 main clusters.

Recursive partitioning cluster one was made up of 12 provenances and its' clustering was determined by the concentration of α-terpineol within the plants (Table 6). The average weight of α-terpineol between the provenances in cluster one ranged between 0.10-0.20 mg/g DW. The lowest level was recorded in Goshern while the highest was reported at North Clifton and Old Coach Rd.

Recursive partitioning cluster two and three were described by the concentration of α & β-eudesmol within the plants. Cluster two was made up of 10 provenances (Table 6) and the average weight of α & β-eudesmol between the provenances in cluster two ranged between 0.41-1.29 mg/g DW with the lowest level being recorded in Ellendale while the highest was reported from Westerway samples.

Recursive partitioning cluster three was made up of 17 provenances (Table 6) and the average concentration of α & β-eudesmol across the provenances in cluster two ranged between 1.93-7.92 mg/g DW and were higher compared to cluster two (Figure 13). The lowest level was recorded in Gog forest, whilst the highest was reported at Mount Dundas.

Table 6: List of the three clusters determined according to their oil constituents that were used to best predict membership of the groups across the 39 provenances.

Cluster 1 (α-terpineol)	**Cluster 2 (α & β-eudesmol)**	**Cluster 3 (α & β-eudesmol)**
Cameron	Catagunya Rd	Alum cliffs
Goshern	Ellendale	World Heritage Apiary site
Longley	Eleven Rd	Courd Rd
Lake Leake Rd	Lymington Rd	Eaglehawk Neck
Meetus Falls Rd	Knocklofty	Frodshams Pass
Murrayfield	Margate	Gog Forest
Moorina	Mt Nelson	Gormanston
North Clifton	Peggy's Beach	Geevston
Old Coach Rd	West Point Rd	Lake Pedder
Pullens Rd, Woodbridge	Westerway	Junee Road
Southeast Cape		Mt Dundas
Wayatinah		Sentinel Range
		The wedge
		Wilmot Power Station
		Waratah
		Florentine
		Henty Dunes

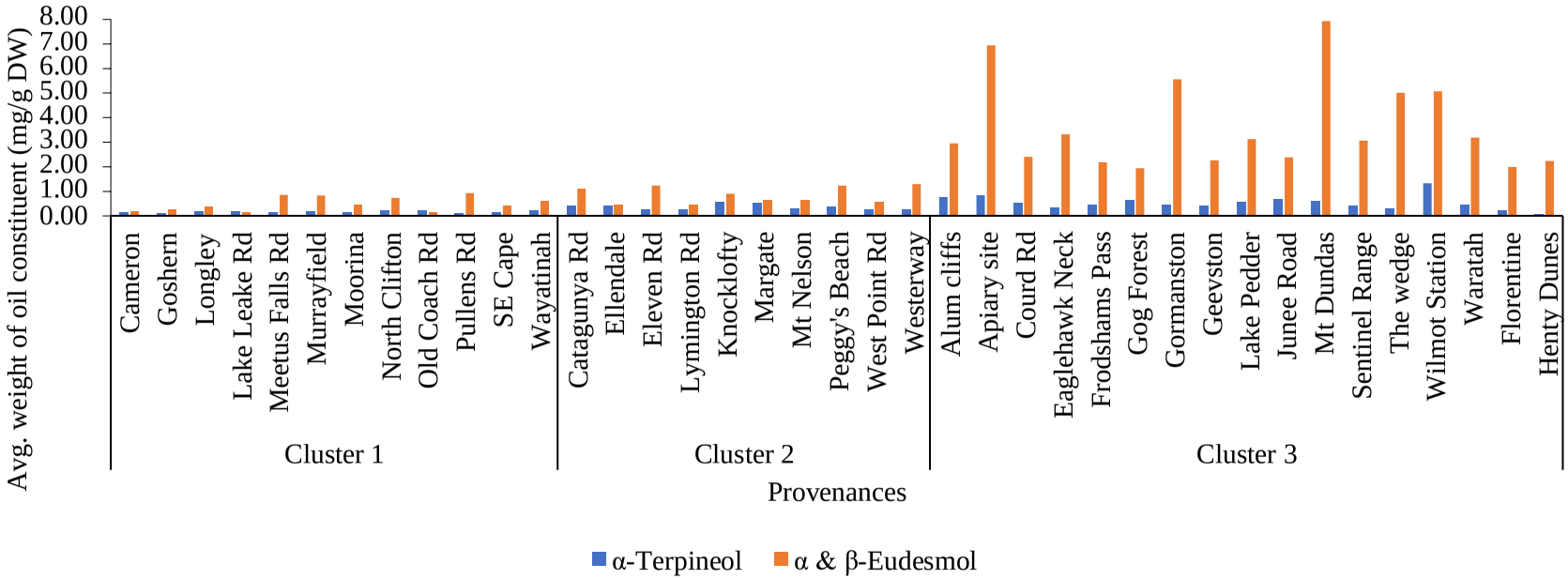


Figure 13: Variation in the average weights of α-terpineol and α & β-eudesmol in the extracts of *L. scoparium* that make up three clusters across the 39 provenances.

Figure 14 shows that the geographical spread of the initial four *k*-means groups and their provenances based on their latitudes and longitudes showed a wide dispersion with no distinctive grouping.

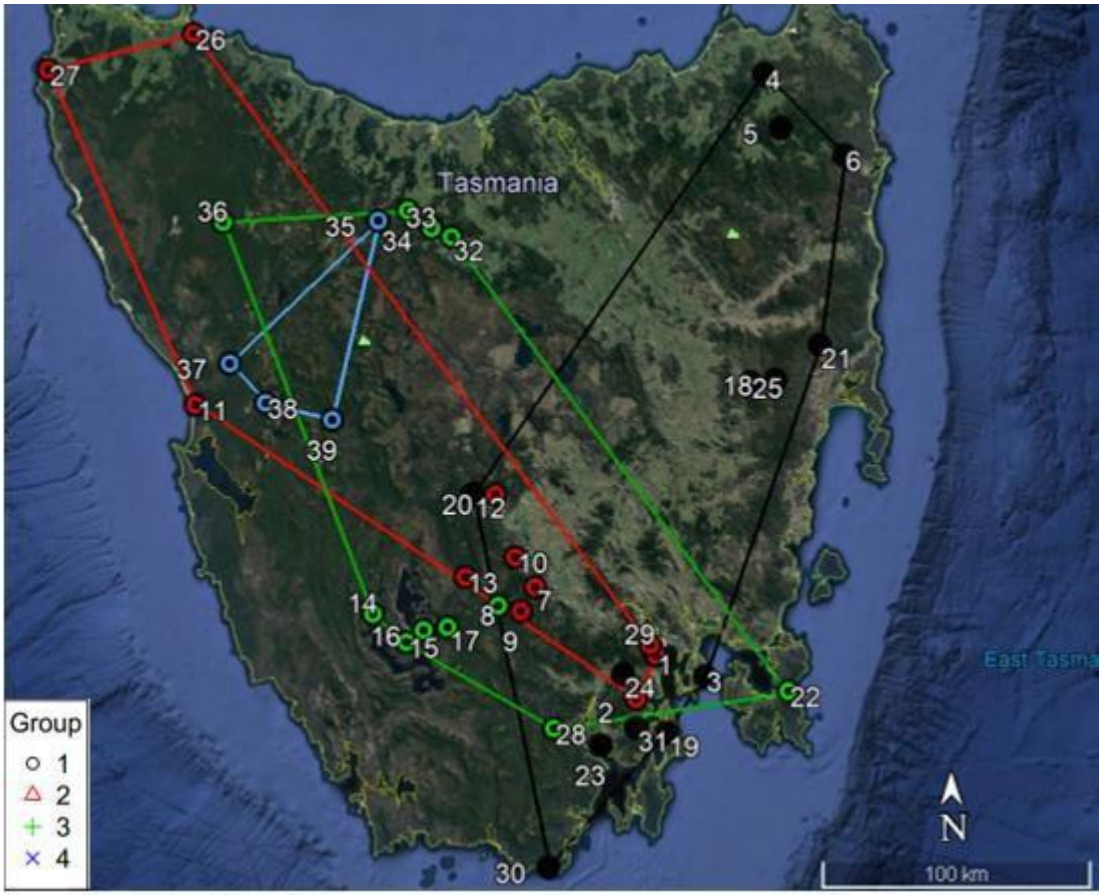


Figure 14: Geographic distribution of the four k-means groups and their provenances across Tasmania.

CHAPTER 5: Discussion

5.1 Optimising solvent extraction

Dry weight analysis revealed that the moisture content of the samples were between 48% - 88%. At higher moisture content, oil recovery gradually decreases due to the unruptured cell walls leading to higher internal resistance within the cells for molecular diffusion and interaction (Seth et al. 2010). Hexane was initially considered as the solvent due to its non-polar characteristics, as most of the terpenes are highly non-polar in nature, thus it is able to extract more non-polar terpenes with very little impurities (Jiang, Kempinski & Chappell, 2016). However, hexane and the water within the plant material are not miscible (Sigma-Aldrich 2020), thus ethanol was chosen as it has sufficient polarity to be miscible with the cell contents yet will still solubilise organic compounds.

Water content of the extraction solvent, ethanol solvent had implications for the use of octadecane ($C_{18}H_{38}$) as an analytical internal standard during GC analysis. Octadecane had a very low solubility at room temperature in ethanol (at 30°C only 13.1% solubility) (Xie et al. 2008) and initial trials from this study confirmed crystallization during refrigerated storage of the standard solution. This would have resulted in inconsistent and inaccurate addition of the internal standard into the extracted samples. Carvone proved to be a suitable internal standard eluting between 8.45 – 8.68 mins in a region where there were no endogenous peaks.

A trial experiment with 12 samples was analysed twice on two following days to investigate the accuracy of the repeatability of the samples. The retention times were slightly shifted between the two days, which is expected, however, the mean peak areas were within ± 5% SD, which was acceptable, thus all the samples were analysed once.

A trial experiment with two extraction times were carried out. It was found that an extraction time of 24 hours yielded 20% more volatiles than a 2 hour extraction period. Indeed, some components were of such low concentrations after a short period of agitation, their concentrations were below the level of quantification.

5.2 Peak identification

GC-MS data of all 172 Tasmanian *L. scoparium* samples revealed there were as many as 70 different oil components. In this study, there was insufficient time to undertake a full steam distillation of vegetative material of Tasmanian *L. scoparium*, however out of the identified 19 peaks, 16 peaks were present in both the ethanol extract processed in this study compared to a Manuka oil sample that had previously been steam distilled from the leaves of *L. scoparium* var. *scoparium*, the genetic stock of which was sourced from New Zealand (source confidential). From the calculated Kovats indices, 15 were an exact match to the published literature that were analysed on similar conditions (2 unknowns). Kovats indices fell within the accepted range and when analysed in conjunction with the mass spectra and alongside a *L. scoparium* sample of known composition, peak identification was assigned with qualified confidence. Absolute identification would require isolation and characterisation and/or purchase of standards for each unknown to allow for comparison of diagnostic characteristics. This was outside the scope of this study.

5.3 Composition of oil constituents in Tasmanian *L. scoparium*

The concentrations of the triketones, such as isoleptospermone and leptospermone plays a major role in New Zealands' claim that their native population of *L. scoparium* is distinct from Australian population (Douglas et al. 2004). Despite there being signs of a peak eluting in the same region in graph A in Figure 7, diagnostic ions for the two triketones were neither present at acceptable levels nor align with those detected in Manuka oil. It should be noted, however, only a few samples were analysed through MS due to limited budget and time restrictions, and any peaks that may have eluted in the same region as leptospermone and isoleptospermone, could not have been distinguished as being the target ketones when analysed by GC FID. In addition, any peaks that may have been attributed to these chemicals would co-elute with γ-eudesmol. A closer examination of every one of the 172 chromatograms may have identified slight shifts and doublets that may have indicated the presence of leptospermone, prompting a re-analyse by GC-MS, however this was not within the scope of this study. Overall, however, the presence of significant quantities of the β-ketones are likely to have been evident during data processing.

The present study supports the findings of Brophy et al. (2000) as no triketones were detected from both Tasmanian *L. scoparium* varieties; var. *eximium* and var. *scoparium.* On the contrary,

triketones; flavesone and leptospermone had been previously reported in var. *eximium* sourced from Tasmania in a study conducted by Perry et al. (1997). This implies that the chemistry of *L. scoparium* is highly variable.

Some of the more interesting features of the ethanol extract was high levels of nerolidol 0 - 3.6 mg/g DW. As a percentage of total volatiles measured, this upper value be in the vicinity of 4% in one individual harvested from the provenance of Goshern, in the NE of Tasmania. The very high level is not reflected in all of the families collected from this area, with one individual recorded as having zero levels of nerolidol. This chemical has been previously reported to be present at levels of 0.2 and 0.4% in Tasmanian *L. scoparium* var. *scoparium* and var. *eximium* respectively (Brophy et al. 2000). On the other hand, no publications have reported the occurrence of this oil constituent in New Zealand *L. scoparium* populations. For example, a comprehensive field study conducted by Douglas et al. (2004) analysed essential oils from 261 *L. scoparium* plants across 87 locations throughout New Zealand and reported 48 different oil constituents, however nerolidol was absent from the list of identified oil constituents. Similarly Christoph, KubecZka & Stahl-Biskup (1999) did not detect nerolidol amongst the 90 different constituents identified in commercial manuka oils. This implies that there is a possibility of Tasmanian *L. scoparium* population being chemically distinctive to New Zealand *L. scoparium* population.

Several publications states that essential oils from Australian *L. scoparium* population have high monoterpene content whilst essential oils distilled from New Zealand population present with high levels of both sesquiterpenes and triketones (Perry et al. 1997; Porter & Wilkins 1998; Christoph, KubecZka & Stahl-Biskup, 1999; Douglas et al. 2004). The present study correlates with these claims as nine strong peaks of monoterpenes were observed between 5 – 20 mins (Figure 6A) whilst low or no peaks were observed in the New Zealand *L. scoparium* oil sample within the same time (Figure 6B). Densely packed sesquiterpene peaks can be seen eluting between 20 – 32 mins in New Zealand *L. scoparium* oil sample. Although there is a large difference in the methods of extraction between solvent and steam distillation, it could be expected that the high levels of monoterpenes observed in the Tasmanian solvent extract would be reflected in a steam distilled oil produced from the same vegetative material due to the volatility of the low molecular weight components. This further point towards Tasmanian *L. scoparium* population being chemically distinctive to New Zealand *L. scoparium* population.

On the other hand, New Zealands' claim that all of its *L. scoparium* population are endemic and genetically exclusive at the variety level, to Australian populations may be pre-mature as the concentrations of sesquiterpenes and triketones in *L. scoparium* populations of northern New Zealand are reported to produce oils with high levels of monoterpenes, lower levels of sesquiterpenes and the triketones present at very low levels or completely absent (Perry et al. 1997). These characteristics are similar to many of the Australian populations and the diversity may be a reflection of a close genetic relationship, rather than variations that have evolved exclusively in New Zealand.

High monoterpene content in northern New Zealand and Australian populations is also associated with fire ecology and Harris (2002) reported varying levels of serotiny in *L. scoparium* plants grown from seeds that were collected from different provenances across New Zealand. Traits adapted to withstand fire is very rare in New Zealand flora except for *L. scoparium*, is recorded as the only species being serotinous (Bond, Dickinson & Mark, 2004; Stephens, Molan & Clarkson, 2005). As the serotinous nature is commonly found in Australian flora, it is suggested that serotinous trait transferred from Australia to New Zealand (Thompson 1989).

Chemotaxonomy results from this present study lead us to one of the most difficult conundrums faced at the present, that is on one hand, serotinous trait present within New Zealand *L. scoparium* population is said to be originated from Australia, while on the other hand some chemicals are possibly unique to the continent, such as nerolidol. This brings us to the claim by Bond, Dickinson & Mark (2004) that Tasmanian population of *L. scoparium* were different from New Zealand *L. scoparium* populations and highlights the need of taxonomic revision of the population.

5.4 Identification of chemotypes

The present study investigated the chemical profiles of plants belonging to 39 provenances and demonstrated three main clusters within Tasmania. Douglas et al. (2004) was able to define 10 distinct chemotypes within New Zealand based on the identification of 48 constituents. Although some of these components, such as α-pinene, caryophyllene/ humulene, and linalool were able to define chemotypes in New Zealand, they were not diagnostic for Tasmanian families or provenances. In the present study, two important chemicals which best predict Tasmanian chemotypes were identified; α & β-eudesmol and α-terpineol. However, the present study was not

able to integrate and identify all the oil constituents present within the Tasmanian *L. scoparium* population due to time restrictions. Analyses of the family replicates in the remaining 4 blocks (780 samples) might further elucidate the genetic relationships.

In New Zealand, the concentration of triketones have been used to define chemotypes (Porter & Wilkins 1998), although, the current study didn't identify triketones, chemotype with high eudesmol has been used by Porter et al. (1998) to define Nelson and Kaiteriteri regions in New Zealand. A similar role is identified to be played by α & β-eudesmol in Tasmania as it was able to predict two chemically distinctive clusters, therefore α & β-eudesmol could play a role in defining chemotypes within Tasmania.

Chemotypes in New Zealand *L. scoparium* population has been used to define geographic boundaries. For example, a study by Porter & Wilkins (1998) defined four different geographical sites based on oil composition. Oils distilled from wild East Cape population contained high levels of triketones, Nelson and Kaiteriteri areas were rich in eudesmol and linalool, Woodstock and Canterbury areas rich in pinenes, whilst a population that was deficient in the triketone, eudesmol and linalool were spread across the rest of New Zealand. This was not true from Tasmanian context, despite being able to define clusters within the state, when the spread of the clusters was plotted geographically, dispersion within the clusters was observed. This means that while it is possible to find distinct groupings of the provenances, they do not correspond to specific geographic locations as illustrated in figure 14. Profiling the chemicals of oils produced by steam distillation when the trial site reaches maturity, may provide more consistencies across families and provenances.

It is also worth noting that, although genotypic control is particularly significant and obvious in the oil compositions within provenances, plant chemistry reflects climatic and season effects (Porter et al. 1998). Similar trends maybe observed in Tasmania, if the current geographical data was overlaid with climatic data.

CHAPTER 6: Conclusions

This study is the first to compare the chemistry of *L. scoparium* from plants collected across Tasmania and grown under similar conditions and hope to compare to the essential oils produced by New Zealand populations.

The present study investigated the genetic variation in the essential oils obtained from *L. scoparium* populations belonging to 39 provenances across Tasmania. Essential oil composition from plants harvested from block one showed a wide variation in the oil constituents.

GC-MS data of all 172 Tasmanian *L. scoparium* samples revealed there were as many as 70 different oil components, out of which 17 were identified. Triketones were not present in the Tasmanian extracts analysed within this study despite the use of SIM to search for diagnostic ions specific to the triketones.

High monoterpene levels were observed in Tasmanian samples, which is similar to *L. scoparium* populations of Northern New Zealand. Both these populations produce oils with high levels of monoterpenes, lower levels of sesquiterpenes and the triketones at very low levels or completely absent. This may reflect a close genetic relationship, rather than variations that have evolved exclusively in New Zealand.

Chemotypic clustering was carried out to identify possible chemical constituents to define clusters across Tasmania and two important oil constituents; α & β-eudesmol and α-terpineol, were identified to best predict Tasmanian chemotypes. Eudesmol has been used to define chemotypes in New Zealand, similar approach can be taken within Tasmania as α & β-eudesmol was able to predict two chemically distinctive clusters. Despite being able to define clusters within the state, when the spread of the clusters was plotted geographically, dispersion within the clusters was observed. This meant that while it is possible to find distinct relationships across provenances, they did not correspond to specific geographic regions.

In this study, there was insufficient time to undertake a full steam distillation of the vegetative material of Tasmanian *L. scoparium* and to identify all the oil constituents present. Any peaks that may have eluted in the same region as leptospermone and isoleptospermone, could not have been

distinguished as being the target ketone using GC FID alone, therefore, a closer examination of every one of the 172 chromatograms may identify slight shifts and doublets that may have indicated the presence of leptospermone, prompting a re-analyse by GC-MS. Lastly, analyses of the family replicates in the remaining 4 blocks (780 samples) might further elucidate the genetic relationships.

References

Adorjan, B & Buchbauer, G 2010, 'Biological properties of essential oils: an updated review', *Flavour and Fragrance Journal*, vol. 25, no. 6, pp. 407–426.

Agnihotri, VK, Thappa, RK, Meena, B, Kapahi, BK, Saxena, RK, Qazi, GN & Agarwal, SG 2004, 'Essential oil composition of aerial parts of *Angelica glauca* growing wild in North-West Himalaya (India)', *Phytochemistry*, vol. 65, no. 16, pp. 2411–2413.

Aldred, EM, Buck, C & Vall, K 2009, ''Chapter 22 - Terpenes'' in *Pharmacology: A Handbook for Complementary Healthcare Professionals*, Churchill Livingstone, Elsevier, pp. 167–174.

Ali, NAA, Wurster, M, Arnold, N, Teichert, A, Schmidt, J, Lindequist, U & Wessjohann, L 2008, 'Chemical Composition and Biological Activities of Essential Oils from the Oleogum Resins of Three Endemic Soqotraen *Boswellia* Species', *Academy of Chemistry of Global Publications*, vol. 2, pp. 6–12.

Alvarez-Suarez, J, Gasparrini, M, Forbes-Hernández, T, Mazzoni, L & Giampieri, F 2014, 'The Composition and Biological Activity of Honey: A Focus on Manuka Honey', *Foods*, vol. 3, no. 3, pp. 420–432.

Baker, GR, Lowe, RF & Southwell, IA 2000, 'Comparison of oil recovered from tea tree leaf by ethanol extraction and steam distillation', *Journal of Agricultural and Food Chemistry*, vol. 48, no. 9, pp. 4041–4043.

Banthorpe, D V, Charlwood, B V & Francis, MJO 1972, 'The Biosynthesis of monoterpenes', *Chemical Reviews*, vol. 72, no. 2, pp. 115–155.

Beavis, L 2018, 'Manuka honey: Tasmanian beekeepers claim "we were first" in battle with New Zealand over product name - ABC News', *ABC News*, viewed 22 september 2020, Available from: https://www.abc.net.au/news/2018-01-11/manuka-honey-name-began-in-tasmania-beekeepers-claim/9318708.

Boland, DJ, Brophy, JJ, Flynn, TM & Lassak, E V. 1982, 'Volatile leaf oils of *Eucalyptus delegatensis* seedlings', *Phytochemistry*, vol. 21, no. 10, pp. 2467–2469.

Bond, WJ, Dickinson, KJM & Mark, AF 2004, 'What limits the spread of fire-dependent vegetation? Evidence from geographic variation of serotiny in a New Zealand shrub', *Global Ecology and Biogeography*, vol. 13, no. 2, pp. 115–127.

Bos, R, Woerdenbag, HJ, Kayser, O, Quax, WJ, Ruslan, K & Elfami, E 2007, 'Essential Oil Constituents of *Piper cubeba* L. Fils. From Indonesia', *Journal of Essential Oil Research*, vol. 19, no. 1, pp. 14–17.

Brophy, JJ, Goldsack, RJ, Bean, AR, Forster, PI & Lepsch, BJ 2000, 'Leaf essential oils of the genus *Leptospermum* (Myrtaceae) in eastern Australia. Part 5. *Leptospermum continentale* and allies', *Flavour and Fragrance Journal*, vol. 15, no. 4, pp. 271–277.

Bureau of Meteorology 2020, *Summary statistics Hobart Airport*, viewed 15 October 2020, Available from: http://www.bom.gov.au/climate/averages/tables/cw_094008.shtml.

Burrell, J 1965, 'Ecology of Leptospermum in Otago', *New Zealand Journal of Botany*, vol. 3, no. 1, pp. 3–16.

Butnariu, M & Sarac, I 2018, 'Essential Oils from Plants', *Journal of Biotechnology and Biomedical Science*, vol. 1, no. 4, pp. 35–43.

Cassel, E, Vargas, RMF, Martinez, N, Lorenzo, D & Dellacassa, E 2009, 'Steam distillation modeling for essential oil extraction process', *Industrial Crops and Products*, vol. 29, no. 1, pp. 171–176.

Chemat, F & Boutekedjiret, C 2015, ''Extraction // Steam Distillation'' in *Reference Module in Chemistry, Molecular Sciences and Chemical Engineering* , Elsevier, Elsevier, pp. 1–12.

Chizzola, R 2013, ''Regular monoterpenes and sesquiterpenes (essential oils)'' in *Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes* , Springer Berlin Heidelberg, Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 2973–3008.

Christoph, F 2001, *Chemische Zusammensetzung und antimikrobielle Eigenschaften der ätherischen Öle von* Leptospermum *scoparium J. R. et G. Forst. und anderer Teebaumöle der Gattungen* Kunzea, *<i>Leptospermum<i/> und* Melaleuca *unter besonderer*

Berücksichti, pp. 1-210.

Christoph, F, KubecZka, KH & Stahl-Biskup, E 1999, 'The composition of commercial manuka oils from New Zealand', *Journal of Essential Oil Research*, vol. 11, no. 6, pp. 705–710.

Cordell, GA 1976, 'Biosynthesis of Sesquiterpenes', *Chemical Reviews*, vol. 76, no. 4, pp. 425–460.

Davies, NW 1990, 'Gas chromatographic retention indices of monoterpenes and sesquiterpenes on methyl silicon and Carbowax 20M phases', *Journal of Chromatography A*, vol. 503, no. C, pp. 1–24.

Dawson, M 2012, 'Australian *Leptospermum* in cultivation: Species and cultivars', *New Zealand Garden Journal*, vol. 15, no. 2, pp. 14–22.

Dhifi, W, Bellili, S, Jazi, S, Bahloul, N & Mnif, W 2016, 'Essential Oils' Chemical Characterization and Investigation of Some Biological Activities: A Critical Review', *Medicines*, vol. 3, no. 4, p. 25.

Douglas, MH, Van Klink, JW, Smallfield, BM, Perry, NB, Anderson, RE, Johnstone, P & Weavers, RT 2004, 'Essential oils from New Zealand manuka: Triketone and other chemotypes of Leptospermum scoparium', *Phytochemistry*, vol. 65, no. 9, pp. 1255–1264.

El-Massry, KF, El-Ghorab, AH & Farouk, A 2002, 'Antioxidant activity and volatile components of Egyptian *Artemisia judaica* L.', *Food Chemistry*, vol. 79, no. 3, pp. 331–336.

Filly, A, Fabiano-Tixier, AS, Louis, C, Fernandez, X & Chemat, F 2016, 'Water as a green solvent combined with different techniques for extraction of essential oil from lavender flowers', *Comptes Rendus Chimie*, vol. 19, no. 6, pp. 707–717.

Frykberg, E 2019, ''Australia quiet on trans-Tasman mānuka honey row '', *RNZ News*,viewed 02 Novembe 2020, Available from: https://www.rnz.co.nz/news/country/401450/australia-quiet-on-trans-tasman-manuka-honey-row.

Georgiou, C, Koutsaviti, A, Bazos, I & Tzakou, O 2010, 'Chemical composition of *Echinophora tenuifolia* subsp. *sibthorpiana* essential oil from Greece', *ACG Publications*, no. 3, pp. 167–170.

González, S, Guerra, PE, Bottaro, H, Molares, S, Demo, MS, Oliva, MM, Zunino, MP & Zygadlo, JA 2004, 'Aromatic plants from Patagonia. Part I. Antimicrobial activity and chemical composition of *Schinus polygamus* (Cav.) Cabrera essential oil', *Flavour and Fragrance Journal*, vol. 19, no. 1, pp. 36–39.

Hamm, S, Lesellier, E, Bleton, J & Tchapla, A 2003, 'Optimization of headspace solid phase microextraction for gas chromatography/mass spectrometry analysis of widely different volatility and polarity terpenoids in olibanum', *Journal of Chromatography A*, vol. 1018, no. 1, pp. 73–83.

Harris, W 2002, 'Variation of inherent seed capsule splitting in populations of Leptospermum scoparium (Myrtaceae) in New Zealand', *New Zealand Journal of Botany*, vol. 40, pp. 405–417.

Bin Jantan, I, Mohd Yassin, MS, Chin, CB, Chen, LL & Sim, NL 2003, 'Antifungal activity of the essential oils of nine Zingiberaceae species', *Pharmaceutical Biology*, vol. 41, no. 5, pp. 392–397.

Jennings, W & Shibamoto, T 1980, 'Qualitative Analysis of Flavor and Fragrance Volatiles by Glass Capillary Gas Chromatography', Academic Press, New York.

Jiang, Z, Kempinski, C & Chappell, J 2016, 'Extraction and Analysis of Terpenes/Terpenoids', *Current Protocols in Plant Biology*, vol. 1, no. 2, pp. 345–358.

Johnson, CT 1980, 'The Leaf Anatomy of Leptospevmum Forst. (Myrtaceae)'.

Johnson, CT 1984, 'The Wood Anatomy of Leptospermum Forst. (Myrtaceae)'.

Kant, MR, Ament, K, Sabelis, MW, Haring, MA & Schuurink, RC 2004, 'Differential timing of spider mite-induced direct and indirect defenses in tomato plants', *Plant Physiology*, vol. 135, no. 1, pp. 483–495.

Kitson, FG, Larsen, BS & McEwen, CN 1996, ‘‘What Is GC/MS?’’ in *Gas Chromatography and Mass Spectrometry* , Elsevier, Elsevier, pp. 3–23.

Van Klink, JW, Brophy, JJ, Perry, NB & Weavers, RT 1999, ‘β-triketones from Myrtaceae: Isoleptospermone from Leptospermum scoparium and papuanone from Corymbia dallachiana’, *Journal of Natural Products*, vol. 62, no. 3, pp. 487–489.

Lloyd, P 2017, ‘Competition in the Manuka Honey Industry in New Zealand’.

Lucero, ME, Estell, RE & Fredrickson, EL 2003, ‘The essential oil composition of *Psorothamnus scoparius* (A. Gray) Rydb’, *Journal of Essential Oil Research*, vol. 15, no. 2, pp. 108–111.

Maddocks-Jennings, W, Wilkinson, JM, Shillington, D & Cavanagh, H 2005, ‘A fresh look at manuka and kanuka essential oils from New Zealand’, *International Journal of Aromatherapy*, vol. 15, no. 3, pp. 141–146.

Manuka Beta Oil β-Triketones n.d., *Manuka Essential Oil*, viewed 18 september 2020, Available from: http://www.mbtk.org.nz/what-msstk/manuka-essential-oil/.

McDonald, CM, Keeling, SE, Brewer, MJ & Hathaway, SC 2018, ‘Using chemical and DNA marker analysis to authenticate a high-value food, manuka honey’, *npj Science of Food*, vol. 2, no. 1, p. 9.

McIntyre, D 1963, ‘Pollen Morphology of New Zealand Species of Myrtaceae*’, *Transactions of the Royal Society of New Zealand*, pp. 83–107.

Morteza-Semnani, K & Saeedi, M 2003, ‘Constituents of the essential oil of *Commiphora myrrha* (Nees) Engl. var. *molmol*’, *Journal of Essential Oil Research*, vol. 15, no. 1, pp. 50–51.

Muturi, EJ, Selling, GW, Doll, KM, Hay, WT & Ramirez, JL 2020, ‘*Leptospermum scoparium* essential oil is a promising source of mosquito larvicide and its toxicity is enhanced by a biobased emulsifier’, *PLoS ONE*, vol. 15, no. 2, pp. 1–14.

O'Grady, AP & Worledge, D 2020, Pittwater manuka *(Leptospermum scoparium)* trial: Experimental design and preliminary results, pp. 1-24.

O'Grady, AP, Worledge, D & Battaglia, M 2005, 'Temporal and spatial changes in fine root distributions in a young *Eucalyptus globulus* stand in southern Tasmania', *Forest Ecology and Management*, vol. 214, no. 1–3, pp. 373–383.

Patel, RN, Manaaki, RNP & Research, W-L 1994, 'Wood anatomy of the dicotyledons indigenous to New Zealand 23. Myrtaceae-subfam. Leptospermoideae (part I) Wood anatomy of the dicotyledons indigenous to New Zealand 23. Myrtaceae*-subfam. keptospermoideae (part I)', *New Zealand Journal of Botany*, vol. 32, pp. 95–112.

Perry, NB, Brennan, NJ, Van Klink, JW, Harris, W, Douglas, MH, McGimpsey, JA, Smallfield, BM & Anderson, RE 1997, 'Essential oils from New Zealand manuka and kanuka: Chemotaxonomy of *Leptospermum*', *Phytochemistry*, vol. 44, no. 8, pp. 1485–1494.

Porter, NG, Smale, PE, Nelson, MA, Hay, AJ, Van Klink, JW & Dean, CM 1998, 'Variability in essential oil chemistry and plant morphology within a leptospermum scoparium population', *New Zealand Journal of Botany*, vol. 36, no. 1, pp. 125–133.

Porter, NG & Wilkins, AL 1998, 'Chemical, physical and antimicrobial properties of essential oils of Leptospermum scoparium and Kunzea ericoides', *Phytochemistry*, vol. 50, no. 3, pp. 407–415.

Price, JN & Morgan, JW 2006, 'Variability in plant fitness influences range expansion of *Leptospermum scoparium*', *Ecography*, vol. 29, no. 4, pp. 623–631.

Retamales, HA 2007, Phylogeny of Myrteae (Myrtaceae) with an emphasis on the Chilean species: Insights into character evolution and historical biogeography.

Reverchon, E & Senatore, F 1992, 'Isolation of rosemary oil: Comparison between hydrodistillation and supercritical CO2 extraction', *Flavour and Fragrance Journal*, vol. 7, no. 4, pp. 227–230.

Ronghua, Y, Mark, AF & Wilson, JB 1984, 'Aspects of the ecology of the indigenous shrub Leptospermum scoparium (Myrtaceae) in New Zealand', *New Zealand Journal of Botany*, vol. 22, p. 483.

Senatore, F & De Feo, V 1999, 'Chemical composition of the essential oil from *Tagetes mandonii* Sch. Bip. (Asteraceae)', *Flavour and Fragrance Journal*, vol. 14, no. 1, pp. 32–34.

Seth, S, Agrawal, YC, Ghosh, PK & Jayas, DS 2010, 'Effect of moisture content on the quality of soybean oil and meal extracted by isopropyl alcohol and hexane', *Food and Bioprocess Technology*, vol. 3, no. 1, pp. 121–127.

Seyer, WF, Patterson, RF & Keays, JL 1944, 'The Density and Transition Points of the n-Paraffin Hydrocarbons', *Journal of the American Chemical Society*, vol. 66, no. 2, pp. 179–182.

Shimadzu n.d., *Linear retention index*, viewed 22 October 2020, Available from: https://www.shimadzu.com/an/sites/default/files/ckeditor/an/qn5042000001j1sm-img/qn5042000001j1vh.jpg.

Sigma-Aldrich 2020, *Solvent Miscibility Table*, viewed 22 september 2020, Available from: https://www.sigmaaldrich.com/chemistry/solvents/solvent-miscibility-table.html.

Southwell, IA & Stiff, IA 1989, 'Ontogenetical changes in monoterpenoids of Melaleuca alternifolia leaf', *Phytochemistry*, vol. 28, no. 4, pp. 1047–1051.

Srivastava, S, Mallavarapu, GR, Rai, SK, Singh, D, Mishra, R, Pandey-Rai, S & Kumar, S 2006, 'Composition of the essential oils of the leaves and flowers of *Rhus mysurensis* Heyne ex Wight & Arn growing in the Aravalli mountain range at New Delhi', *Flavour and Fragrance Journal*, vol. 21, no. 2, pp. 228–229.

Stephens, JMC, Molan, PC & Clarkson, BD 2005, 'A review of *Leptospermum scoparium* (Myrtaceae) in New Zealand', *New Zealand Journal of Botany*, vol. 43, no. 2, pp. 431–449.

Storer, JR, Elmore, JS & Van Emden, HF 1993, 'Airborne volatiles from the foliage of three cultivars of autumn flowering chrysanthemums', *Phytochemistry*, vol. 34, no. 6, pp. 1489–1492.

Sylvestre, M, Pichette, A, Longtin, A, Couppé, M-A, Martin, DK, Rodin Bercion, S & Legault, J 2007, 'Chemical Composition of Leaf Essential Oil of *Hedyosmum arborescens* and Evaluation of Its Anticancer Activity', *Natural Product Communications*, vol. 2, no. 12, pp. 1269–1272.

Tan, ST, Wilkins, AL, Holland, PT & Molan, PC 1988, 'Extractives from New Zealand Honeys. 1. White Clover, Manuka, and Kanuka Unifloral Honeys', *Journal of Agricultural and Food Chemistry*, vol. 36, no. 3, pp. 453–460.

Thompson, J 1989, 'A revision of the genus Leptospermum (Myrtaceae)', *Telopea*, vol. 3, no. 3, pp. 301–449.

Tongnuanchan, P & Benjakul, S 2014, 'Essential Oils: Extraction, Bioactivities, and Their Uses for Food Preservation', *Journal of Food Science*, vol. 79, no. 7, pp. 1231–1249.

Weston, RJ 1984, 'Composition of essential oil from leaves of *Eucalyptus delegatensis*', *Phytochemistry*, vol. 23, no. 9, pp. 1943–1945.

Wilson, MB, Barnes, BB & Boswell, PG 2014, 'What Experimental Factors Influence the Accuracy of Retention Projections in Gas Chromatography-Mass Spectrometry?', *J Chromatogr A*, vol. 1373, pp. 179–189.

Wilson, PG, O'Brien, MM, Gadek, PA & Quinn, CJ 2001, 'Myrtaceae revisited: A reassessment of infrafamilial groups', *American Journal of Botany*, vol. 88, no. 11, pp. 2013–2025.

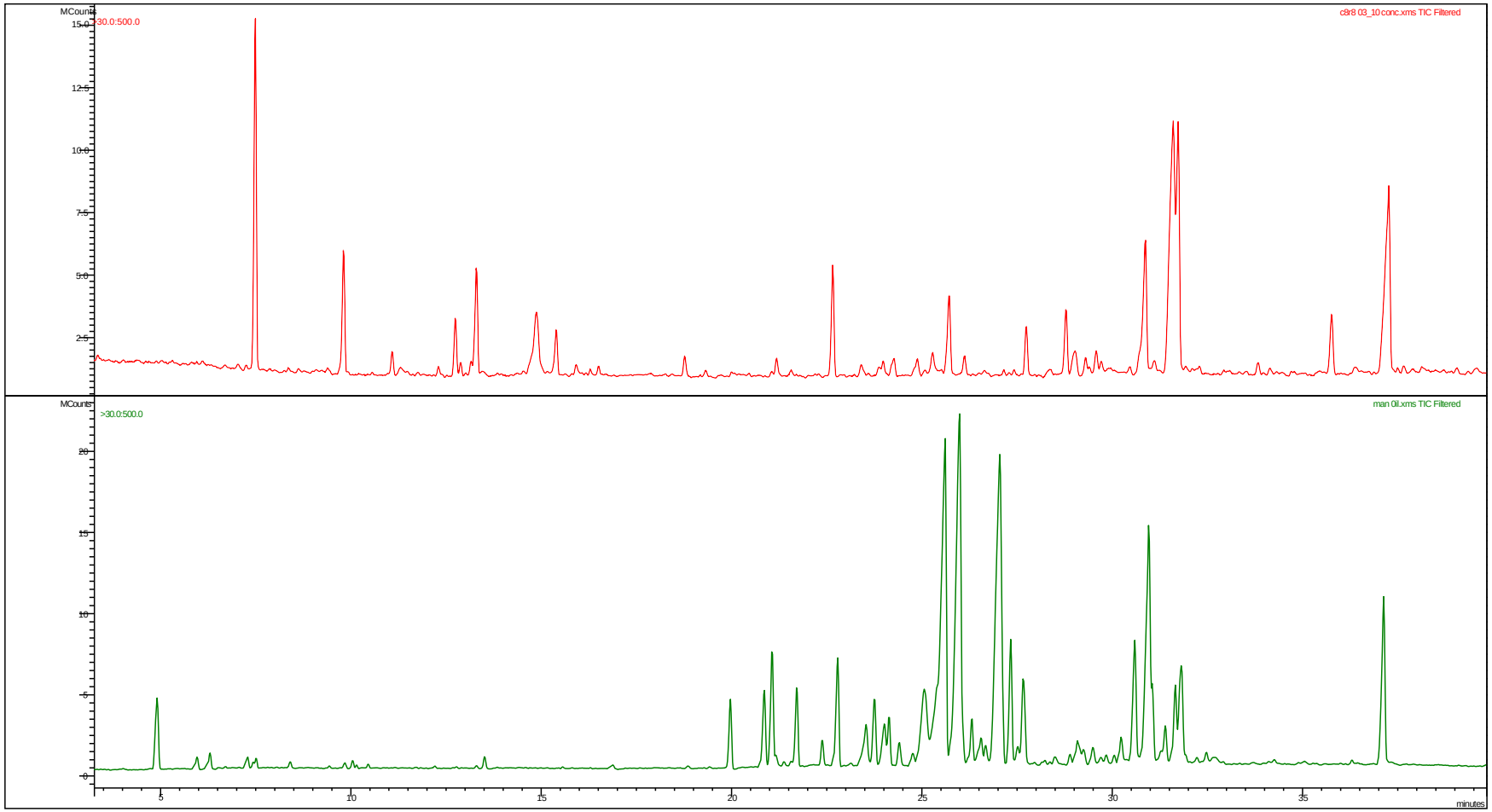
Wu, S, Zorn, H, Krings, U & Berger, RG 2007, 'Volatiles from submerged and surface-cultured beefsteak fungus, *Fistulina hepatica*', *Flavour and Fragrance Journal*, vol. 22, no. 1, pp. 53–60.

Xiao, JF, Zhou, B & Ressom, HW 2012, 'Metabolite identification and quantitation in LC-MS/MS-based metabolomics', *Trends in Analytical Chemistry*, vol. 32, pp. 1–14.

Xie, B, Liu, G, Jiang, S, Zhao, Y & Wang, D 2008, 'Crystallization behaviors of n-octadecane in confined space: Crossover of rotator phase from transient to metastable induced by surface freezing', *Journal of Physical Chemistry B*, vol. 112, no. 42, pp. 13310–13315.

Zellner, B d'Acampora, Bicchi, C, Dugo, P, Rubiolo, P, Dugo, G & Mondello, L 2008, 'Linear retention indices in gas chromatographic analysis: a review', *Flavour and Fragrance Journal*, vol. 23, no. 5, pp. 297–314.

Appendix



Appendix 1: GC-MS chromatograms of oil constituents of a Tasmanian *L. scoparium* leaf extract (graph A) and a New Zealand *L. scoparium* oil sample (graph B).