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Phytochemistry 65 (2004) 1255–1264
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Essential oils from New Zealand manuka: triketone and other chemotypes of *Leptospermum scoparium* ☆

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Received 17 December 2003; accepted 17 March 2004

Dedicated to the memory of our seven Crop & Food Research colleagues killed in a plane crash, 6 June 2003
Available online 23 April 2004

Abstract

The triketone chemotype of manuka, *Leptospermum scoparium* (Myrtaceae), is commercially important because of its antimicrobial activity. Oils from 36 individual plants on the East Cape of New Zealand all showed similar high triketone contents (>20% total triketones) with little seasonal variation. Analyses of oils from 261 individual manuka plants collected from 87 sites throughout New Zealand showed that the high triketone chemotype was localised on the East Cape, although oils with triketone levels up to 20% were found in the Marlborough Sounds area of the South Island. Cluster analysis revealed other chemotypes localised on other areas. Ten further chemotypes are described: α-pinene; sesquiterpene-rich with high myrcene; sesquiterpene-rich with elevated caryophyllene and humulene; sesquiterpene-rich with an unidentified sesquiterpene hydrocarbon; high geranyl acetate; sesquiterpene-rich with high γ-ylangene + α-copaene and elevated triketones; sesquiterpene-rich with no distinctive components; sesquiterpene-rich with high *trans*-methyl cinnamate; high linalol; and sesquiterpene-rich with elevated elemene and selinene. Some of the chemotypes contained aroma compounds at relatively high levels, with a geranyl acetate-rich oil being most notable. Possible origins for this complex array of chemotypes are proposed.

Keywords: *Leptospermum scoparium*; Myrtaceae; Manuka; Essential oil; Chemotype; Triketones; Sesquiterpenes; Monoterpenes; Geranyl acetate; Methyl cinnamate

1. Introduction

Manuka, *Leptospermum scoparium* J.R. et G. Forst. (Myrtaceae), grows as a shrub or small tree throughout New Zealand (Wardle, 1991) and in eastern Australia (Brophy et al., 1999b). In New Zealand it is valued for its essential oil (Porter, 2001). There is growing international interest in triketone-rich manuka oils due to their activity against Gram-positive bacteria including antibiotic-resistant strains (Christoph et al., 2000, 1999; Harkenthal et al., 1999; Kim and Rhee, 1999; Lis-Balchin et al., 2000; Porter and Wilkins, 1998). However, there are major variations in the chemical composition of oils from this one species, leading to potential confusion in the marketplace.

In our previous work on *Leptospermum* essential oils we found that oils from Australian *L. scoparium* had higher monoterpene levels than most New Zealand *L. scoparium*, and low or no triketones (Perry et al., 1997). Brophy et al. (1999b) also found no triketones in oils from Australian *L. scoparium* var. *scoparium* and

☆ Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2004.03.019.

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doi:10.1016/j.phytochem.2004.03.019

var. *eximum* but did find triketones (Brophy et al., 1999a, 2000) in the closely aligned *Leptospermum* subgroups of Thompson (1989). Within New Zealand, our original investigations were based on analyses of plants grown from seed collected from 15 sites around the country and grown at one site. Therefore, environmental effects on oil composition were eliminated and it was possible to sample all the plants on the same day to eliminate possible seasonal effects. From this work we identified three regional chemotypes: high α-pinene (mean 22.5%) in the far North, high triketones (mean total 32.5%) on the East Cape, and a type containing a complex of sesquiterpenes (mean total 64.7%) over the rest of the North and South Islands (Perry et al., 1997). This study showed that manuka oil composition was largely genetically controlled since oil compositions of plants grown at the study site were similar to those of plants growing at the seed source site (Perry et al., 1997).

Table 1
Composition of *L. scoparium* oils from the East Cape seasonal study[a]

GC peak	Compound name	Mean (SD)	Minimum	Maximum
1	α-Pinene	0.7 (0.9)	0.0	3.6
2	β-Pinene	0.3 (0.4)	0.0	1.9
3	Myrcene	0.3 (0.3)	0.0	1.3
4	*p*-Cymene	0.3 (0.2)	0.0	0.9
5	1,8-Cineole	0.7 (0.4)	0.0	1.8
6	β-Ocimene	0.2 (0.2)	0.0	0.7
7	γ-Terpinene	nd	–	–
8	α-Terpinolene	nd	–	–
9	Linalol	0.3 (0.1)	0.1	0.8
10	Terpinene-4-ol	0.1 (0.1)	0.0	0.2
11	α-Terpineol	0.1 (0.1)	0.0	0.4
13	Citronellol	0.0 (0.0)	0.0	0.1
14	Citronellyl formate	0.2 (0.2)	0.0	0.8
15	Methyl citronellate	nd	–	–
16	*cis*-Methyl cinnamate	0.0 (0.1)	0.0	0.1
17	Methyl geranate	0.0 (0.1)	0.0	0.1
18	Citronellyl acetate	nd	–	–
19[b]	*trans*-Methyl cinnamate/α-cubebene	2.8 (1.2)	0.4	5.3
20+21[c]	γ-Ylangene + α-copaene	5.7 (3.0)	2.0	13.6
22	Geranyl acetate	0.0 (0.0)	0.0	0.1
23	β-Elemene	0.4 (0.4)	0.0	1.6
24	α-Gurjunene	0.8 (0.7)	0.1	4.1
25	β-Caryophyllene	1.5 (1.2)	0.0	3.9
26	$C_{15}H_{24}$	0.3 (0.2)	0.0	1.1
27	Aromadendrene	1.7 (1.3)	0.0	5.5
28	$C_{15}H_{24}$	1.3 (2.3)	0.0	7.9
29	α-Humulene	3.6 (3.3)	0.4	11.9
30	$C_{15}H_{24}$	0.6 (0.4)	0.0	1.4
31	α-Amorphene	2.6 (1.5)	0.0	6.1
32	β-Selinene	1.8 (1.3)	0.0	5.8
33	$C_{15}H_{24}$	2.4 (2.3)	0.0	9.1
34[d]	α-Selinene/viridiflorene	3.0 (1.6)	1.1	8.2
35	α-Muurolene	0.9 (0.2)	0.5	1.4
36	γ-Cadinene	0.2 (0.2)	0.0	0.7
37	*trans*-Calamenene	15.6 (3.7)	7.9	22.7
38	δ-cadinene	4.5 (1.2)	2.7	8.6
39	Flavesone	8.2 (2.0)	4.9	12.3
40	Cadina-1,4-diene	0.0 (0.0)	0.0	0.1
41	Calacorene	0.2 (0.1)	0.0	0.4
42	Not identified	0.4 (0.1)	0.0	1.3
43	Caryophyllene epoxide	0.7 (0.4)	0.3	1.9
44	Not identified	0.2 (0.2)	0.0	1.2
45	Isoleptospermone	6.2 (1.9)	2.7	11.5
46	Leptospermone	16.6 (4.4)	10.6	29.4
47	β-Eudesmol	0.8 (0.2)	0.4	1.3
48	α-Eudesmol	0.0 (0.0)	0.0	0.2
50	Grandiflorone	nm	–	–

[a] GC peak areas as % of total peak area (nd, not detected; nm, not measured).
[b] Not resolved but ^{1}H NMR spectra showed no *trans*-methyl cinnamate in East Cape samples.
[c] Resolution varied so peaks summed.
[d] Not resolved.

Porter and Wilkins (1998) proposed a fourth chemotype rich in linalol and eudesmols, found in the Nelson area of the South Island. They also proposed a fifth chemotype high in myrcene and eudesmols, but considered it a variant of the linalol–eudesmol chemotype.

Leptospermum scoparium grows throughout New Zealand in habitats ranging from lowland to sub alpine areas (Wardle, 1991) and our previous sampling (Perry et al., 1997) was not representative of this range. The aim of this research was to determine if the triketone-rich chemotype was present in other New Zealand locations and define the boundaries of this chemotype around the East Cape. Before undertaking this survey we needed to know whether seasonal variation in essential oil composition was important, as it would be logistically impossible to collect samples in a national survey all at the same time. Work on manuka in the Nelson area of the South Island showed seasonal variation in the leaf oil composition of young plants (<6 year old); significantly higher relative levels of monoterpenes were present during the spring/summer period of foliage growth (Porter et al., 1998). We

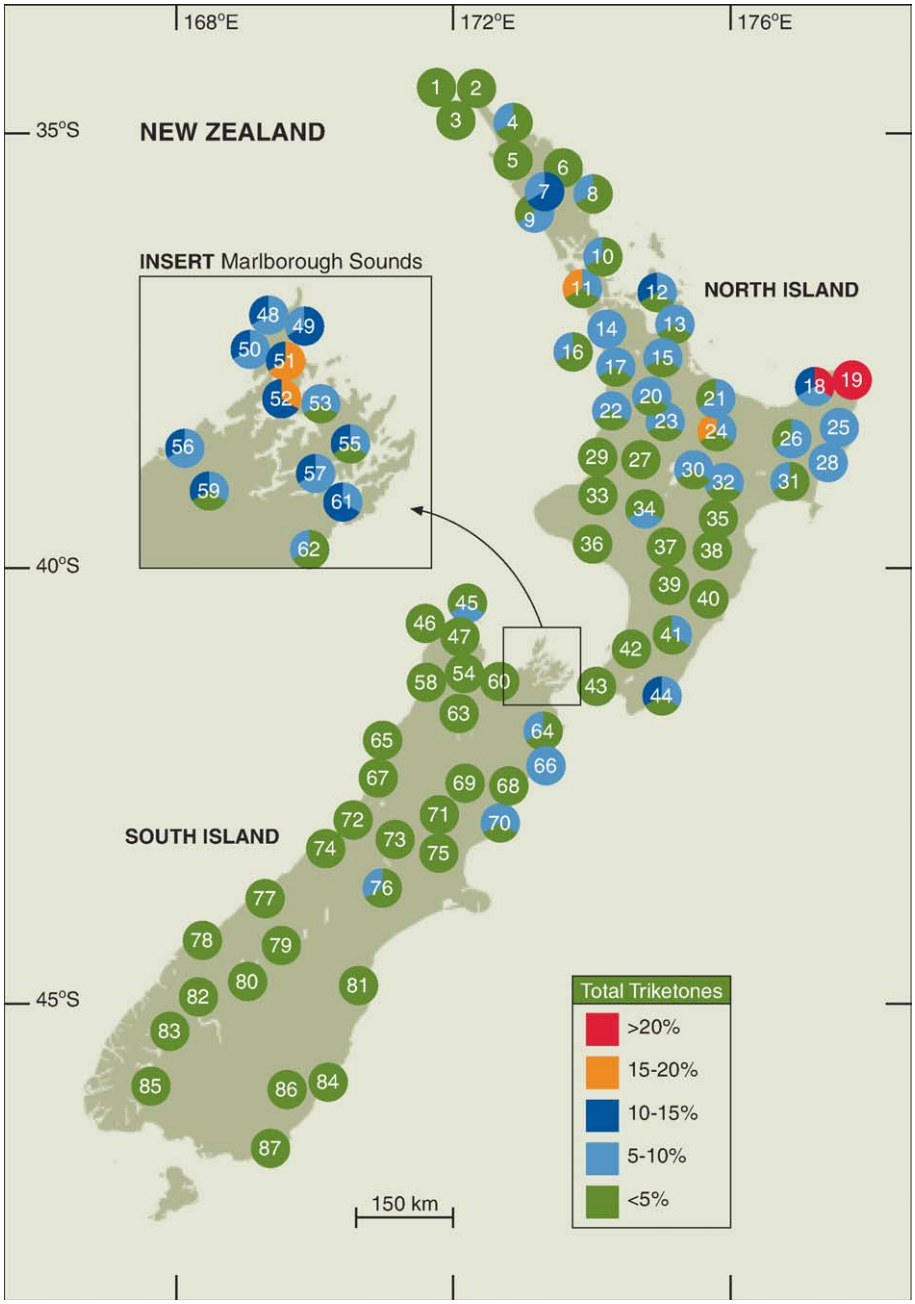

Fig. 1. Sampling sites and triketone levels in New Zealand *L. scoparium*.

therefore investigated the seasonal variation of the triketone-rich oil from East Cape manuka before sampling countrywide. We now report here the results of this seasonal study and of a national survey of the essential oil composition of manuka, covering many habitats throughout the North and South Islands of New Zealand.

2. Results

Manuka foliage samples were collected and oils extracted using standardised methods. Forty-eight GC peaks were quantified, with two reference peaks used for retention time correction (Perry et al., 1997). We have now identified most of the peaks (Table 1) by GC–MS and correlations with the results of Porter and Wilkins (1998).

2.1. Seasonal and individual variation within the East Cape triketone chemotype

An East Cape population (close to site 19, Fig. 1) was sampled at approximately monthly intervals from October 1996 to September 1997, with foliage from four different individual plants harvested each time. The composition of the oils from 36 individual plants is summarised in Table 1.

The mean levels of total triketones ranged from about 25% to about 35%, with leptospermone the main triketone in all the oils. Triketone levels did not show any obvious trends with the seasons (Fig. 2). Statistical analysis using a SAS General Linear Model showed only a few significant ($P < 0.05$) differences in triketone composition over the study period. For example, total triketone levels in the November samples were significantly ($P < 0.05$) higher than in the March and September samples. The mono- and sesquiterpene components of the oil samples also did not show any clear-cut seasonal patterns. We found significant differences in oil yield over the study period (data not shown) due primarily to seasonal differences in leaf/twig ratio.

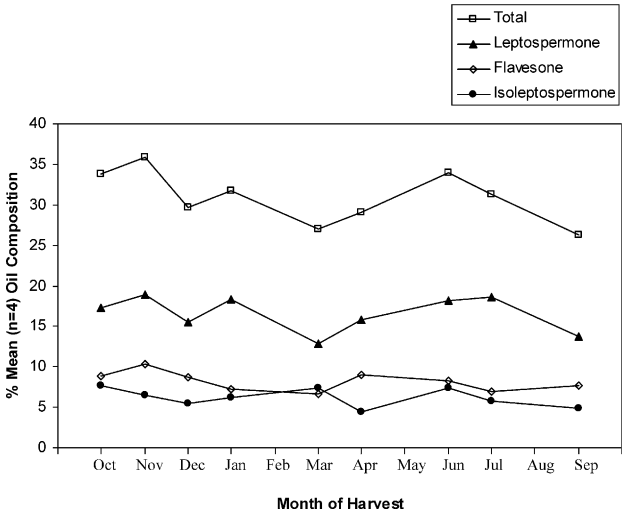

Fig. 2. Triketone levels in *L. scoparium* oils from the East Cape seasonal study.

2.2. Triketone levels throughout New Zealand

In the regional variation study, 43 sites in the South Island were sampled during January–February 1999 and January–February 2000, and 44 sites in the North Island during February–March 2001. The 87 collection sites are indicated on Fig. 1, which also summarises triketone levels, with one segment for each of the three oils analysed from each site.

There were major differences in triketone levels, both between sampling sites and between individuals at a given site. As expected, all three plants at the East Cape site 19 had total triketones >20% (Fig. 1), as found previously (Perry et al., 1997) and in the seasonal study (see above). These were the highest levels of triketones found and there was a rapid drop-off in triketone levels at neighbouring sites. For example, 75 km South at site 25 all three plants had <10% total triketones, though one of the plants from site 18 (50 km W) had total triketones >20%.

The South Island sampling also showed a very localised "hot spot" for high triketone oils in the Marlborough Sounds region, with three plants on D'Urville Island (sites 51 and 52) having 15–20% total triketones (Fig. 1).

2.3. Other chemotypes in New Zealand

To search for other chemotypes within this complex dataset of 261 oils with 48 GC peak levels quantified, we used non-hierarchical cluster analysis. This is an exploratory technique used to identify and separate like groups by computation, based on Euclidean metric (Krzanowski and Marriott, 1995). The number of clusters separated within the data set is arbitrary, and we have chosen 10 because these correspond to distinct chemotypes, as shown in Table 2 and Fig. 3 and summarised below:

Cluster 1, **α-Pinene Chemotype**, oils were found in the North of both islands. **Cluster 2**, **Sesquiterpene/Myrcene Chemotype**, included only five of the plants surveyed and was very localised in the Waikato region. **Cluster 3**, **Caryophyllene/Humulene Chemotype**, oils contained mostly sesquiterpenes. **Cluster 4**, **Sesquiterpene 33 Chemotype**, was found only in the North Island. **Cluster 5**, **Geranyl Acetate Chemotype**, included only three plants in the survey and is a previously unreported manuka chemotype. **Cluster 6**, **γ-Ylangene/**

Table 2
Compositional clusters for *L. scoparium* oils (mean GC peak areas as % of total peak area)

Compound	Cluster number (number of plants in each cluster)									
	1 (26)	2 (5)	3 (51)	4 (28)	5 (3)	6 (22)	7 (46)	8 (18)	9 (35)	10 (27)
α-Pinene	**21.5**[a]	0.6	1.4	1.9	1.2	1.0	1.8	1.6	4.1	1.5
β-Pinene	5.8	0.1	0.7	0.8	0.1	0.8	0.2	0.4	3.7	0.3
Myrcene	1.3	**20.0**	1.5	0.8	0.9	0.8	1.2	1.6	2.3	1.5
p-Cymene	1.1	0.2	0.3	0.5	0.1	0.3	0.3	0.6	0.4	0.3
1,8-Cineole	8.5	0.3	0.7	1.2	0.1	1.9	0.7	3.2	3.0	1.9
β-Ocimene	0.1	0.0	0.0	0.1	0.0	0.3	0.8	0.3	0.3	0.3
γ-Terpinene	1.1	0.3	0.5	0.6	0.2	0.3	0.5	0.7	0.4	0.3
α-Terpinolene	0.2	0.0	0.0	0.0	0.0	0.1	0.1	0.2	0.1	0.1
Linalol	8.5	2.2	1.1	0.8	1.0	3.5	3.3	3.7	**12.6**	3.2
Terpinene-4-ol	0.9	0.1	0.2	0.3	0.1	0.3	0.2	0.4	0.5	0.3
α-Terpineol	1.9	0.1	0.2	0.4	0.1	0.6	0.2	0.8	0.9	0.5
Citronellol	0.3	0.0	0.0	0.0	0.1	0.2	0.1	0.5	2.1	0.5
Citronellyl formate	0.7	0.0	0.2	0.2	0.0	2.7	0.6	1.0	1.8	2.3
methyl citronellate	0.5	0.2	0.1	0.1	0.3	0.7	0.1	0.2	1.2	0.2
cis-Methyl cinnamate	0.4	0.2	1.1	1.4	0.8	0.6	1.0	0.4	0.4	0.2
Methyl geranate	3.4	1.0	0.8	0.2	0.9	1.5	0.5	1.1	2.1	1.5
Citronellyl acetate	0.5	0.2	0.0	0.0	0.1	0.4	0.1	1.3	1.4	0.6
trans-Methyl cinnamate/α-cubebene	2.1	6.0	5.3	5.3	3.4	7.3	6.0	**17.0**[b]	9.0	10.6
γ-Ylangene + α-copaene	4.6	0.8	2.3	2.1	0.1	**25.6**	5.4	4.0	5.1	6.0
Geranyl acetate	0.1	0.5	0.4	0.5	**48.6**	0.2	0.1	0.5	0.3	0.7
β-Elemene	1.1	5.9	4.4	2.6	1.0	2.6	0.6	6.1	4.1	**10.8**
α-Gurjunene	0.2	0.6	1.0	0.5	0.3	0.6	1.2	0.9	0.6	0.6
β-Caryophyllene	5.2	7.7	**8.3**	2.7	0.9	3.2	5.4	4.0	4.7	3.2
$C_{15}H_{24}$	0.1	0.1	0.3	1.4	1.9	0.2	0.5	0.3	0.2	0.5
Aromadendrene	0.3	0.2	0.7	0.9	0.2	2.4	3.9	1.0	0.6	1.2
$C_{15}H_{24}$	0.8	0.4	1.0	2.4	0.8	1.5	2.2	1.0	0.6	0.8
α-Humulene	1.6	6.6	**9.1**	3.1	2.5	3.1	4.8	6.4	3.3	2.6
$C_{15}H_{24}$	0.3	0.3	0.5	2.0	1.3	0.8	1.1	1.1	0.9	0.9
α-Amorphene	1.1	1.2	3.1	2.0	0.9	2.0	5.5	1.9	1.4	2.8
β-Selinene	3.5	3.2	6.0	1.3	3.1	4.4	5.5	3.6	3.6	**10.0**
$C_{15}H_{24}$ (sesquiterpene JJ)	0.6	1.5	1.8	**14.5**	4.4	0.8	1.4	2.1	0.9	1.3
α-Selinene/viridiflorene	3.3	5.0	8.6	10.8	2.3	5.8	7.0	7.3	5.6	**13.5**
α-Muurolene	0.4	1.9	1.7	4.5	3.8	1.0	1.0	1.9	1.4	3.1
γ-Cadinene	0.9	0.5	0.8	1.6	0.8	0.8	1.6	0.8	0.5	0.5
trans-Calamenene	2.7	4.1	7.2	4.5	2.5	3.9	7.0	5.1	4.3	3.3
δ-Cadinene	1.1	2.1	3.7	4.1	0.9	2.1	5.2	2.6	1.7	1.1
Flavesone	0.9	1.4	3.1	2.7	1.3	1.6	**3.6**	1.7	1.1	0.7
Cadina-1,4-diene	0.2	0.1	0.4	1.1	0.6	1.0	1.4	0.2	0.2	0.1
Calacorene	0.2	0.1	0.2	0.3	0.1	0.4	0.3	0.4	0.5	0.3
Not identified	0.1	0.0	0.1	0.1	0.1	0.1	0.2	0.2	0.3	0.4
Caryophyllene epoxide	0.2	0.3	0.4	0.6	0.1	0.3	0.5	0.2	0.1	0.2
Not identified	0.4	0.2	0.4	0.3	0.3	0.4	0.5	0.3	0.2	0.3
Isoleptospermone	0.3	0.5	0.9	0.5	0.1	1.3	**1.8**	0.3	0.4	0.3
Leptospermone	1.0	3.1	1.7	0.8	0.3	3.4	**4.5**	0.8	1.2	0.7
β-Eudesmol	1.1	3.9	2.2	1.1	0.3	0.7	0.8	0.8	1.3	0.5
α-Eudesmol	1.0	3.8	1.6	0.7	0.6	0.5	0.5	0.5	1.2	0.9
Grandiflorone	0.0	0.6	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Tm	**56.3**	25.5	8.2	8.4	**53.8**	15.3	11.0	18.0	37.3	15.9
Ts	30.9	50.6	**65.6**	**65.1**	29.5	**64.3**	**63.6**	52.5	43.3	**64.7**
Tt	2.2	5.0	5.8	4.0	1.6	6.3	**9.9**	2.9	2.8	1.7
Oil yield[c]	0.5	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.4	0.4

Tm, total monoterpene; Ts, total sesquiterpenes; Tt, total triketones.
[a] Bold numbers refer to the distinguishing features of each cluster.
[b] Identified as *trans*-methyl cinnamate by ^{1}H NMR in all cluster 8 oil samples.
[c] ml per 100 g dry weight.

α-Copaene Chemotype, includes the 15–20% triketone oils from the Marlborough Sounds, but the high ylangene/copaene level distinguishes it from the East Cape high-triketone chemotype. **Cluster 7**, **Sesquiterpene plus East Cape Triketone Chemotype**, included the East Cape triketone chemotype and a chemotype with

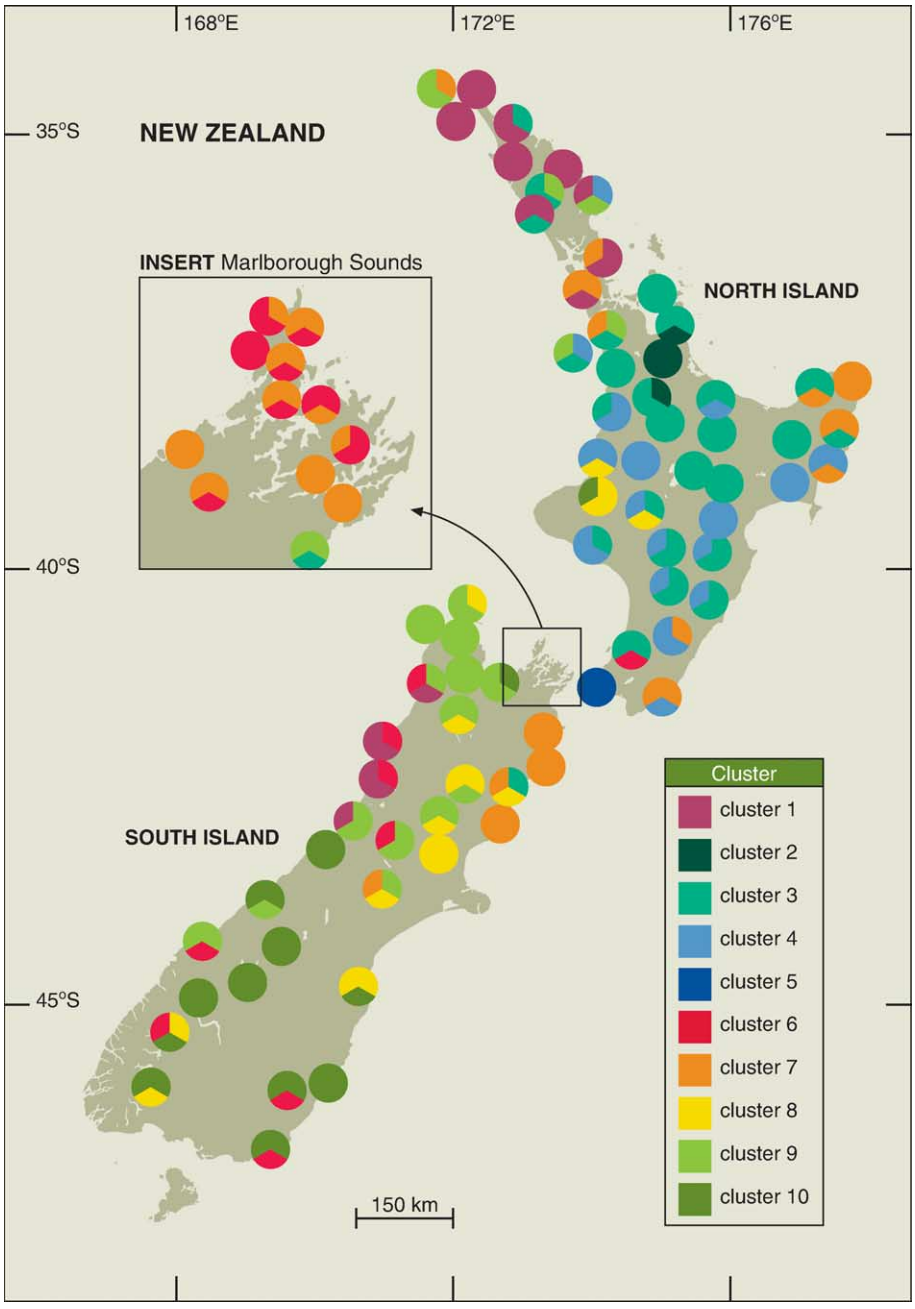

Fig. 3. Cluster analysis of essential oil composition of *L. scoparium* in New Zealand.

a complex of sesquiterpenes at similar levels. The sesquiterpene plants were mostly in the North of the South Island. **Cluster 8**, **Methyl-Cinnamate/Sesquiterpene Chemotype**, oils were analysed by ^{1}H NMR to confirm the presence of high levels of *trans*-methyl cinnamate since this compound co-eluted with δ-cubebene on GC. **Cluster 9**, **Linalol Chemotype**, oils also had higher levels of citronellol, citronellyl formate and methyl citronellate that contributed to a noticeable citrus aroma. **Cluster 10**, **Elemene/Selinene Chemotype**, oils were found predominantly in the South Island and only one site in the North Island.

2.4. Inter-relationship of clusters

Based on the cluster means, a dendrogram was constructed that allowed the similarity of each cluster to its nearest neighbour to be placed in context (Fig. 4). Of interest is the close alignment of the three mostly South Island clusters, 8, 9 and 10, which are linked with cluster 6, also predominantly in the South Island. North Island clusters 2 and 3 are closely aligned, while cluster 7, which is present in both islands, is similar to clusters 2, 3 and 6. Clusters 1 and 5, the two high monoterpene chemotypes, are the most different.

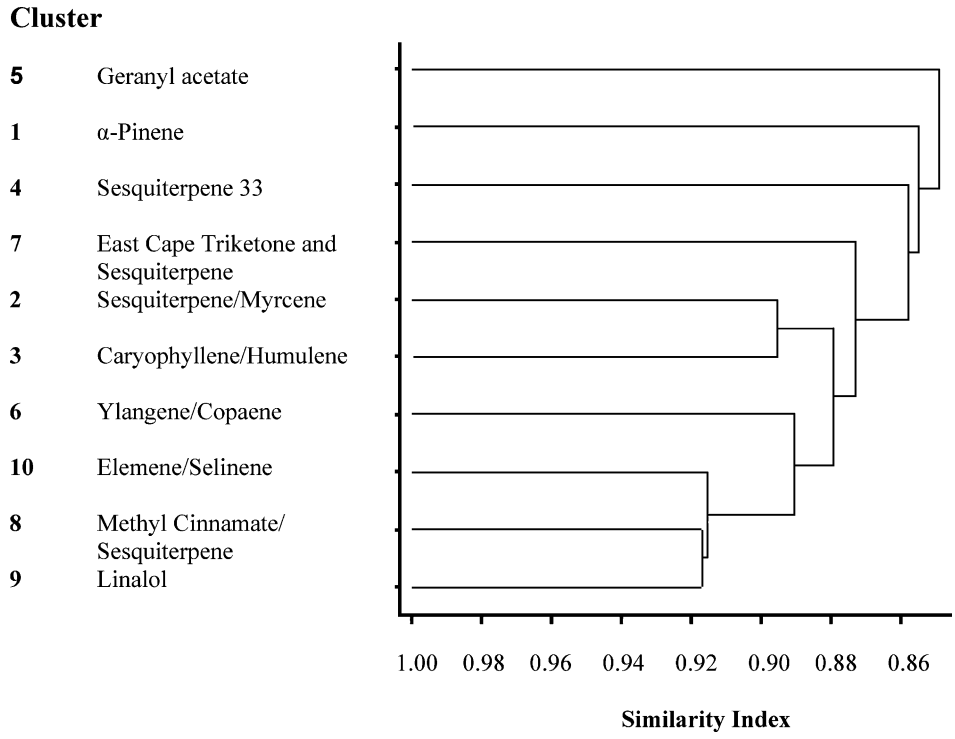

Fig. 4. Dendrogram of the cluster analysis.

3. Discussion

We have shown that the volatile oil composition of manuka, *L. scoparium*, in New Zealand is extremely variable. We propose 11 chemotypes with intriguing patterns in their geographic distribution (Figs. 1 and 3). These chemotypes are distinguished by different levels of four classes of natural products: monoterpenes and sesquiterpenes, both products of the isoprenoid biosynthetic pathway; methyl cinnamate, from the phenylpropanoid pathway; and triketones, from an as yet uncharacterised biosynthetic pathway.

We have shown that the high triketone (>20% total triketones) chemotype previously identified (Perry et al., 1997; Porter and Wilkins, 1998) has a very limited distribution, being found only on the East Cape of the North Island (Fig. 1). There were only slight seasonal differences in triketone levels (Fig. 2). This means that commercial producers of antibacterial manuka oils could harvest throughout the year, as long as this was consistent with maximising foliage yield and regrowth of the plants.

The only other area of New Zealand that yielded relatively high triketone manuka oils was the Marlborough Sounds (Fig. 1), although the level of triketones was lower than on the East Cape and generally below the standard expected for good antimicrobial activity (Christoph et al., 2000). We do not know why both the East Cape, and the Marlborough Sounds should have manuka with higher levels of triketones. One potential link could be by Maori travelling to D'Urville Island in the Marlborough Sounds (Thomson, 1918), since Maori sometimes took plants and seed on their journeys (Brailsford, 1997). Another, perhaps stronger, linkage is that both high-triketone regions are known to have been plant refugia, being less affected by past environment obliteration (as discussed by McGlone (1985) and colleagues (McGlone et al., 2001)). As noted, Brophy et al. (Brophy et al., 1999b) did not find triketones in oils from their Australian samples of *L. scoparium*, but did find leptospermone in an oil from *L. glabrescens* (Brophy et al., 1999a).

A high α-pinene chemotype in manuka from the North of the North Island has previously been proposed (Perry et al., 1997) and we now confirm this chemotype and report that it is also present in an area on the West Coast of the South Island (cluster 1, Fig. 3). Porter and Wilkins (1998) also identified a pinene-rich chemotype type III on the eastern side of the South Island, but because the α-pinene level was so high (63%), this was probably a sample of *Kunzea ericoides* that had been misidentified as manuka (N. Porter, personal communication). Volatile monoterpenes have been related to fire ecology (Owens et al., 1998), and we suggest that the presence of high monoterpene oils in Northland and on the Westland fringe could be explained by frequent firing and regeneration cycles (Ogden et al., 1998; Burrows et al., 1979). In contrast to Australia, fire adaptation is not a common feature of the New Zealand flora (Ogden et al., 1998), although manuka has serotinous capsules

that release seeds *en masse* after fire, and of all the native woody plants manuka (together with kanuka (*K. ericoides*)) regrows most strongly after disturbance (Wardle, 1991). In Northland, the *L. scoparium* examined by Harris (2002) had strongly serotinous capsules, but his Westland samples did not.

We have discovered a new monoterpene chemotype that is high in geranyl acetate and very localised in the South of the North Island (cluster 5, Fig. 3). Monoterpene acetates are less flammable than monoterpene hydrocarbons (Owens et al., 1998), so this is less likely to relate to fire adaptation. Geranyl acetate is a perfumery ingredient (Arctander, 1969), so this chemotype might be commercially useful. Brophy et al. (2000) found geranyl acetate (4–11%) in oils from *L. variabile*. We have also identified a mixed monoterpene + sesquiterpene chemotype (cluster 9, Fig. 3) with elevated levels of linalol, another aroma compound. This chemotype is similar to the type-II north-west Nelson chemotype described by Porter and Wilkins (1998).

The general sesquiterpene chemotype previously described (Perry et al., 1997) is here subdivided into different chemotypes based on particular major compounds. These chemotypes are not likely to have any commercial use, because these sesquiterpene hydrocarbons are not generally distinguished by biological activity or aroma. However, our discovery of relatively high levels of methyl cinnamate in many South Island samples, at levels up to 30%, is interesting because this compound is a perfume and flavour ingredient (Arctander, 1969). Methyl cinnamate has been noted in several Australian *Leptospermum* species, with the highest levels in an oil from *L. riparium* (Brophy et al., 1999a).

Overall, the volatile oils of *L. scoparium* in New Zealand are complex mixtures showing a very high degree of infraspecific variation with some geographical patterns. The oils from Australian *Leptospermum* species are similarly complex mixtures, variable between species and occasionally within species (Brophy et al., 1999b), but the infraspecific variation has not been explored in the same detail as reported here. There is also large variability in morphological characters of *L. scoparium*, both in Australia (Thompson, 1989) and in New Zealand (Yin Ronghua et al., 1984; Wardle, 1991). Both morphological and metabolic variability might be explained by polyphyletic origins for New Zealand's *L. scoparium*.

The first myrtaceous pollens in New Zealand and Australia that are similar to *Leptospermum* are recognisable in the Palaeocene some 60 million yeas ago (Lee et al., 2001). Since that time about 82 species have evolved, predominantly in eastern Australia (McGlone et al., 2001; Thompson, 1989). Thompson, in her definitive study of the *Leptospermum* genus (Thompson, 1989), notes that *L. scoparium* in New Zealand is not a primitive *Leptospermum* and cannot be an ancient Myrtaceae plant of the New Zealand Palaeocene as detailed by Fleming (1975). During the Cenozoic period, the land shape and environment of New Zealand changed dramatically with tectonic uplift, volcanism, sea inundation and long term shifts in climate (Lee et al., 2001; McGlone, 1985; McGlone et al., 2001). Considering the Pleistocene extinction of such Australian genera as *Eucalyptus* and *Acacia*, there is a possibility that the Palaeocene *Leptospermum* was replaced by more recent genetic stock following long distance dispersal (Pole, 1994; McGlone et al., 2001). A phylogenetic study could help to explain chemotype differences in *L. scoparium* as well as giving answers to the evolution and dispersal of the genus *Leptospermum*.

4. Experimental

4.1. Plant materials

In October 1996 a population of *L. scoparium* near Te Araroa on the East Cape of New Zealand was chosen for a seasonal study. Each month foliage (leaf and stem) samples were collected from four individual plants. Samples were not collected in January, May and August of 1997 due to extreme weather conditions at those times.

For the regional variation study, three manuka plants were sampled for foliage (leaf and stem) at each site. The South Island survey was undertaken from January to April 1999, (site nos. 45–47 and 63–87), with nine additional sites (nos. 48–62) harvested in the Marlborough Sounds in January 2000 (Fig. 2). The North Island sampling was undertaken in February–March 2001 (site nos. 1–44, Fig. 2). The plants at each site were generally within 50 m of each other, but at four sites (1, 12, 17 and 34; Fig. 2) a single plant was at a greater distance.

Foliage samples (3–5 kg) from both seasonal and regional studies were bagged, numbered and sent by courier post to the Invermay Research Centre. Voucher samples were retained, while the bulk samples were slowly dried at 3 °C and 17% relative humidity for 20 days prior to distillation

4.2. Distillation method

Each dried foliage sample was chopped into approximately 25 mm lengths and steam distilled under standardised conditions for 2 h. The distillation was essentially the same as that method reported previously (Perry et al., 1997). However, due to frothing of the condensate, the set-up was modified to include a hot-water jacketed separating funnel, which improved oil recovery and measurement by helping the oil particles coalesce. Oil samples were stored at −20 °C in glass vials.

4.3. Essential oil analyses and component identifications

GC analyses were performed on a Perkin–Elmer Autosystem gas chromatograph (under the control of PE-Omega software) equipped with a split injector (100:1, 260 °C) and a 10 m J &W DB-1 column with a 0.25 mm ID and 0.25 μm film. Oil samples (10 μl) were diluted in cyclohexane (1 ml) containing 0.5% *n*-dodecane (C_{12}) and *n*-octadecane (C_{18}), and 0.5 μl subsamples were injected using an autosampler. The carrier gas used was hydrogen with a linear velocity of 50 cm/s. For the seasonal studies, the column temperature was programmed 80–160 °C at 5 °C/min. For the regional variation study the upper limit on the temperature program was extended to 180 °C in order to detect grandiflorone. Peaks were detected by a flame ionisation detector (350 °C). Levels of 48 peaks were recorded (Table 2). The alkanes C_{12} and C_{18} were used as reference peaks to correct for retention time fluctuations and to help in peak matching.

GC–MS data were collected on a Finnigan GC8000, 70 eV, detector voltage 250 V, fitted with a 30 m J &D B-1 column (0.25 mm ID, 1 μm film thickness) and helium carrier gas with a flow rate of 1.5 ml/min, split ratio 10:1. The oven temperature program was set at 50–100 °C at 3 °C/min, then 100–180 °C at 5 °C/min then 180–250 °C at 30 °C/min, followed by a 10 min hold at 250 °C. Peak scans were compared with those in a series of libraries (NBS, WILEY, LIBTX) on the Finnigan MASLAB data system, using the reverse fit factor for peak matching. Peaks were matched to those reported earlier (Porter and Wilkins, 1998). To determine of Kovats retention indices (RIs), an altered temperature program (50–250 °C at 5 °C/min) was used. Indices were calculated by comparison with a separate injection of a standard *n*-alkane (even number) mixture.

Total monoterpenes in each oil were calculated by summing the levels of peaks 1–11, 13–15, 17, 18 and 22; total sesquiterpenes by summing peaks 20 + 21, 23–38, 40–44, 47 and 48; and total triketones by summing peaks 39, 45, 46 and 50.

4.4. Statistical analyses

The data for the seasonal trial was analysed using SAS (SAS Institute, version 6.15) software. The data for the regional variation study was subjected to non-hierarchical classification (*k*-means clustering) in which the Euclidean distance between classes is maximised (Krzanowski and Marriott, 1995). From the cluster means a dendrogram was drawn from the minimum spanning tree obtained from single linkage cluster analysis. The implementation of the clustering algorithms used was in GenStat release 6.1 (Genstat, 2003).

Supplementary material (Tables 5 and 6) is available which details sampling sites, the mean levels of monoterpenes, sesquiterpene and triketones, and the cluster placement for each plant oil analysed.

Acknowledgements

We thank J.-P. Dufour for GC–MS analyses; N. Porter, J. Douglas, J. Follett, N. and H. Fulton, H., A. and J. Douglas, and M. Kerr and staff at Tairawhiti Pharmaceuticals for field assistance; and N. Porter, J. Douglas and W. Harris for discussions. This research was supported by the New Zealand Foundation for Research, Science and Technology, the Ministry of Agriculture and Fisheries Sustainable Farming Fund and New Zealand Manuka Products Ltd.

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