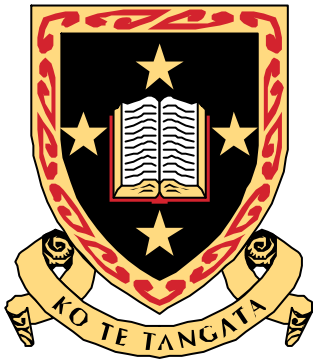
A Chemical Investigation of New Zealand Unifloral Honeys



The University of Waikato

Te Whare Wānanga o Waikato

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by

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Abstract

The diethyl ether-extracted organic compounds of 155 samples of unifloral grade New Zealand kamahi and honeydew honeys, and New Zealand and Norwegian erica honeys, together with a series of active and inactive manuka honeys were analysed using combined gas chromatography/mass spectrometry. It was found that Kamahi honey is characterized by the presence of 2,6-dimethylocta-3,7-diene-2,6-diol, meliracemoic acid, and kamahines A-C and these compounds were typically present at average levels of 31, 14, and 73 mg/kg of honey, respectively. 2,6-Dimethylocta-3,7-diene-2,6-diol was isolated and the structure of this compound was defined using one- and two-dimensional NMR analyses.

The only recognizably distinct peak present in the honeydew honey profile was indole acetic acid. In this honey, a relatively low to moderate level of indole acetic acid, ranging from 0.9 to 9.1 mg/kg honey was detected.

In the New Zealand erica honey samples, ericinic acid, isoericinic acid isomers (average levels 363 and 34 mg/kg respectively), *trans,cis* and *trans,trans*-abscisic acid isomers (average levels 302 and 224 mg/kg respectively) and benzoic acid (average level 6950 mg/kg) were identified as floral marker compounds. Ericinic acid was isolated and the structure of this acid was defined using one-and two-dimensional NMR analyses.

Low levels of ericinic and isoericinic acids (average levels of 1.1 and 0.32 mg/kg respectively) were detected in the Norwegian erica-rich honeys. The results presented here indicate that ericinic and isoericinic acids are likely to be universally present in erica honeys at levels which may range from as low as 1 mg/kg or less, as found in some Norwegian samples, to more than 100 mg/kg in some New Zealand samples.

Two groups, namely a fingerprint pattern which characterized active manuka honeys, and a fingerprint pattern that characterized inactive manuka honeys were identified. Some substances contributing to the GC/MS profile were found as marker compounds for the presence of unidentified substances responsible for the UMF

activity. A statistically significant correlation was found between a small set of marker compounds (*i.e.* phenylacetic acid, 2-methoxyacetophenone, 2-methoxybenzoic, phenyllactic, octanedioic, *cis*-cinnamic, *trans*-cinnamic, nonanedioic, 4-methoxyphenyllactic and decanedioic acids and methyl syringate) and UMF activity of manuka honey. The best-fit marker compound regression equation (R = 0.92) was obtained for a set of pooled 30 moderate to high activity (UMF > 14.1) samples. It was shown that the marker compound regression equation is capable of predicting the approximate UMF activity in both active and inactive manuka and kanuka honey samples.

The leaf oil profiles of manuka (*L. scoparium*) plants that yielded active and inactive manuka honeys were characterized using an adaption of the micro-scale extraction and GC/FID or GC/MS, technique developed by Brophy *et al.* (1989). Six major groups of volatile (steam distillable) compounds (monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes [excluding eudesmols], eudesmols, triketones, and nor-triketones) and 3 groups of non-volatile or semi-volatile compounds (flavonoids, grandiflorone and nor-grandiflorone) were recognized in the leaf oil components. The active manuka honeys do not appear to be derived uniquely, or predominantly, from a single leaf oil chemotype.

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List of Abbreviations

Ac	acyl
Ac_2O	acetic anhydride
~	approximately
aq	aqueous
°C	degree centigrade
ca	approximately (circa; about)
$CDCl_3$	deuterochloroform
°C/min	centigrade per minute
COSY	correlation spectroscopy
CV	coefficient of variance
2D	two-dimensional
DEPT	distortionless enhancement by polarisation -transfer
Δδ	chemical shift difference (ppm)
DMAP	4-dimethylaminopyridine
DMSO	dimethyl sulfoxide
d	doublet
E	East
e.g.	for example
et al.	and others
etc.	and so forth
FID	flame ionization detector
HCOOH	formic acid
FT	fourier transform
g	gram
GC	gas chromatography
GC/FID	integrated gas chromatography/flame ionisation detection
GC/MS	integrated gas chromatography/mass spectrometry
GLC	gas-liquid chromatography
h	hour
HC	hydrocarbon
HPLC	high-performance liquid chromatography
HPLC/MS/MS	high-performance liquid chromatography-tandem mass -Spectrometry
HPLC-APCI-MS/MS	high performance liquid chromatography – atmospheric -pressure chemical ionization - tandem mass spectrometry
HMBC	heteronuclear multiple bond coherence (long-range ^{1}H-^{13}C correlation)
HMQC	heteronuclear multiple quantum coherence (^{1}H-^{13}C correlation)
HP	Hewlett Packard
HSQC	heteronuclear single quantum coherence (^{1}H-^{13}C correlation)
i.d.	inside diameter
int std	internal standard
Hz	hertz (s^{-1})

J	coupling constant (Hz)
kg	kilogram
LC	liquid chromatography
LD_{50}	dose that is lethal to 50% of test subjects
log	logarithm to the base 10
Ltd.	limited
m	meter
M^+	the ionised molecule
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (mass spectroscopy)
MHz	MegaHertz
Me	methyl
mg	milligram
µL	microlitre
µm	micrometer
MDL	minimum detection limits
min	minute(s)
mL	millilitre
mL/min	millilitre per minute
mol	moles
m.p.	melting point
MS	mass spectrometry
mS/cm	milliSiemens per centimetre (conductivity)
MQL	minimum quantification level
m/z	mass/charge ratio
Mr	molecular weight (relative molecular mass)
N	North
n	total number of individuals or variates
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NZ	New Zealand
OMe	methoxyl
Ph	phenyl
pH	hydrogen ion concentration
PLC	preparative layer chromatography
ppm	parts per million
psi	pounds per square inch
p	probability
Py	pyridine
q	quartet
R	regression coefficient
recv std	recovery standard
RF	response factor
s	singlet
sec	second
SIM	selected ion monitoring mode
spp.	species
std	standard
Stdev	standard deviation
Σ	summation

t	triplet
TIC	total ion chromatogram
TLC	thin layer chromatography
TOCSY	totally correlated spectroscopy
tr	trace
TS	Trypticase Soy
UMF	Unique Manuka Factor
UV	ultraviolet
v/v	volume-to-volume ratio
vs.	versus

Chapter One

Introduction and Review

Chapter One

Introduction and Review

1.1. Chemical Characterization of Honeys

During the last century intensive efforts have been made to develop scientific methodologies which can be used to identify the plant sources from which honeys are derived. It has long been known that honeys exhibit taste, aroma and colour and other physical (organoleptic) characteristics, which depend on composition variations within the nectar and/or honeydew collected from different floral sources.

Numerous researchers, including Maurizio (1951), Demianowicz (1964), Sawyer (1975) and Moar (1985) have reported descriptions of pollen grains found in honeys and shown that the geographical and floral origin of honeys can in, many cases, be reliably inferred from pollen analyses. This approach is known as melissopalynology.

A significant limitation of melissopalynology is that pollen frequencies vary from as low as 10 000 grains of pollen/10 g honey to as much as 200 000 grains of pollen/10 g honey, depending on pollen levels in source nectars. Where a honey is derived mainly from low-pollen nectar, melissopalynology struggles to define the floral integrity of such a honey, since as little as 20% of the pollens grains present in the honey may have originated from the dominant floral source.

1.2. Early Chemical Investigation

1.2.1. Carbohydrate Analyses

It has long been believed that it might be possible to define floral source-specific chemical parameters which might assist in defining the origin of a honey. In early investigations, Thomson (1936) and White (1975b) determined a variety of physical

parameters (*e.g.* moisture, ash, refractive index, density and pH), carbohydrate levels (*e.g.* sucrose, glucose and maltose etc.), enzyme levels and mineral levels. While these investigations defined typical ranges for the appropriate parameters, these ranges did not greatly assist in the distinction of one floral source from another.

By the 1960's it was envisaged that chromatographic techniques might contribute to floral source distinction. In 1964, Pourtallier reported the application of thin-layer chromatography to carbohydrate analysis of honey (Pourtallier, 1964). Subsequently Siddiqui (1970) analysed 95 Canadian honey samples using a paper-chromatographic procedure. While this investigation afforded fructose, glucose and oligosaccharide data, it did not identify the floral source(s). Pourtallier (1967) and Echigo (1970) have reported the use of gas/liquid chromatography to determine the carbohydrate content of honeys.

1.2.2. Organic Acids

A variety of organic compounds, other than carbohydrates, are known to be present in honey samples. Formic acid (HCOOH) was the first recognised in honey by Vogel in 1882 (White, 1975a). Subsequently a variety of other organic acids including acetic, butyric, citric, formic, gluconic, lactic, maleic, oxalic, pyroglutamic and succinic acids have been identified in honeys (Nelson and Mottern, 1931; Stinson *et al*., 1960). Maeda *et al.* (1962) suggested gucollic, α-ketoglutaric and pyruvic acids were present in honeys.

1.2.3. 5-Hydroxymethylfurfuraldehyde (HMF)

Since 1908 the presence in honeys of 5-hydroxymethylfurfuraldehyde (HMF) has received considerable attention (Fiehe, 1908; Bremer and Sponnagel, 1909). HMF may be formed by the decomposition of fructose in the presence of acid. The quantity of HMF in a honey is now known to be dependent on the extent of heating during extraction, and or storage conditions (White, 1975b). Merz (1963) found HMF to be the principal peak in poor flavour honey (based on GLC analyses of ether extracts). Merz (1963) also observed that the quantity of HMF exceeded that of other high-boiling point

components in honey. It was originally believed that HMF was not present in raw honey (hive honey that has not been heated), however other investigations have shown that low levels of HMF may be present in raw honey (White, Kushnir and Subers, 1964).

1.2.4. Floral Source-Associated Toxic Substances

The belief that some floral source-specific compounds might be present in honeys was supported by historical accounts of toxic reactions from the ingestion of certain types of honeys (Palmer-Jones, 1947; 1965), especially honey from the *Ericacea* spp. (White, 1975a). Scott *et al*. (1971) have reported the isolation of toxic substances (*e.g.* grayanotoxins) from toxic British Columbia honey samples, however they were not able to identify the origin of the compounds.

In New Zealand, honey from the Coromandel district, collected during the blooming period of *Coriaria arborea* (tutu, tutin) was found to be periodically toxic during late 1940s. For example, Palmer-Jones *et al.* (1947) described an outbreak of honey poisoning during which the periodic toxicity of tutin honeys and tutin plant parts was correlated with oral toxicity results observed using guinea pigs. Chemical isolation revealed tutin to be the poisonous substance in toxic tutin honeys. Palmer-Jones *et al*. (1947) also isolated a trace of tutin, and a considerable amount of hyenanchin (mellitoxin) (Figure 1.1), from *C. arborea* honeydew. Later, Hodges and White (1966) isolated tutin and hyenanchin from a toxic honey.

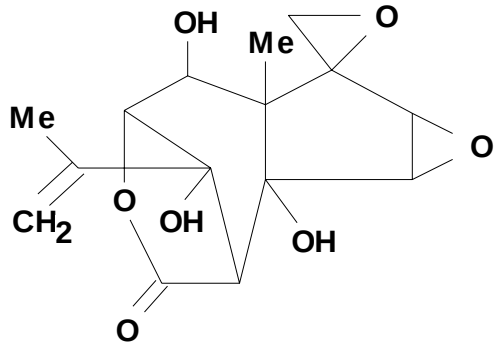


Figure 1.1. Chemical structure of hyenanchin (mellitoxin).

Clinch *et al*. (1968) estimated the levels of toxins in 150 honey samples collected from hives during the period 1962-1967 using oral dosing of guinea pigs, intracerebral injection of mice, and thin-layer chromatography. Samples found to be toxic by the mouse test (Clinch, 1966) showed spots corresponding to hyenanchin alone, or both hyenanchin and tutin. Some indications of other similar substances were obtained.

Blunt *et al.* (1979) reported ^{13}C NMR spectroscopic data for tutin and some related, less toxic, substances. ^{13}C NMR micro-sample probe techniques were used to show the presence of traces of dihydrotutin and dihydrohyenanchin in toxic honeydew honeys (Blunt *et al.*, 1979). In a more recent study, Love *et al.* (1986) reported an HPLC method for the determination of tutin and hyenanchin in toxic honeys.

1.3. Development of GC/FID and GC/MS Methodologies

Various investigators, including Tillmans and Kiesgen (1927), Lothrop (1932), Dorrscheidt and Freidrich (1962) have suggested chemical analysis of minor organic compounds may be useful in characterising or fingerprinting honey samples. The development of gas chromatography in the 1960s led to increased interest in fingerprinting techniques, since gas chromatography enabled the components of complex mixtures to by identified *in/situ*. Bonaga and Giumanini (1986) have suggested that some of the chemical substances in honey, which originate from a particular floral source, may correlate with nectar components, or be derived from them by biochemical modifications carried out by the bee.

The advent of high performance capillary columns and the increased sensitivity of modern mass spectrometers have greatly facilitated the development of head space and extractable organic finger-printing methodologies.

1.3.1. Head Space Methods

Deshusses and Gabbai (1962) used thin-layer chromatography to examine the diethyl ether extract of the vacuum distillate from 3 kg of honey, however little useful information was obtained. Later Deshusses and Gabbai (1962) analysed honey samples by the direct injection in the vapour phase above the honey samples into a gas chromatograph (GC) fitted with a packed column and flame ionization detector (FID). They suggested that the resulting chromatograms might serve as 'fingerprints' for the identification of the honey types. Hoopen (1963) reported the results of an investigation in which volatile carbonyl compounds were converted to the corresponding dinitrophenylhydrazones and analysed using column and thin-layer chromatography and GC methods.

Cremer and Reidmann (1964; 1965) subsequently reported the results of investigations performed using a gas chromatograph fitted with a capillary column instead of a packed column. They identified 22 volatile compounds, amongst which only three compounds (formaldehyde, propionaldehyde and acetone) were common to all honey samples.

Bicchi *et al*. (1983) extracted the volatile components of Italian Piedmont honey using four different methods. For example, honey was extracted for 2 h with Me_2CO (3 cycles), after which the combined extracts were concentrated, volatile compounds were recovered by vapour-distillation using CH_2Cl_2 for 5 h. The CH_2Cl_2 extracts were concentrated and analysed by gas chromatography using an OV-1 capillary column. Fifty-two volatile compounds were identified.

Bouseta *et al.* (1992) characterised 47 volatile compounds in 84 unifloral honeys (from 14 unifloral sources in 10 countries) using a dynamic headspace GC/MS system. Bouseta *et al.* (1992) pointed out some compounds (such as caproaldehyde, acetone, diketones, sulfur compounds and alkanes) appeared to be characteristic of the floral source and proposed that some of the identified compounds (*e.g.* branched aldehydes,

furan derivatives) reflected the microbiological purity and processing and storage conditions of the honeys, rather than their floral origins.

Overton *et al*. (1994) used a purge-and-trap (P and T) thermal desorption technique for identification and quantification of volatile and semi-volatiles in honeys. The adsorbent traps were analysed by thermal desorption-gas chromatography-mass spectrometry (TD/GC/MS). The P and T technique can be used to analyses a wider range of both volatile and semi-volatile organic compounds. This technique is more sensitive, by a factor of at least 100, compared to the headspace technique (Overton *et al.*, 1994).

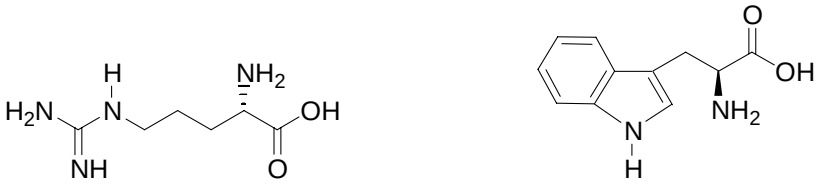
1.3.2. Extractable Organic Compounds

Amino Acids as Floral Source Marker Compounds

Early work was directed towards the possibility that amino acids (Figure 1.2) might serve to characterize the floral sources from which honeys are derived. For example, Davies (1975; 1976) attempted to correlate floral origin with the free amino acids composition. Although these investigations showed that nectar and honeydew amino acid profiles persisted in honeys, the results did not identify any simple relationships between floral sources and the resulting honey. Kanematsu *et al*. (1982) determined the levels of free amino acids in 112 samples of honey from 15 countries (*e.g.* Japan and Europe) and showed that free amino acid patterns and certain ratios (*e.g.* proline/phenylalanine) could be used to characterise the floral origins of honey. Bonanga and Guimanini (1986) attempted to correlate free amino acids and floral sources. Steeg and Montag (1987) used combined capillary GC/MS to detect phenylalanine decomposition products in honeys derived from various floral sources.

Recently, Pirini *et al*. (1992) suggested that the presence of arginine is an important discriminating factor useful for the characterization of chestnut honey, whereas acacia honey only contains tryptophan. Pirini *et al*. (1992) also reported that the proline content of chestnut honey is higher than that of the other honeys analysed. Bouseta *et al*. (1996) found higher concentrations of proline and phenylalanine in lavender honeys than in eucalyptus honeys. In addition, a higher content of amino acids were found in thyme

honeys than in castanea honeys, which in turn were richer in amino acids than eucalyptus honeys (Conte *et al*., 1998).



arginine tryptophan

proline phenylalanine

Figure 1.2. Some of amino acids found in honeys (arginine, tryptophan, proline and phenylalanine).

On the basis of the results obtained for 56 honey samples from three Argentinian regions, Cometto *et al*. (2003) suggested that free amino acids profile can be used to verify the floral, or geographical, origin of a honey. Cometto *et al*. (2003) suggested that the amino acid profile is characteristic of the flora in the immediate vicinity of the apiary rather than the overall floral characteristic of the geographic region from which the honey was collected.

Iglesias *et al*. (2004) determined the free amino acid content of 46 honey samples (vipers bugloss, multiflower, heather, rosa bush, rosemary and honeydew honey samples) collected from central Spain. Glutamic acid and tryptophan were considered to be good floral source and/or honeydew marker substances.

Amino Acids as Geographical Indicators

Bosi and Battaglini (1978) and Gilbert *et al*. (1981) were able to distinguish the geographical origin of some European honeys on the basis of free amino acids composition. Gilbert *et al*. (1981) determined the levels of 17 amino acids in 45 samples

of honey obtained from the United Kingdom, Australia, Argentina and Canada using gas chromatography and found that some groups of honey samples originating from different countries could be distinguished from each other on the basis of their amino acid profiles. Davies *et al*. (1982) investigated the amino acid content of 36 English, Welsh and foreign honeys and also found that amino acid levels could be used to distinguish British and foreign honeys. Cometto *et al*. (2003) found that under some circumstances (see above) amino acid profiles can be used to verify the geographical origin of Argentinian honeys.

Amino Acids (Biochemical Modification)

Bonanga and Guimanini (1986) attempted to determine if honey amino acid profiles could be correlated with biochemical modification(s) of amino acids carried out by honeybees, however no such correlations were identified.

Methyl Anthranilate (2-Aminobenzoic Acid Methyl Ester)

Recent proposals that classes of compounds, other than amino acids, might be floral source markers were in part prompted by the early accounts of the occurrence of methyl anthranilate (Figure 1.3) in orange blossom and some other citrus type honeys (Deshusses and Gabbai, 1962; White, 1966). Methyl anthranilate was one of the first compounds to be identified as a possible floral source marker compound in citrus type honeys (Deshusses and Gabbai, 1962).

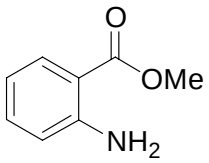


Figure 1.3. Chemical structure of methyl anthranilate.

Deshusses and Gabbai (1962) and White (1966) investigated levels of methyl anthranilate in Spanish orange flower honey and in some European citrus honey respectively. Knapp *et al*. (1967) also suggested that the level of methyl anthranilate in honeys could be used as a possible characteristic for distinguishing citrus honeys. This compound is known to impart a distinctive aroma to citrus honeys. In a later

investigation, methyl N-methylanthranilate was detected in some Japanese citrus honeys (Tsuneya *et al*., 1988).

Serra-Bonvehi *et al.* (1995) have reported the content of methyl anthranilate in Spanish blossom honey to be in the range 1.8-3.6 mg/kg. When stored, this level decreases by about 9% per month. Ferreres *et al*. (1994) determined methyl anthranilate in 18 citrus honey samples produced in Mediterranean Spain and, in accord with the conclusions of earlier workers, reported that this compound was the dominant marker in citrus honeys.

More recently, Nozal *et al*. (2001) have determined methyl anthranilate levels in some Dutch (Netherland) honeys while Sawai *et al*. (2004) have found methyl anthranilate in honeys derived from Japanese cultivars of varieties (var. *assamica*).

Other Extractable Organic Compounds

In the 1980's several accounts of the recovery of extractable compounds from honeys appeared, including studies reported by Graddon *et al.* (1979), Steeg and Montag (1987a; 1987b; 1988). These studies prompted the suggestion that floral source compounds might occur in New Zealand native honeys (*e.g.* manuka, kanuka and rewarewa honeys). Subsequently, in the late 1980's, Waikato University researchers developed an analytical procedure, which was used to determine the extractable organic fingerprints of some New Zealand unifloral honeys (Tan *et al*., 1989). The procedure involved solubilization of a honey sample in distilled water, followed by liquid/liquid extraction with diethyl ether, concentration of the extracts, methylation using diazomethane and GC/FID or GC/MS analyses.

1.3.3. Extractable Organic Substances from New Zealand Unifloral Honeys

The diethyl ether extracts of a variety of New Zealand unifloral grade honeys, including white clover, manuka, kanuka, vipers bugloss, noddling thistle, rewarewa, ling-heather, thyme, willow and kamahi honeys have been reported in theses by Tan (1988), Vallentine (1992), Hynik (1998), Sun (1995) and Broome (1998), and in a series of

papers (Tan *et al.*, 1988; Wilkins *et al.*, 1993a; 1993b; 1995a; 1995b). More than 200 GC volatile extractable organic compounds (mainly aliphatic, aromatic, and degraded carotenoid-like substances) were identified in these investigations.

These studies identified some substances which were proposed as floral markers for unifloral grade New Zealand honeys. These substances, and typical levels of these compounds, are listed in Table 1.1. Other honeys, such as clover honey, were found to be characterised only by the presence of low levels of extractable organic substances which did not serve to characterise the floral source.

New Zealand Manuka (Leptospermum scoparium) and Kanuka (Kunzea ericoides) Honeys

Previous investigations of the organic compounds present in the diethyl ether extracts of New Zealand unifloral manuka (*Leptospermum scoparium*) honey have revealed the presence of a range of substances which appear to floral source specific (Tan *et al*., 1988; 1989a; Wilkins *et al*., 1993a).

In addition to the prominent marker compounds listed in Table 1.1, an array of other acids or neutral organic compounds including, 4-methoxyphenaldehyde, 3,4-dimethoxy-phenaldehyde, acetophenone, 2-methoxyacetophenone and the methyl esters of benzoic acid, 3,4-dimethoxybenzoic acid and 3-(3',4'-dimethoxyphenyl) *trans*-prop-2-enoic acid were also detected in the methylated diethyl ether extracts of manuka honeys (Tan *et al*., 1988; 1989a; Wilkins *et al*., 1993a).

Manuka and kanuka (*Kunzea ericoides*) are members of the same family and genus and their chemical profiles cannot be distinguished (Tan *et al*., 1988).

Table 1.1. Substances proposed as floral markers for New Zealand unifloral grade honeys.

honey type	possible marker compounds	range (mg/kg)
manuka[a]	phenyllactic acid	420-1120
	4-methoxyphenyllactic acid	30-46
	methyl syringate	8-230
	2-methoxyacetophenone	10-40
	2-methoxybenzoic acid	2-40
vipers bugloss[b]	1,4-dihydroxybenzene	16-28
noddling thistle[c]	(*E*)-2,6-dimethyl-6-hydroxy-2,7-octadienoic acid	6-30
	(*E*)-2,6-dimethyl-3,7-octadiene-2,6-diol	1-6
	(*Z*)-2,6-dimethyl-6-hydroxy-2,7-octadienal	2-11
	(*Z*)-2,6-dimethyl-2,7-octadiene-1,6-diol	2-12
	α,5-dimethyl-5-ethenyl-2-tetrahydrofuran acetaldehydes (lilac aldehydes)	1-5
	β,5-dimethyl-5-ethenyl-2-tetrahydrofuran ethanols (lilac alcohols)	1-3
rewarewa[d]	2-methoxybutanedioic acid	2-3
	4-hydroxy-3-methyl-*trans*-2-pentenedioic acid	1-4
ling-heather[e]	4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one	100-180
	4-(3-oxo-1-butenylidene)-3,5,5-trimethyl-cyclohex-2-en-1-one	27-36
	4-hydroxy-4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one	30-60
	4-hydroxy-4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one	1-2
	4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one	1-2
thyme[f]	3,5,6-trihydroxy-5,6-dihydro-β-ionone	25-110
	3-hydroxy-5,6-epoxy-5,6-dihydro-β -ionone	1-5
	3-hexanoic acid	4-8
	3-aminoacetophenone	1-5
willow[g]	*trans*,*cis*-abscisic acid	100
	trans,*trans*-abscisic acid	40
	2-methoxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione	1
kamahi[h]	kamahines A-C[i]	10-150
	meliracemoic acid[j]	0.5-10
erica[k]	ericinic acid	120

[a]Tan *et al*., 1988; [b]Wilkins *et al*., 1993a; [c]Wilkins *et al*., 1995b; [d]Wilkins *et al*., 1993b; [e]Wilkins *et al*., 1995a; [f]Tan *et al*., 1989a; [g]Tan *et al*., 1990; [h]Tan, 1989; [i]Broom, 1998; [j]Ede *et al*., 1993; [k]Hyink, 1998.

New Zealand Thyme (Thymus vulgaris) Honeys

Cyclohexanetriol derivatives (*e.g.* 3,5,6-trihydroxy-5,6-dihydro-β-ionone), 3-hexenoic acid and 3'-aminoacetophenone (Table 1.1) were considered to be indicative of thyme (*Thymus vulgaris*) honey (Tan, 1989; Tan *et al*., 1990). Other components found in thyme honey were aliphatic-diacids, pyridinecarboxylic acid and abscisic acid isomers (Tan *et al*., 1989a).

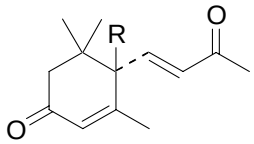
New Zealand Kamahi (Weinmannia racemosa) Honeys

Several components detected in extracts of New Zealand kamahi (*Weinmannia racemosa*) honey were suggested to be degraded carotenoids (Tan, 1989). Tan (1989) demonstrated that the extracts of kamahi honey contained a number of apparently related compounds of unknown structure, which exhibited mass spectra similar to that determined for 3,5,5-trimethylcyclohex-2-en-1-one.

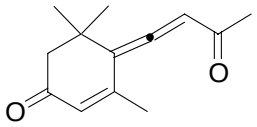
Subsequently, Broome *et al*. (1992; 1994) elucidated the structures of kamahines A-C and Ede *et al*. (1993) identified a novel nor-sesquiterpenoid, meliracemoic acid. A number of other studies have extended the list of extractable organic compounds present in New Zealand kamahi honeys (Tan, 1989; Hyink, 1998) (Table 1.1).

New Zealand Ling-Heather (Calluna vulgaris) Honeys

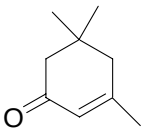
Tan *et al*. (1989b) has reported that New Zealand ling-heather (*Calluna* spp.) honey is characterised by the presence of some degraded carotenoid-like compounds (Table 1.1), the most dominant of which is 4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one (dehydrovomifoliol) (Figure 1.4). Hausler and Montag (1989; 1991) have reported that a series of degraded carotenoid-like compounds are present in European heather honeys (see Section 1.3.4). In addition, appreciable levels of an array of early eluting substances including benzoic acid, phenylacetic acid and phenyllactic acid (2-hydroxy-3-phenylpropionic acid) were identified in heather honey extracts, together with very low levels of *trans*,*cis* and *trans*,*trans*-abscisic acids (Tan, 1989a).



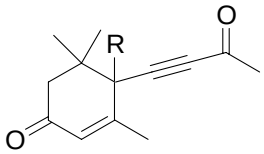
R = OH: dehydrovomifoliol
R = H: 3-oxo-α-ionone



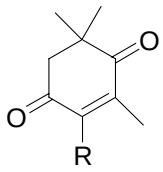
4-(3-oxobut-1-enylidene)- 3,5,5-trimethylcyclohex-2-en-1-one



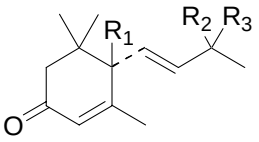
3,5,5-trimethylcyclohex-2-en-1-one



R = OH: 4-hydroxy-4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one
R = H: 4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one



R = H: 3,5,5-trimethylcyclohex-2-ene-1,4-dione
R = OCH_3: 2-methoxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione

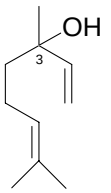


$R_1 = R_2 = OH$, $R_3 = H$ (or $R_1 = R_3 = OH$, $R_2 = H$) (isomers): 4-hydroxy-4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one.

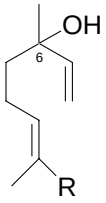
Figure 1.4. Chemical structures of some compounds (degraded carotenoids) found in extracts of New Zealand ling-heather honeys (Tan *et al*., 1989b).

New Zealand Noddling Thistle (Carduus nutans) Honeys

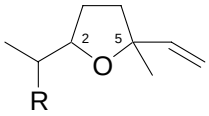
A series of linalool derivatives have been identified in the diethyl ether extracts of New Zealand nodding thistle (*Carduus nutans*) honeys (Tan *et al*., 1989; Wilkins *et al*., 1993b). Wilkins *et al*. (1993b) reported that some of the linalool derivatives (Figure 1.5) appeared to be unique to noddling thistle honeys. In addition to these compounds, an array of aromatic acids including benzoic acid, phenylacetic acid, phenyllactic acid, methyl syringate, *cis* and *trans* cinnamic acid isomers were detected in the extracts of nodding thistle honeys, together with an array of common fatty acids (*e.g.* myristic and palmitic acids) and diacids (*e.g.* hexanedioic and octanedioic acids) (Wilkins *et al*., 1993b).



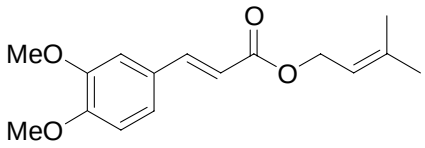
linalool
(3,7-dimethyl-1,6-octadiene-3-ol)



R = CH_2OH: 2,6-dimethyl-2,7-octadiene-1,6-diol
R = CHO: 2,6-dimethyl-6-hydroxy-2,7-octadienal
R = $COOCH_3$: methyl 2,6-dimethyl-6-hydroxy-2,7-octadienoate



R = CH_2OH: lilac alcohol
R = CHO: lilac aldehyde
R = $COOCH_3$: lilac methyl ester



prenyl 3-(3′,4′,-dimethoxyphenyl)-*trans*-propenoate

Figure 1.5. Chemical structures of some compounds found in extracts of New Zealand noddling thistle honeys (Wilkins *et al*., 1993b).

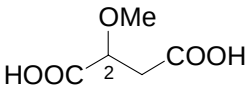
New Zealand Vipers Bugloss (Echium vulgare) Honeys

In addition to the marker compounds listed in Table 1.1, the most dominant of which was 4-hydroxyphenol, Tan (1989) and Wilkins *et al*. (1995a) noted the presence of lesser levels of 2,5-cyclohexadiene-1,4-dione (benzoquinone) and a number of *para*-disubstituted aromatic compounds (*e.g.* 4-methoxyphenol, 4-methoxyphenaldehyde and methyl 4-methoxybenzoate). Low levels of phenaldehyde, methyl benzoate, phenylacetaldehyde and array of aliphatic fatty acids and diacids (*e.g.* octanedioic and nonanedioic acids) together with moderate levels of C_{10}-semialdehydes were also detected in the extracts of viper bugloss honeys (Wilkins *et al*., 1995a).

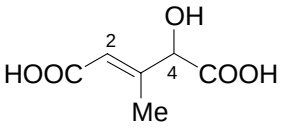
New Zealand Rewarewa (Knightia excelsa) Honeys

In addition to the major floral source related compounds reported in Table 1.1, the most dominant of which were 2-methoxybutanedioic acid and 4-hydroxy-3-methyl-*trans*-2-pentenedioic acid, thirty-two aliphatic dicarboxylic acids ranging in size (molecular weight) from malonic acid to tridecanedioic acid were detected at levels which ranged from 64 to 111 mg/kg (Wilkins *et al*., 1995b). Compounds identified included

butanedioic acid, decanedioic acid, pentanedioic acid, octanedioic acid, 2-decanedioic acid, 2-methoxybutanedioic acid, 2-furancarboxylic acid, benzoic acid and phenylacetic acid (Wilkins *et al*., 1995b).



2-methoxybutanedioic acid
(2-methylmalic acid)



4-hydroxy-3-methyl-*trans*-2-pentenedioic acid

Figure 1.6. Chemical structures of some compounds found in extracts of New Zealand rewarewa honeys (Wilkins *et al*., 1995b).

1.3.4. Extractable Organic Compounds from European Honeys

Speer and Montag (1984) have proposed that heather honey could be distinguished from other honeys by their high phenylacetic acid and benzoic acid levels, while Hausler and Montag (1989; 1991) have reported the presence of degraded carotenoid-like substances (*e.g.* 4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-ene-1-one ((S)-(+)-dehydrovomifoliol)) in Spanish, German and French heather (*Calluna* spp.) honeys.

More recently, Ferreres *et al*. (1996) have proposed ellagic acid and *cis,trans*-abscisic acid and *trans,trans*-abscisic acid as the main floral marker compounds in Portuguese heather honeys derived from (*Erica arborea*) rather than (*Calluna* spp.). Guyot *et al*. (1999) have also investigated floral marker compounds in *Calluna vulgaris* and *Erica arborea* heather honeys and they found benzoic acid and decanoic acid were the indicators of *Ericacea* family honeys. They also reported that phenylacetic acid, dehydrovomifoliol, and 4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one as specific markers of honeys derived from *Calluna vulgaris*.

Speer and Montag (1987) used a GC/MS method to quantitatively determine benzoic acid, phenylacetic acid and other compounds believed to be degradation products of phenylalanine in 4 acacia, 4 lime, 5 clover, 4 rape, 2 dandelion, 2 Tasmanian

leatherwood, 4 chestnut and 27 heather honeys from different countries. Later, Steeg and Montag (1988) also used GC/MS to determine the levels of minor extractable organic substances (aldehydes, alcohol, phenols etc.) in buckwheat, dandelion, rape, heath, forest, and conifer honeys.

Guyot *et al.* (1998) have reported that European chestnut honey is characterised by high concentrations of acetophenone, 1-phenylethanol and 2-aminoacetophenone while lime tree honey is characterised by the presence of an unidentified ethylmethylphenol isomer, 4-*t*-butylphenol, estragole and 4-methylacetophenone. Homogentisic acid (2,5-dihydroxyphenylacetic acid) was also identified as a marker substance for Italian strawberry-tree (*Arbutus unedo*) (Guyot *et al.*, 1998). Giuseppe *et al.* (2000) have reported the presence of a series of 3-keto C_9-, C_{10}- and C_{13}-norisoprenoids in strawberry-tree honeys.

3-Methylbutanal, 3-hydroxy-4,5-dimethyl-2(5H)-furanone and (*E*)-*β*-damascenenone have been identified as the major characteristic aroma-active components of buckwheat honey (Zhou *et al*., 2002).

1.3.5. Extractable Organic Compounds from Some Japanese Unifloral Honeys

In the 1950's the levels of pantothenic acid in some Japanese honeys was determined by Watanabe *et al*. (1956; 1959). Nitrogen containing compounds have been detected as aroma compounds in some unifloral Japanese honeys. For example, N-β-phenylethylformamide and N-β-phenylethyl-2-formylpyrrole have been detected in shina and clover honey aromas, N-β-phenylethylacetamide was detected in shina, mikan, and clover honey aromas, indole and methyl anthranilate were detected in mikan and tochi honey aromas (Tsuneya *et al*., 1988).

1.3.6. Extractable Organic Compounds from Some Australian Unifloral Honeys

In an early investigation, Graddon *et al.* (1979) identified more than 100 volatile constituents in some unifloral Australian honeys using capillary GC/MS. A surprising range of hydrocarbons and oxygenated compounds were found and some of them were

believed to floral source unique. More recently D'Arcy *et al*. (1997) have reported the results of investigations in which Australian blue gum (*Eucalyptus leucoxylon*) and yellow box (*Eucalyptus melliodora*) honeys were extracted by stirring with portions of ethyl acetate, and the resulting extracts were analysed using GC/MS. D'Arcy *et al*. (1997) found 55 compounds including norisoprenoids, monoterpenes, benzene derivatives, aliphatic compounds and Maillard reaction products, and 13 of them were quantitatively identified for the first time in honeys.

Yao *et al*. (2003) suggested that some phenolic acids (gallic and coumaric acids) and abscisic acid could be useful for the identification of honey floral origin of Australian Leptospermum honeys (jelly bush honeys). Yao *et al*. (2004a) also investigated Australian monofloral Eucalyptus honeys (black box, bloodwood and stringybox honeys) and determined the levels of phenolic acids, including gallic, chlorogenic, coumaric and caffeic acids, and two abscisic acid isomers in these honeys. Yao *et al*. (2004a) have concluded that HPLC procedures, like GC/MS methods, can be used to identify honey floral sources.

1.3.7. Flavonoids

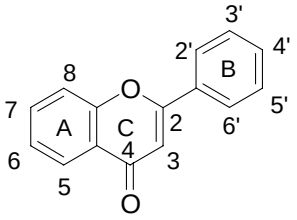
Previous studies have shown that flavonoid analysis could lead to the determination of the floral and geographical origin of honey (Amiot *et al*., 1989; Ferreres *et al*., 1991; Sabatier *et al*., 1992). Berahia *et al*. (1993) found pinocembrin as the major flavonoid in sunflower honeys collected from France and they also reported that GC/MS provided flavonoid patterns, which could be useful in the determination of the floral origin.

In another study, Ferreres *et al*. (1993) found that hesperetine (hesperetin-7-rutinoside) was the major flavonoid detected in citrus honey and suggested that this compound could be a useful floral marker for citrus honey. Ferreres *et al*. (1994) also suggested that the most characteristic flavonoids found in Portuguese (*Ericaceae*) heather honey, namely myricetin (3,5,7,3',4',5'-hexahydroxyflavone), myricetin 3'-methyl ether,

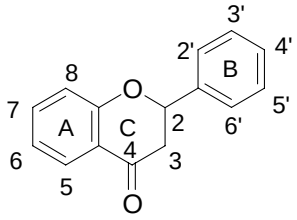
myricetin 3-methyl ether and tricetin, could probably be used as markers for the botanical origin of this honey.

Martos *et al*. (2000) and Yao *et al*. (2004b) found tricetin (5,7,3',4',5'-pentahydroxyflavone), quercetin, luteolin (5,7,3',4'-tetrahydroxyflavone), myricetin and kaempferol to be present in Australian monofloral Eucalyptus honey and suggested that this flavonoid profile could be used to verify the botanical origin of Eucalyptus honey. On the other hand, Yao *et al*. (2003), found myricetin, luteolin and tricetin to be the main flavonoids in Australian jelly bush honey (*Leptospermum polygalifolium*).

More recently, Yao *et al.* (2004c) have investigated the flavonoid composition of Australian tea tree, crow ash, brush box, heath and sunflower honeys and found these compounds could be useful floral markers for these honeys. For example, myricetin, tricetin, quercetin, luteolin and an unknown flavonoid were found to be the main flavonoids in Australian crow ash honey, while quercetin, quercetin 3,3'-dimethyl ether, myricetin and luteolin were characteristic only for the Australian sunflower honey.



Flavone



Flavanone

Quercetin: 3, 5, 7, 3′, 4′ = OH
Luteolin: 5, 7, 3′, 4′ = OH
Kaempferol: 3, 5, 7, 4′ = OH

Pinocembrin: 5, 7 = OH
Hesperetin: 5, 7, 3′ = OH; 4′ = OCH_3
Myricetin: 3, 5, 7, 3′, 4′, 5′ = OH

Figure 1.7. Flavonoids found in some Australian honeys (courtesy of http://www.fst.uq.edu.au/staff/bdarcy/honey).

1.4. Antibacterial Properties of Honey

The natural antibacterial property, one of the intrinsic features of honey, has long been recognized *in vivo* (Aristotle, 350 B.C). A Dutch scientist, Van Ketel, demonstrated that honey has antibacterial effects for the first time in 1892 (Dustmann, 1979). *In vitro*

studies have identified different aspects of the antibacterial properties of honey including those attributable to low pH (acidity), high osmolarity (or high sugar content) (Sakett, 1919), the presence of hydrogen peroxide (White *et al*., 1962; 1963a; 1963b; 1964a; 1964b) and compounds derived from floral sources (Verge, 1951; Schuler and Vogal, 1956). Two types of antibacterial agents have been recognized, namely; heat and light sensitive agents (*e.g.* hydrogen peroxide produced by honey glucose oxidase), and heat and light stable non-peroxide agents (residual antimicrobial activity when hydrogen peroxide is destroyed by addition of a catalase during assay).

In an extensive study of the antibacterial activity of unifloral New Zealand honeys performed at The University of Waikato, some manuka (*Leptospermum scoparium*) honeys display antibacterial activity which was significantly greater than that which could be ascribed merely to the acidity, high osmolarity and the presence of hydrogen peroxide (Russell, 1983; Molan and Russell, 1988; Tan *et al*., 1988; Russell *et al*., 1990).

1.4.1. Possible Sources of Non-peroxide Antibacterial Agents in Manuka Honey

There are several sources from which the non-peroxide antibacterial agents could originate. These include the floral source (nectar), honeydew, beeswax, propolis, or the metabolism (modification) of substances by the bee.

Floral Source

Molan and Russell (1988) proposed that the non-peroxide antibacterial activity of honeys might be due to honey components derived from the floral source while this deduction was supported by the observation that only some of the manuka honeys collected from specific regions, particularly the East Coast region of the North Island of New Zealand, possessed non-peroxide antibacterial activity (Molan, 1995) (Figure 1.8).

Perry *et al*. (1997) have described three chemotypes of New Zealand manuka (*Leptospermum scoparium*), namely pinene rich, sesquiterpenes rich and leptospermone (triketone) rich chemotypes, based on chemical analyses of the essential oil steam distilled from the leaves of manuka plants.

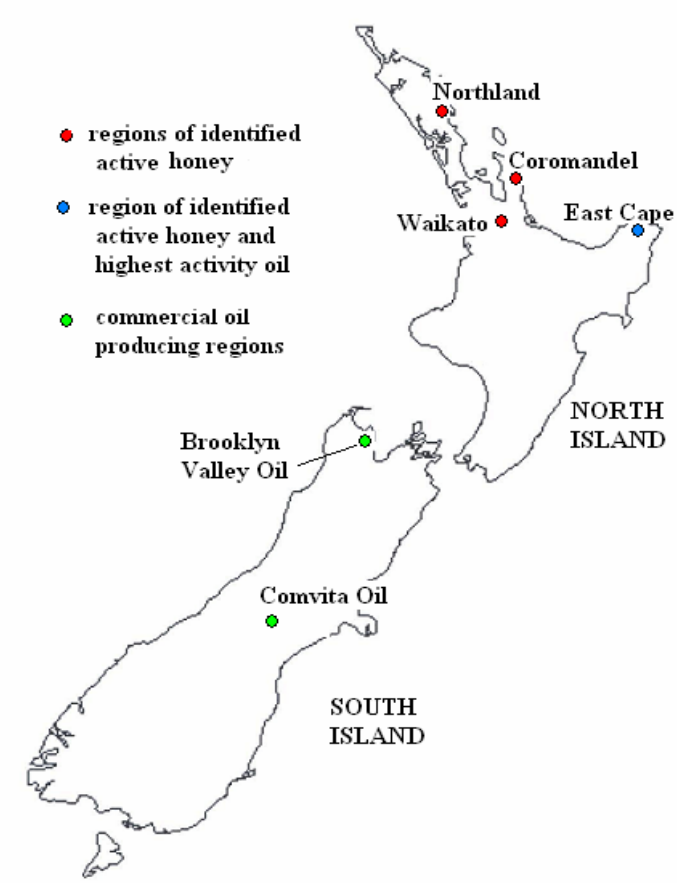


Figure 1.8. The regions of New Zealand showing the origin of active manuka oils and non-peroxide antibacterial active manuka honeys.

Porter and Wilkins (1998) subsequently described five chemotypes, including three described by Perry *et al*. (1997) together with eudesmol rich and linalool rich variants. The chemotype with a high proportion of leptospermone occurs almost exclusively in the East Coast region. The triketone rich oil derived from the East Coast chemotype has the greatest antimicrobial activity of the five chemotypes described by Porter and Wilkins (1998).

In an endeavour to link essential oil activity and honey antimicrobial of manuka honeys, Tan *et al*. (1988) and Wilkins *et al*. (1993a) investigated the possible occurrence of leptospermone and related triketones in manuka honeys, however leptospermone was not identified as component of the diethyl ether extracts of any honeys (including those from the East Coast). Weston *et al*. (2000) also attempted to detect leptospermone in a

manuka honeys with high non-peroxide activity, using three methods [chromatography on XAD-2 resin; extraction with 2-butanol; and liquid/liquid extraction with diethyl ether], but failed to detect leptospermone in any of the extracts. Weston *et al*. (2000) suggested that because of the insolubility of leptospermone in water it was not likely that it would be present in nectar, and therefore it would not be found in honeys.

Honeydew

Tan *et al*. (1988) showed that some of the distinctive extractable aromatic components, such as phenyllactic acid, 4-methoxyphenyllactic acid, 2-methoxybenzoic acid and 2'-methoxyacetophenone, found in manuka honeys were not present in extracts of manuka flowers and suggested that they may have been derived from the honeydew which characteristically coats the trunks of manuka trees, since manuka is frequently infested by scale insects (*Eriococcus orariensis* and *Coelostomidia* spp.), which produce a sticky honeydew secretion (Figure 1.9). None of the foregoing compounds are considered to be responsible for the antibacterial activity of manuka honey, although the possibility that the might have a synergic effect cannot be excluded.

Figure 1.9. The scale insect *Coelostomidia* spp. which produces a sticky honeydew secretion on manuka plants (courtesy of Airborne Honey Ltd, 2003).

Wax, Propolis and Bee Metabolites

Bogdanov (1997) suggested from his experiments with sugar-adulterated honey that a major part of the non-peroxide antibacterial activity was of bee origin. In addition, Russell *et al*. (1990) suggested that other sources of the aromatic acids could be due to residual pollen present in the honey or absorbed into beeswax, both of which have been found to have some non-peroxide antibacterial activity (Lavie, 1960). Flavonoids, which are major constituents of propolis, are known to possess a range of biological activities, (see below) however they do not appear to be responsible for the non-peroxide activity of manuka honeys.

1.4.2. Previous Attempts to Identify Non-peroxide Antibacterial Compounds in Manuka Honey

Despite many years of research, the major non-peroxide antibacterial components of manuka honey have not been isolated or identified, other than for possible low level contributions from some aromatic components such as methyl syringate (Molan and Russell, 1988) (see Section 7.2.2), to date efforts by Waikato University researchers, Bogdanov (1997), Weston (2000) and others have also failed to identify active substances.

Aromatic and Phenolic Compounds

Russell *et al*. (1990) attempted to isolate some of the components responsible for the antibacterial activity of manuka honey. Bioassay-directed separation of the diethyl ether extracts of some active manuka honeys led to the identification of methyl syringate (4-hydroxy-3,5-dimethoxybenzoic acid methyl ester), and 3,4,5-trimethoxybenzoic acid in one of the active fractions, however these compounds were found to be only minor contributors to the observed activity. The levels of these compounds in manuka honeys were less than those of some other aromatic acids such as phenyllactic acid and 4-methoxyphenyllactic acid (Wilkins *et al*., 1993a).

Later, Weston *et al*. (1999) found that methyl syringate and phenyllactic acid failed to inhibit the growth of the bacteria at levels of 0.04-1.6 mg/disk, and suggested

that this may be due to their insolubility in the aqueous diffusion medium. However, while these substances exhibited some antibiotic activity towards a range of bacteria, they are not likely to be the compounds which are responsible for the high residual non-peroxide antibacterial activity of the manuka honeys since they also occur in several European honeys (Steeg and Montag, 1987), none of which have been reported to exhibit non-peroxide antibacterial activity.

The possibility that flavonoids present in manuka honey might be responsible, at least in part, for the non-peroxide antibacterial activity of manuka honey has been investigated. Markham *et al*. (1996) showed that pinocembrine, pinobanksin, chrysin and flavonone, (Figure 1.7) were present in propolis from New Zealand hives, including propolis recovered from manuka honey hives. Some flavonoids have been reported to contribute to the antibacterial activity of propolis (Marcucci, 1995). Weston *et al*. (1999) has reported that phenolic components including phenolic derivatives of benzoic acids, cinnamic acids and flavonoids were not responsible for the non-peroxide antibacterial activity of manuka honey, however these compounds individually and collectively do exhibit some antibacterial activity.

Weston (2000) has reported that the HPLC chromatograms of the phenolic, flavonoid containing, fractions recovered from Amberlite XAD-2 resin chromatography of non-peroxide active and inactive manuka honeys were identical.

Carbohydrates

More than 95% of honey solids are carbohydrates. Weston and Brocklebank (1999) hypothesized that active manuka honey may have had a unique oligosaccharide similar to the tetrasaccharide known as sialyl Lewis X ((sLe^X): [Neu5Acα2-3Galß1-4(Fucα1-3)GlcNAc]) (Figure 1.10). This tetrasaccharide is the antigenic agent which mediates the adhesion of the bacterium, however Weston and Brocklebank (1999) found that the oligosaccharide composition of active and inactive manuka honeys were not different.

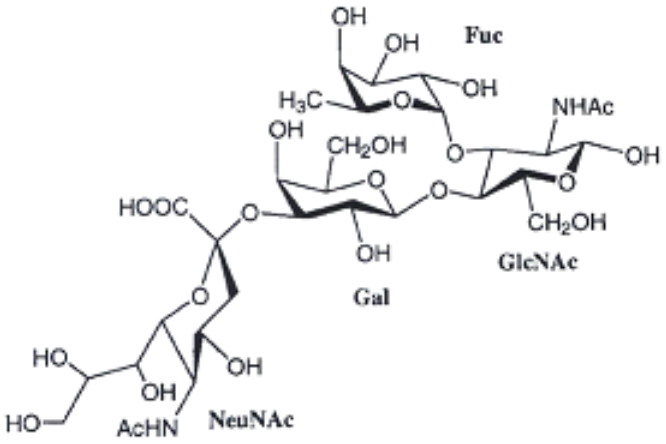


Figure 1.10. Chemical structure of the sialyl Lewis^X tetrasaccharide (Pichierri, 2002).

Weston *et al.* (1999) also concluded that all of the non-peroxide activity is associated with the carbohydrate fraction of the honey, other than monosaccharides (glucose and fructose) which have no antibacterial properties.

Peptides and Proteins

Other compounds which could contribute to the non-peroxide activity of manuka honey are antibiotic peptides (*e.g.* abaecin, apidaecin and lysozymes) present in the body fluid of bees which had been injured and injected by bacteria (Casteels *et al*., 1989; 1990; 1993; Fujiwara, *et al*., 1990; Hultmark, 1996). Weston *et al.* (2000) hypothesized that if peptides, which possess strong antibacterial activity, were present in honey they could contribute significantly to the non-peroxide activity of manuka honey, however these peptides only occurred in infected bee fluids and not in honeys.

Tan (1989) suggested that the antibacterial substances responsible for the additional activity in manuka honeys might not be detected in GC analysis since, either they were not volatile under the analysis conditions, or because the antibacterial substances were not extracted using diethyl ether. Tan (1989), Snow (2001) and Farr (2005) have found that most of the antibacterial activity is retained in the aqueous layer (*e.g.* it is not destroyed by the extraction procedure). Sealey (1988) has reported that the antibacterial substances in manuka honey have molecular weights of less than 1000 Daltons.

1.5. Objectives of This Research

The principal objectives of the present work were to:

(1) Produce publication standard accounts of the extractives of unifloral grade New Zealand kamahi and honeydew honeys, and New Zealand and Norwegian erica honeys,

(2) Determine if there was a statistical difference between the extractive organic profiles of non-peroxide active and inactive manuka honeys,

(3) Determine if leaf oil chemotype information, determined using the micro-scale technique developed by Brophy *et al*. (1989) provided data which was comparable to steam distillate data, and if the leaf oil profiles of manuka plants collected from areas which afforded active and inactive honeys were correlated.

Objective 1

Hitherto some data concerning extractable organic compounds present in kamahi (Broom *et al*., 1992; 1994), honeydew (Hyink, 1998) and erica (Hyink, 1998) honeys has been obtained using samples collected during the period 1984-2000, however a definitive account of the extractable organic substances present in these honeys, and the identification of possible marker compounds, has not been presented in the literature.

A major object of the present work was to obtain definitive, publishable quality data, using modern honey samples, supplied by either Airborne Honey Ltd., Leeston, Canterbury (honeydew and kamahi honeys), or sourced from New Zealand and Norwegian Bee-Keepers (erica honey).

Because New Zealand quarantine regulations prohibit the importation of foreign honeys, the Norwegian erica honey samples were extracted in Norway and the extracts (after confirmation of their non-biological nature) were forwarded to New Zealand for chemical analysis.

There is some confusion in the literature concerning the extractive and marker substance, which characterise heather honey, since heather honeys are often considered to be derived from either or both of *Calluna* and *Erica* spp. In New Zealand, ling-heather honey is produced from *Calluna vulgaris*, and has an extractable organic profile which differs from that of the single erica honey sample that has been examined to date (Hyink, 1998). A recent paper (Ferreres *et al*., 1996a) has reported data for some Spanish heather honeys derived from *Erica* spp. which differs from that previously reported for New Zealand (Tan *et al*., 1989b) and some other European (Steeg and Montag, 1984) Calluna heather honeys.

In particular, the proposal that abscisic acid isomers were marker substances for heather honeys (Ferreres *et al*., 1996) conflicts with other literature accounts of heather extractives (Steeg and Montag, 1984; Tan *et al*., 1989b; Guyot *et al*., 1999). It was therefore of interest to explore the possibility that the Spanish data might correspond with those determined for New Zealand and Norwegian honey derived from *Erica* spp. rather than *Calluna* spp.

Where appropriate quantities of substances sufficient for structure elucidation were isolated, one and two-dimensional NMR data were obtained. Kamahi, honeydew, New Zealand erica and Norwegian erica data are reported in Chapters 3, 4, 5 and 6 respectively.

Objective 2

Previous studies on the chemical profiles of manuka honey have shown that, non-peroxide active manuka honey profiles could not be distinguished from the inactive honey profiles (Tan, 1989; Weston, 2000). However in these studies, visual inspection of the GC/MS profiles of a series of active and inactive manuka honeys has been an increasing interest in finding active and inactive manuka honeys might differ to a degree that could be correlated with bioactivity. Although it is known that substances responsible for the non-peroxide activity of manuka honeys are not extracted into diethyl ether, the substances contributing to the GC/MS profiles might be considered as activity

related marker substances for the presence of unidentified compounds responsible for the non-peroxide activity.

The one of the principle attempts made in the present study is to determine if there are any significant differences between GC/MS profiles of active manuka honey and inactive manuka honey. The other important aim is to determine if a correlation exists between the non-peroxide activity (UMF values) and the levels of organic substances present in manuka honey.

Ninety-six active and inactive unifloral manuka honey samples were analysed to evaluate their content of various array of aliphatic and aromatic organic substances and the levels of these organic substances were correlated with UMF values using statistical analysis (*e.g*. multiple regression analysis). The use of statistical analysis to correlate UMF values and levels of organic substances of manuka honey is a novel approach and therefore this appears the first time such an analysis has been attempted for New Zealand manuka honeys. The results obtained from statistical analysis are discussed in Chapter 7.

Objective 3

It known many varietal forms of manuka exist, and that there are variations in their steam distilled leaf oil composition. It was of interest to ascertain if active manuka honeys were derived predominantly from a single manuka leaf oil chemotype, or from plants with differing leaf oil finger-prints. Hitherto a limiting factor in investigating this issue has been the requirement to collect a substantial amount of manuka leaf (*ca* 5 kg) in order to obtain a quantity of oil sufficient for GC/MS analysis. The possibility that the micro-scale technique by Brophy *et al.* (1989), which requires only 20 leaves, might be applied to New Zealand manuka (*Leptospermum scoparium*) in the same manner as it was originally applied to Australian tea tree leaf was therefore investigated. This investigation unexpectedly leads to the identification of a new nor-triketone chemotype, in addition to the well-known East Coast triketone chemotype (Perry *et. al*., 1997 and Porter and Wilkins, 1998).

Cluster analysis of leaf oil profiles determined using an adaptation of the Brophy micro-scale technique was undertaken (see Chapter 8).

Additional objectives were:

(i) To determine if the diethyl ether extraction, based on the procedure developed by Tan (1988) would adequately recover extractable organic compounds with high water affinities.

(ii) To determine if the GC/MS profiles of unifloral grade kanuka and manuka honeys could be distinguished.

Whilst overnight (16 h) extraction was considered by Tan (1988) to be an adequate time to fully recover extracts with both high and low water affinities, some evidence from recent observations have suggested that a longer extraction was required to quantitatively recover some aromatic acid compound such as benzoic acid. Investigation of this hypothesis showed that a longer extraction was indeed required to quantitatively recover this compound, and the extraction time was therefore extended to 24 h.

Kanuka and Manuka

A further secondary objective was to determine if the GC/MS profiles of unifloral grade kanuka and manuka honeys could be distinguished. Although botanically distinct, kanuka and manuka afford similar pollens, thus they cannot be distinguished by melissopalynology. During the course of the present investigation, 7 samples considered to be unifloral grade kanuka (sourced from an area where kanuka had been definitively identified as the dominant floral source) became available. Results are presented in Appendixes (i) & (ii).

Chapter Two

Methods, Materials and Modification of Extraction Procedure

Chapter Two

Methods, Materials and Modification of Extraction Procedure

2.1. Honey Samples

A total of 155 *Weinmannia racemosa* (kamahi), *Nothofagus solandri*/*Nothofagus fusca* (beech honeydew), *Erica arborea* (erica) and *Leptospermum scoparium* (manuka) honey samples were supplied by beekeepers or commercial companies. These samples were considered by the suppliers to be predominantly unifloral grade honeys. On receipt in the laboratory, samples were stored in the airtight plastic containers at refrigerator temperature (*ca* 5 ºC). Samples were extracted and analysed as described in Sections 2.2. and 2.4.

2.2. Honey Extractions

2.2.1. Standard Extraction Procedure

Honey samples were extracted using the methodology developed by Tan (1989) with the modifications described below. Typically, 5 g of honey (weighed accurately) was dissolved in 150 mL of distilled water in a 250 mL beaker, at room temperature, with the assistance of a magnetic stirrer for 10-15 min. and the resulting solution was transferred to a 250 mL continuous liquid-liquid extractor. The beaker was rinsed with 2 x 50 mL portions of distilled water and then with 2 x 50 mL portions of diethyl ether (boiling point 34.6 °C), each of which was also transferred to the liquid-liquid extractor. Diethyl ether was then added to the extractor, such that 150 mL of diethyl ether was typically present in the 250 mL rounded bottom flask. Typically a total of 300 mL of diethyl ether was required for each extraction.

For manuka honey samples 500 μL of a 5.0 mg/mL solution of *n*-heptadecanoic acid (17:0 fatty acid) in dichloromethane was introduced into the extractor as a surrogate (recovery) standard prior to commencement of the extraction. For other honey samples, 100 μL of a 5.0 mg/mL solution of *n*-heptadecanoic acid (17:0 fatty acid) in dichloromethane was introduced into the extractor prior to

commencement of the extraction. Honey samples were extracted for 24 h, after which 500 µL of a 5.0 mg/mL solution of *n*-heptadecanoic acid ethyl ester (17:0 Et ester) (5 mg/mL) (manuka honey samples), or 100 µL of a 5.0 mg/mL solution of *n*-heptadecanoic acid ethyl ester for other honey samples, as internal standard was added to the diethyl ether extractive solution.

Thereafter the ether extract was dried over anhydrous Na_2SO_4 and filtered through a cotton wool plug prior to evaporation to *ca* 2 mL on a rotary evaporator (40 ^oC). The concentrated diethyl ether solution was transferred to a 5 mL sample vial and stored at 5 °C pending methylation using an ethereal solution of diazomethane (see Section 2.3).

2.2.2. Time Series Extractions

Typically, 10 g of honey samples which were subjected to time series extractions, were extracted using a 400 mL continuous liquid-liquid extractor and sequentially extracted for 2, 4, 6, 8, 10, 16 and 24 h (total extraction time = 70 h), with replacement of the diethyl ether extractive solution after each time period. Other extraction conditions were as described in Section 2.2.1. For manuka honey samples 500 µL of a solution of a 5.0 mg/mL solution of *n*-heptadecanoic acid (17:0 fatty acid) in dichloromethane was introduced into the extractor at the commencement of each extractor period. For clover honey samples, 100 µL of a solution of a 5.0 mg/mL solution of *n*-heptadecanoic acid (17:0 fatty acid) in dichloromethane was introduced into the extractor at the commencement of each extractor period. At the completion of each time period 500 µL of a 5.0 mg/mL solution of *n*-heptadecanoic acid ethyl ester (17:0 Et ester) for manuka honey samples, [or 100 µL of a 5.0 mg/mL solution of *n*-heptadecanoic acid ethyl ester (17:0 Et ester) for clover honey samples], was added to the diethyl ether extractive solution. Thereafter the diethyl ether extractive solutions were dried over anhydrous Na_2SO_4, filtered through a cotton wool plug, evaporated to *ca* 2 mL on a rotary evaporator (40 ^oC), transferred to a 5 mL sample vial and stored at 5 °C pending methylation using an ethereal solution of diazomethane.

2.3. Preparation of Diazomethane and Methylation of Extracts

In order to give better resolution by gas chromatography, methylation of acidic components in the extracts is required since carboxylic acids and other polar compounds give poor peak shapes on the GC/MS. Methylation was performed using an ethereal solution of diazomethane. Since diazomethane is a very volatile and carcinogenic compound which may explode if residues are ground between glass joints, etc, it was prepared and used in a fume-hood, and methylation reactions were performed in screw-capped vials.

Typically, 1 g of N-nitrosomethyl urea was added to a two-phase mixture of diethyl ether (30 mL) and 25% aqueous sodium hydroxide (30 mL) in a one-piece diazomethane distillation apparatus. Diazomethane formation occurred at the aqueous-diethyl ether interface and the resulting yellow ethereal diazomethane solution was distilled off by gentle heating using a water bath (*ca* 60 ºC) and collected in a 30 mL sample vial. Ethereal diazomethane (2 mL) was added to each of the honey extracts (extracted from 10 g of honey sample) and the ethereal solution was stored at 5 ºC for *ca* 12-16 h (overnight). Methylated samples were concentrated by air-drying to *ca* 1 mL and stored in the dark at refrigerator temperature (5 ºC) until required for analysis.

2.3.1. Preparation of Diazoethane and Ethylated Honey Extracts

Ethylation of honey extracts with diazoethane was used to ascertain the presence of methyl esters in the honey extracts prior to methylation. Diazoethane was prepared as described above for diazomethane, other than that N-nitrosoethyl urea was used instead of N-nitrosomethyl urea. Honeys extracts were ethylated as described above for methylated extracts.

2.4. GC/MS Analyses of Methylated Extracts

GC/MS analyses were performed using He as the carrier gas (column inlet pressure 16 psi; carrier gas flow 1.5 mL/min) and a 30 m x 0.25 mm id ZB-5 column (Phenomex) installed in a HP6890 (Hewlett Packard) GC coupled to a HP5973 mass selective detector (MSD). The GC injector, MS interface and MS ion source were maintained at 250 ^{o}C, 280 ^{o}C and 200 ^{o}C respectively. Mass spectral data were acquired in total ion chromatogram (TIC) mode, scanning the range *m*/z 42 – 450 Daltons. The GC/MS oven temperature was programmed as follows: 20 sec isothermal at 50 ^{o}C, then increased to 75 ^{o}C at 30 ^{o}C/min, then raised at 8 ^{o}C/min to the final temperature of 290 ^{o}C which was held for 25 min to elute any wax components from the column. The injector temperature was 280 ^{o}C. Aliquots of the methylated extractive solutions (typically 1-2 μL) were injected using a HP7683 auto-sampler and the Grob split/splitless technique. The splitless time (column load period) was typically 0.1 min. Compounds were identified using a combination of retention time and mass spectral data.

2.4.1. GC/MS Analyses of Methylated Resin Acids

GC/MS of methylated resin acids was performed using selected ion monitoring mode (SIM) rather than TIC detection and the following GC oven programme: 30 sec isothermal at 150 ^{o}C, then increased to 200 ^{o}C at 25 ^{o}C/min., then raised at 10 ^{o}C/min to the final temperature of 285 ^{o}C which was held for 8 min to elute any wax compounds. SIM analyses were performed using the quantification and confirmation ions listed in Table 2.1.

Table 2.1. Quantification and confirmation ions used to identify methylated resin acids.

compound	quantitative ion (*m/z*)	conformation ions (*m/z*)
1. pimaric acid methyl ester	*m/z* 121	*m/z* 316, 180
2. sandaracopimaric acid methyl ester	*m/z* 121	*m/z* 316, 180
3. dehydroabietic methyl ester	*m/z* 239	*m/z* 314, 299
4. abietic acid methyl ester	*m/z* 256	*m/z* 316, 121
5. isopimaric methyl ester	*m/z* 256	*m/z* 316, 241
6. secodehydroabietic acids 1 and 2 methyl esters	*m/z* 146	*m/z* 316
7. *n*-heptadecanoic acid methyl ester (recv std)	*m/z* 74	-
8. *n*-heptadecanoic acid ethyl ester (int std)	*m/z* 88	-

2.5. Quantification Procedure

TIC or SIM ion profiles were integrated using a Hewlett Packard ChemStation (Version 1.4). Quantification was performed relative to *n*-heptadecanoic acid ethyl ester as internal standard. Relative response factors were determined for methyl benzoate, dimethylpimelate, methyl palmitate, dimethyl sebacate, 4-methoxyacetophenone, methyl 3,4,5-trimethoxybenzoate and methyl 3-phenyl-2-hydroxypropanoate relative to *n*-heptadecanoic acid ethyl ester. Unknown aromatic acids, aliphatic acids, and aliphatic diacid components were quantified as benzoic acid, palmitic acid, or pimelic/sebacic acid (average value) equivalents respectively. Other unknown substances were quantified using a unit response factor relative to *n*-heptadecanoic acid ethyl ester. Results are reported as mg/kg (ppm) of honey (fresh weight).

Resin acids, detected as the corresponding methyl esters, were quantified relative to the *m/z* 88 ion of *n*-heptadecanoic acid ethyl ester as internal standard. SIM relative response factors were determined for pimaric/sandaracopimaric acid methyl esters (*m/z* 121) (average value), isopimaric methyl ester (*m/z* 256), dehydroabietic methyl ester (*m/z* 239), and abietic acid methyl ester (*m/z* 256) relative to *n*-heptadecanoic acid ethyl ester (*m/z* 88). Results are reported as mg/kg (ppm) of honey (fresh weight).

Relative response factors were calculated for the class of compounds using a known concentration of primary standard solution and mixture of compounds. Relative response factor (RRF) was calculated relative to the primary standard as follows:

RRF_A = (peak $area_A$ /$weight_A$) / (peak $area_{17:0\ Et}$/$weight_{17:0\ Et}$)

Where: RRF_A is the relative response factor of target compound,
peak $area_A$ = peak area of target compound (A)
$weight_A$ = weight of target compound (A)
peak $area_{17:0\ Et}$ = peak area of internal standard (17:0 Et)
$weight_{17:0\ Et}$ = weight of internal standard (17:0 Et)

The concentration (mg/kg or ppm) of the target compound was calculated as follows:

$$\text{conc}_A\,(\text{mg/kg}) = \left(\frac{(\text{area}_A/\text{RRF}_A) \times (\text{weight std (mg)} / \text{area std})}{\text{weight honey (g)}} \right) \quad \text{x } 1000$$

where: area_A = area of the target peak (compound A)
RRF_A = relative response factor of compound A
weight std = weight (mg) of internal standard (17:0 Et ester) added
area std = area of the internal standard peak (17:0 Et ester)
weight honey = weight of honey (g) extracted (typically 5 g)
1000 = factor to convert conc_A from mg/g to mg/kg

2.6. Determination of the Recovery of 17:0 Fatty Acid

Analyte recovery was monitoried by determining the % recovery of *n*-heptadecanoic acid (added to the honey solution prior to extraction) relative to that of *n*-heptadecanoic acid ethyl ester (internal standard, added to the diethyl extractive solution after the completion of the liquid-liquid extraction of the honey sample).

The recovery of *n*-heptadecanoic acid from the honey solution was determined by comparing the ratio of the peak areas determined, for *n*-heptadecanoic acid methyl ester (17:0 Me) and *n*-heptadecanoic acid ethyl ester (17:0 Et) in a methylated honey extractive solution as revealed by GC/MS analyses (Section 2.6.4) with the ratio of peak areas determined for a reference mixture of *n*-heptadecanoic acid methyl ester (17:0 Me) and *n*-heptadecanoic acid ethyl ester (17:0 Et).

Preliminary analyses, and results previously reported by Tan (1989) showed that while some fatty acids (*e.g. n*-hexadecanoic acid, stearic acid and oleic acid etc.) were significant components of honey extracts, *n*-heptadecanoic acid was typically present in only trace amounts, and that *n*-heptadecanoic acid could therefore be used as a 'recovery standard'. It was anticipated that the recovery of *n*-heptadecanoic acid from the honey solutions would normally be close to 100%.

2.6.1. n-Heptadecanoic Acid Standard Solution

A standard solution was prepared by weighing *n*-heptadecanoic acid (517 mg) into a 100 mL volumetric flask which was made up to the mark, at room temperature, with distilled chloroform. Typically, 500 μL (manuka honey samples), or 100 μL (other honey samples) was added to the honey solution prior to liquid-liquid extraction.

2.6.2. n-Heptadecanoic Acid Ethyl Ester Standard Solution

A standard soution of *n*-heptadecanoic acid ethyl ester was prepared by weighing *n*- heptadecanoic acid (514 mg) into a 100 mL volumteric flask. Distilled chloroform (*ca* 5 mL) was added into the flask and the resulting solution was reacted with an ethereal solution of diazoethane for 12-16 h at refrigerator temperature (*ca* 5 ºC), after which the standard ethylated solution was made up to the mark of a 100 mL volumetric flask, at room temperature, with distilled chloroform. Typically, 500 μL (manuka honey samples), or 100 μL (other honey samples) was added to diethyl ether extracts immediately after extraction and prior to the concentration of the extractive solutions using a rotary evaporator.

2.6.3. Reference Mixture of n-Heptadecanoic Acid and n-Heptadecanoic Acid Ethyl Ester

Identical volumes of the standard *n*-heptadecanoic acid (17:0) and *n*-heptadecanoic acid ethyl ester (17:0 Et) solutions (500 μL for manuka honey samples or 100 μL other honey samples) were added to a 14 mL vial and the resulting mixture methylated with ethereal diazomethane solution for 12-16 h at refregerator temperaure (*ca* 5 ºC). Methylated reference mixtures were concentrated by air-drying to about 1 mL and stored in the dark (5 ºC) until required. This solution was used to determine the response factor of 17:0 Me relative to 17:0 Et.

2.6.4. Recovery Calculations

The methylated reference mixture and honey samples were analysed using the GC/MS procedure described in Section 2.4. The % recovery of *n*-heptadecanoic acid was calculated as follows:

$$\% \text{ recv} = 100 \times \frac{\text{area}_{17:0\ Me}\ (\text{honey sample})/\text{area}_{17:0\ Et}\ (\text{honey sample})}{\text{area}_{17:0\ Me}\ (\text{ref mix})/\text{area}_{17:0\ Et}\ (\text{ref mix})}$$

where: area $_{17:0\ Me}$ and area $_{17:0\ Et}$ are the integrated peak areas determined for *n*-heptadecanoic methyl ester and *n*-heptadecanoic acid ethyl ester respectively in the methylated honey extracts, and for the corresponding reference mixture.

2.6.5. Recovered Levels of Compounds in Time Series Extractions

Two sub-samples of manuka honey and two sub-samples of clover honey were subjected to time series extraction since they represented the honey samples containing high and low levels of extractable constituents respectively. The concentrations (mg/kg) of phenyllactic acid, methyl syringate, succinic acid, benzoic acid, 2-methoxyacetophenone and decanedioic acid in the manuka honey extracts, and succinic acid, nonanoic acid, 4-methoxybenzoic acid, octanedioic acid, decanedioic acid and heptacosane in the clover honey extracts recovered after each time period (see Section 2.2.2) were calculated relative to *n*-heptadecanoic acid ethyl ester as internal standard (Section 2.5) (Table 2.2).

The levels of target compounds recovered during individual times are reported in Table 2.2. Total levels are presented in Figure 2.1. The accumulated recovery, expressed as % of the total recovery of selected compounds, is presented in Figures 2.2 and 2.3 (manuka and clover honeys respectively).

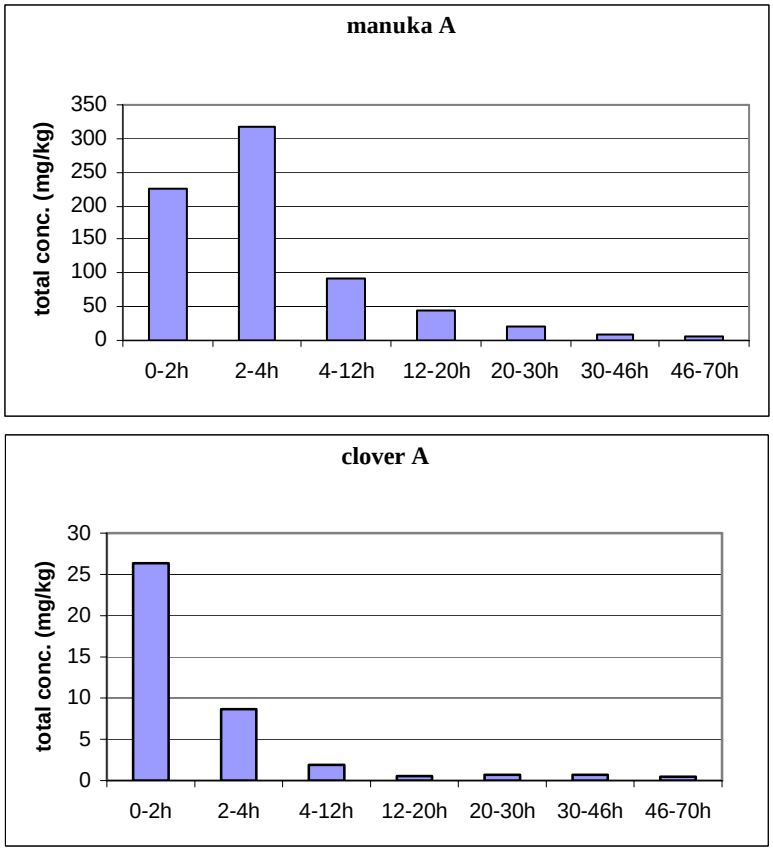


Figure 2.1. Total levels of target recovered during individual times.

The results show that some aromatic acids such as benzoic acid and small aliphatic dicarboxylic acids such as succinic acid are poorly recovered using the 16 h diethyl ether extraction procedure developed by Tan (1988).

The results presented in Table 2.2 and Figures 2.1, 2.2 and 2.3 show that long extraction times (*ca* 50 h) are required to quantitatively recover compounds with high water affinities (*e.g.* benzoic acid and succinic acid).

Table 2.2. Concentrations (mg/kg) of selected compounds (in general detected as the corresponding methyl esters) in diethyl ether extracts of manuka and clover honeys recovered in time series experiments (2-70 h).

time interval (h)	0-2	2-6	6-12	12-20	20-30	30-46	46-70	(sum)
manuka A								
phenyllactic acid	154	274	87	37	14	4.0	0.9	572
methyl syringate	63	34	0.6	0.4	0.6	0.2	0.7	99
succinic acid	0.5	2.1	3.0	6.1	4.7	3.5	4.1	24
benzoic acid	1.6	1.0	0.7	1.5	0.4	0.4	0.5	6.1
2-methoxyacetophenone	4.1	1.9	0.1	0.01	0.3	0.1	0.1	6.6
decanedioic acid	3.2	4.4	0.8	0.5	0.4	0.3	0.2	9.7
total	226	317	92	46	21	9.0	6.4	718
manuka B								
phenyllactic acid	178	262	119	25	3.8	0.00	0.04	589
methyl syringate	63	42	3.1	0.1	0.2	0.1	1.1	110
succinic acid	0.9	3.2	5.1	4.2	5.2	4.4	6.4	29
benzoic acid	1.7	1.3	0.9	0.3	0.2	0.3	0.7	5.4
2-methoxyacetophenone	4.2	3.8	0.2	0.1	0.1	0.00	0.1	8.5
decanedioic acid	4.2	4.4	0.4	0.1	0.1	1.0	0.1	10
total	253	317	129	30	9.6	5.8	8.5	753
clover A								
succinic acid	0.02	0.1	0.2	0.00	0.04	0.00	0.00	0.4
nonanoic acid	0.9	0.2	0.00	0.00	0.00	0.00	0.00	1.1
4-methoxybenzoic acid	1.1	0.3	0.02	0.00	0.00	0.00	0.00	1.4
octanedioic acid	0.8	1.5	0.3	0.03	0.00	0.02	0.00	2.6
decanedioic acid	3.1	3.1	0.5	0.1	0.05	0.05	0.03	6.9
heptacosane	20	3.4	0.9	0.4	0.6	0.6	0.4	27
total	26	8.7	1.9	0.5	0.7	0.7	0.4	39
clover B								
succinic acid	0.000	0.001	0.000	0.000	0.000	0.001	0.000	0.002
nonanoic acid	0.7	0.00	0.00	0.00	0.00	0.00	0.00	0.7
4-methoxybenzoic acid	0.5	0.1	0.01	0.00	0.00	0.00	0.00	0.6
octanedioic acid	0.6	0.6	0.3	0.2	0.05	0.02	0.00	1.7
decanedioic acid	3.3	1.6	0.4	0.3	0.1	0.03	0.00	5.6
heptacosane	16	1.7	0.5	0.8	0.1	0.1	0.3	20
total	21	4.0	1.1	1.3	0.2	0.2	0.3	29

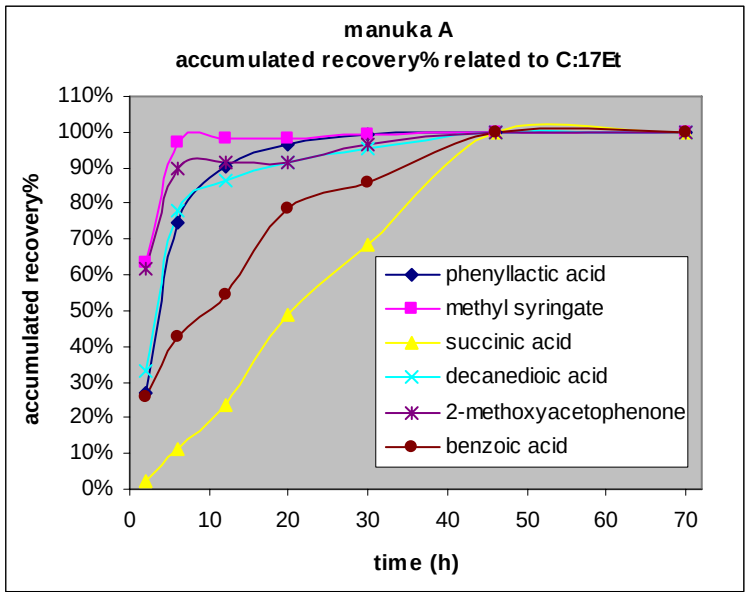


Figure 2.2. The accumulated recovery, expressed as % of the total recovery of phenyllactic acid, methyl syringate, succinic acid, decanedioic acid, 2-methoxyacetophenone and benzoic acid in diethyl ether extracts of manuka honey sub-sample (A) with extractive time (h).

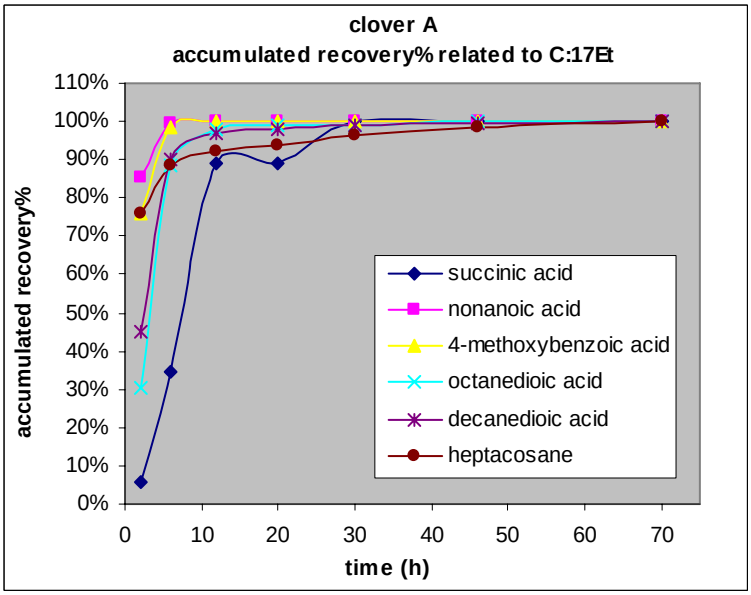


Figure 2.3. The accumulated recovery, expressed as % of the total recovery of succinic acid, nonanoic acid, 4-methoxybenzoic acid, octanedioic acid, decanedioic acid and heptacosane in diethyl ether extracts of clover honey sub-sample (A) with extractive time (h).

2.6.6. Modification of Continuous Liquid-Liquid Extraction Procedure

Since a 50 h extraction period is not convenient for practical applications, the volume of the continuous liquid-liquid extractor was therefore decreased from 400 mL to 250 mL and the amount of honey was also decreased from 10 g to *ca* 5 g, while maintaining the original solvent reflux rate, thereby increasing the solvent turn over rate relative to the aqueous volume, and reducing the time required for complete extraction (Figure 2.4).

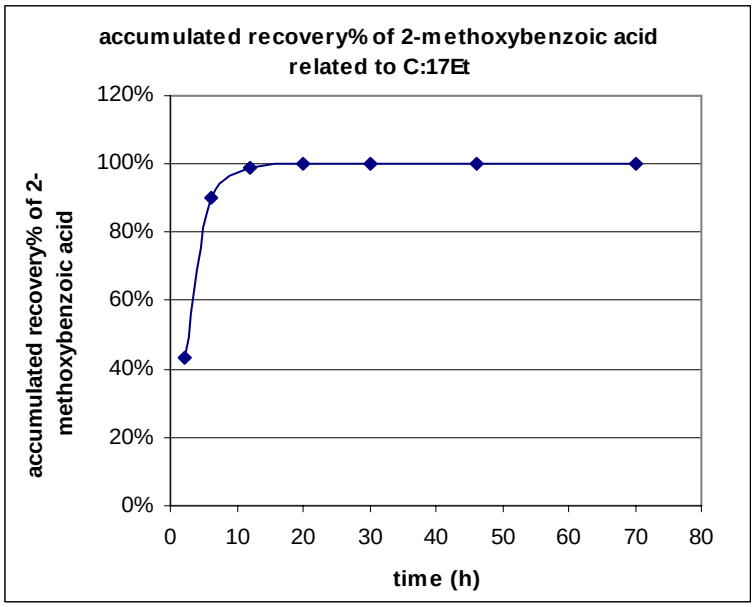


Figure 2.4. The accumulated recovery, expressed as % of the total recovery of 2-methoxybenzoic acid in diethyl ether extracts of manuka honey sub-sample when using a 250 mL extractor and 5 g honey.

Figure 2.4 exhibited that 24 h was an adequate extraction time to recover 2-methoxybenzoic acid. Accordingly, in this investigation the extraction procedure described by Tan (1989) was modified by the use of a smaller extractor (250 mL), a lesser quantity of honey (typically 5 g) and a minimum 24 h extraction period.

2.7. MS Detector Linearity

The linearity of the detector (mass spectrometer) response was assessed using a series of samples prepared from a set of standard solutions containing 5 mg/mL of each of benzoic acid, pimelic acid (C_7 diacid), palmitic acid (16:0 fatty acid), phenyllactic acid, acetophenone and syringic acid. 50, 100, 200, 400, 800 and 1800 μL portions of each of the standard solutions were added to separate vials. A fixed amount (200 μL) of a standard *n*-heptadecanoic acid ethyl ester solution (5 mg/mL) was added to each of the vials. The resulting solutions were made up to *ca* 3 mL and methylated with an ethereal solution of diazomethane. Sub-samples of the respective solutions were analysed using the GC/MS methodology described in Section 2.4.

Detector linearity was assessed by plotting the ratio of the target analyte peak area divided by the peak area determined for the *n*-heptadecanoic acid ethyl ester, against the volume ratio of the target analyte/*n*-heptadecanoic acid ethyl ester (standard), used to prepare the respective solutions, with a linear least squares fit forced through the origin (Table 2.3 and Figure 2.5).

Table 2.3. MS detector linearity data.

	peak area ratio (target analyte/17:0 Et)					
volume[a] ratio	benzoic acid	pimelic acid	palmitic acid	phenyllactic acid	acetophenone	syringic acid
0.25	0.17	0.07	0.09	0.03	0.05	0.01
0.5	0.26	0.21	0.50	0.11	0.24	0.04
1.0	0.69	0.53	1.22	0.39	0.58	0.42
2.0	1.25	1.79	2.70	1.20	1.39	1.16
4.0	2.78	2.89	4.64	2.12	3.97	2.88
9.0	4.58	9.98	(6.99)[b]	6.86	8.91	7.52

[a]volume ratio of target analyte/*n*-heptadecanoic acid ethyl ester (standard); [b]deviant point (omitted from least squares fit).

One of the palmitic acid data points was recognised as an outlier at the 95% confidence level, based on Dixon Test's where a normal distribution of results is expected for the area/conc. value for the series of standard samples.

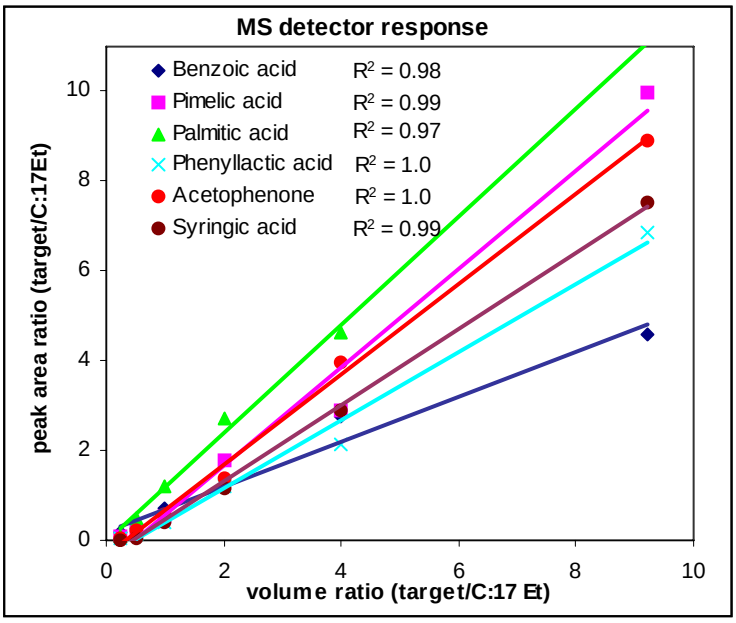


Figure 2.5. Mass spectrometer detector linear response of the series of analytical samples prepared from a set of standard solutions.

Figure 2.5 showed that the peak area ratio of the target/*n*-heptadecanoic acid ethyl ester (for benzoic acid, pimelic acid, palmitic acid, phenyllactic acid, acetophenone and syringic acid) correlated to the volume ratio of the target/*n*-heptadecanoic acid ethyl ester of each of the standard solutions and this results concluded that the mass spectrometer detector was operating linearly within its linear dynamic range.

2.8. GC/MS Reproducibility

Analytical (instrumental) reproducibility was assessed by six replicate injections (GC/MS) of the methylated sub-sample prepared using 200 µL of each of benzoic acid, pimelic acid (C_7 diacid), palmitic acid (16:0 fatty acid), phenyllactic acid, acetophenone, syringic acid and *n*-heptadecanoic acid ethyl ester (internal standard), prepared as described above (Section 2.7).

Instrument (GC/MS) reproducibility was assessed by calculating the ratio of the peak area of target analyte divided by the peak area determined for the *n*-heptadecanoic acid ethyl ester. Calculated peak area ratios (analyte/17:0 Et), mean

values, standard deviation and CV (%) for benzoic acid, pimelic acid, palmitic acid, phenyllactic acid, acetophenone and syringic acid are given in Table 2.4

Table 2.4. Peak area ratios of target analyte peak/17:0 Et peak, mean values, standard deviation and CV (%) for selected analytes.

		peak area ratio (target analyte /17:0 Et)					
injection number	volume ratio target/17:0 Et	benzoic acid	C_7 diacid	16:0 FA	phenyllac. acid	aceto -phenone	syringic acid
injection 1	1	0.85	1.06	1.94	1.01	1.40	1.34
injection 2	1	0.98	1.08	2.00	0.97	1.45	1.34
injection 3	1	0.96	1.03	1.94	1.00	1.45	1.36
injection 4	1	0.92	1.06	1.93	1.08	1.47	1.28
injection 5	1	0.91	1.12	1.90	1.07	1.39	1.26
injection 6	1	0.95	1.07	1.92	1.12	1.55	1.29
average		0.93	1.07	1.94	1.04	1.45	1.31
stdev		0.05	0.03	0.04	0.06	0.06	0.04
CV (%)		5.01%	2.93%	1.82%	5.43%	4.00%	2.91%

The results given in Table 2.4 showed that the values of coefficient variance (CV%) of benzoic acid, pimelic acid (C_7 diacid), palmitic acid (16:0 fatty acid), phenyllactic acid, acetophenone and syringic acid are varied by only less than 6% in respective runs. This result demonstrated that the degree of reproducibility associated with GC/MS analysis is acceptable as these CV% values are in the desirable range (under 10%).

2.9. Honey Extraction Reproducibility

In order to determine the extraction reproducibility, three sub-samples (*ca* 5 g) from the prepared homogenous* manuka honey solution were liquid-liquid extracted for 24 h with diethyl ether as described in Section 2.2.1 (Being limited to quantity of active manuka honey availability, three sub-samples were being extracted). The levels of fifteen compounds (2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, 3,5-dimethoxybenzoic acid, 3,4-dimethoxybenzoic acid, *cis*-cinnamic acid,

trans-cinnamic acid, 4-methoxyphenyllactic acid, decanedioic acid, tetradecanoic acid, methyl syringate, palmitic acid, 8-octadecenoic acid, stearic acid and ricinoleic acid) present in methylated diethyl ether extracts of three manuka sub-samples were quantitatively analysed relative to *n*-heptadecanoic acid ethyl ester using the GC/MS methodology described in Sections 2.4 and 2.5 (Table 2.5).

(*Extraction of sub-samples from one container of honey is not a good representation of the reproducibility of the extraction process as it cannot be assumed that honey samples are homogenous. Because of this reason, approximately 15g (weighted accurately) of manuka honey was dissolved in 300 mL of distilled water, and 100 mL aliquots were taken from the homogenous honey solution).

Table 2.5. Concentrations (mg/kg) of 15 compounds present in methylated diethyl ether extracts of three sub-samples of active manuka honey.

	name of compound	concentration (mg/kg)					
		1	2	3	mean	stdev	CV (%)
1	2-methoxyacetophenone	3.5	4.0	3.4	3.6	0.32	8.8%
2	2-methoxybenzoic acid	8.5	7.6	7.5	7.9	0.55	7.0%
3	phenyllactic acid	323	343	329	332	10.3	3.1%
4	3,5-dimethoxybenzoic acid	2.2	1.8	2.2	2.1	0.23	11%
5	3,4-dimethoxybenzoic acid	2.1	2.5	2.1	2.2	0.23	10%
6	*cis*-cinnamic acid	0.8	0.9	1.0	0.9	0.10	11%
7	*trans*-cinnamic acid	0.7	0.6	0.6	0.6	0.06	9.1%
8	4-methoxyphenyllactic acid	6.5	6.7	6.5	6.6	0.12	1.8%
9	decanedioic acid	1.7	1.5	1.4	1.5	0.15	10%
10	tetradecanoic acid	1.7	2.0	1.6	1.8	0.21	12%
11	methyl syringate	37	36	34	36	1.5	4.3%
12	palmitic acid	6.9	5.9	7.3	6.7	0.72	11%
13	8-octadecenoic acid	21	25	23	23	2.0	8.7%
14	stearic acid	2.4	2.5	2.0	2.3	0.26	11%
15	ricinoleic acid	9.1	8.3	7.6	8.3	0.75	9.0%

The determination of extraction reproducibility is the most important, since it represents the best measure of the reliability of all the other extractions carried out during the experimental work.

Table 2.5 confirmed a reasonable reproducibility for most of compounds (≤ 10 %CV), including 2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, cinnamic acid isomers, 4-methoxyphenyllactic acid and syringic acid. Thus, the

results obtain in this study demonstrated that this reproducibility test was reasonably representative of the sample as whole.

2.10. Bulk Extraction and Compound Separation (Chapters Three and Five)

Bulk extraction of selected honey samples was carried out in order that the more dominant extractives could be isolated in sufficient amounts (1-5 mg) for structural characterisation using one- and two-dimensional ^{1}H and ^{13}C NMR methods. A number of compounds (*e.g.* ericinic acid: see Chapter 5) were isolated from the diethyl ether extracts of a bulk honey sample of appropriate floral type using HPLC and/or silica layer radial chromatography, and their structures were established using GC/MS and one-and two-dimensional NMR spectroscopic data.

Bulk honey samples were liquid-liquid extracted with diethyl ether as described for analytical samples (Section 2.2.1) using a 750 mL extractor.

The resulting extracts were concentrated using a rotary evaporator, and extracts were separated using either radial chromatography, or HPLC. For radial chromatography, extracts were derivatised with diazomethane and applied to a 2 mm silica layer coated on a 20 cm diameter plate. Radial plates were developed using petroleum ether/diethyl ether and diethyl ether/ethanol mixtures, starting with 100% petroleum ether, followed by petroleum ether/diethyl ether mixtures to 100% diethyl ether, and (if required to elute polar compounds) diethyl ether/ethanol mixtures. A 50:50 mixture of diethyl ether: ethanol was used to flush the plate for subsequent use. The solvent gradient and quantities used for separation of particular compounds are given in Chapters 3 and 5. Underivatised extracts were used for HPLC. Analytical conditions, including column type and solvent gradients used, are given in Chapter 5.

Each of the collected radial chromatography or HPLC fractions was analyzed by GC/MS to identify compounds present in the fractions. Selected fractions (those containing the target compounds) were combined and allowed to evaporate to dryness under a fume-hood, followed by vacuum/freeze drying. Thereafter the dried extracts

were dissolved into $CDCl_3$ (low polarity compounds) or $CDCl_3$ /DMSO (high polarity compounds) and NMR spectral data were acquired.

NMR Spectroscopy

NMR data of target compounds were obtained using Bruker DRX 300 and 400 MHz spectrometers fitted with conventional and inverse 5 mm $^{13}C/^{1}H$ probeheads respectively. Bruker-supplied pulse programs were used to acquire ^{1}H, ^{1}H coupled and decoupled ^{13}C, DEPT135, COSY, TOCSY, NOE-difference and gradient selected HMQC, HSQC and HMBC spectral data.

2.11. Determination of Kovat's Indices on a ZB-5 Column (Chapter Three)

Retention indices of compounds detected in honey extracts were determined by interpolation of GC retention times relative to those determined for a series of reference *n*-alkanes (C_9-C_{30} mixture) under identical conditions.

A linear relationship between log retention time and Kovat's number (or carbon number) is observed when GC analyses are conducted under isothermal conditions, however a non-linear relationship is observed when the GC oven is temperature programmed (Heeg *et al*., 1979). A 3rd order polynomial equation of the form $y = ax^3 + bx^2 + cx + d$, can be fitted to data acquired under temperature programme conditions. An illustrative 3rd order polynomial plot determined for a reference hydrocarbon mixture is presented in Figure 2.6. Kovat's indices for kamahi honey components are reported in Chapter 3.

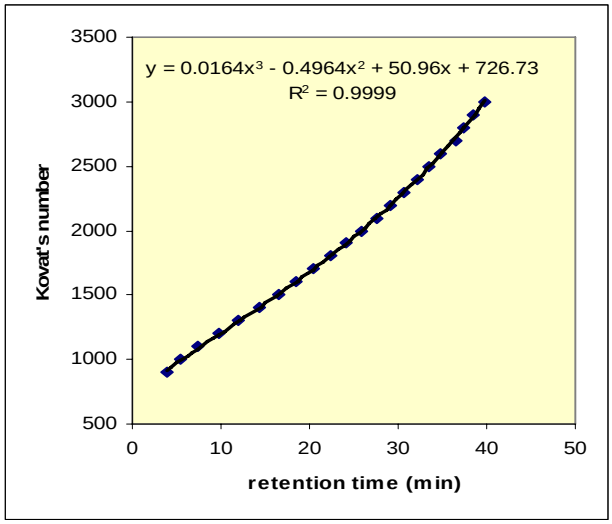


Figure 2.6. Plot of retention time *vs* Kovat's number on a ZB-5 column determined for a reference hydrocarbon mixture.

2.12. Assessment of Non-Peroxide Activity (UMF values) of Active Manuka Honeys (Chapter Seven)

The antibacterial activity (UMF value) of manuka honey samples was assayed by an agar well diffusion method and the method used in this study was modified from that published by Allen *et al.* (1991).

The micro-organism, *Staphylococcus aureus* was chosen as it was sensitive to the non-peroxide antibacterial activity and not affected by the acidity or osmolarity of the honeys (Molan and Russell, 1988).

2.12.1. Inoculum Preparation

A freeze-dried culture of *Staphylococcus aureus* (ATCC 9144) obtained from ESR was reconstituted in Trypticase Soy (TS) broth (Merck 1.05459) according to the instructions supplied, and incubated at 37˚C for 18 hours. A loopful of the broth culture was subcultured onto blood agar plates (Blood Agar Base, Merck 1.10886, with 5% sheep's blood) incubated for 24 hours at 37˚C and used to inoculate 7 x Microbank vials (Pro-Lab Diagnostics PL.160) for long term storage at -70˚C.

Working cultures were obtained by placing one bead from the preserver ampoule stock into 10 mL of TS broth and incubating for 18 hours at 37˚C. A further

working culture was prepared by inoculating a 200µl volume of the prepared culture from the previous day, into another 10 mL TS broth. This was incubated for approximately 5 hours at 37ºC. This culture was then adjusted to an absorbance of 0.5 measured at 540 nm using sterile TS broth as a blank and a diluent and a cuvette with a 1 cm pathway. A volume of 100 µl of the culture adjusted to 0.5 absorbance was used to seed 150 mL nutrient agar to make the assay plates.

2.12.2. Culture quality control

A new freeze dried culture was obtained from the ESR every 6 months. At the end of 6 months the new culture was reconstituted and placed on beads as above. This was then tested and compared with the previous culture to ensure compatible results.

2.12.3. Plate preparation

To prepare the assay plates 150 mL nutrient agar (23g/l Difco 213000) was sterilised then held at 50˚C for 30 minutes before seeding with 100 µl of *S. aureus.* culture adjusted to 0.5 absorbance as above. The agar was swirled to mix thoroughly and poured into a large square assay plate (Corning 431111 - 245x245x18mm) which had been placed on a level surface. As soon as the agar was set the plates were placed upside-down at 4˚C overnight before using the next day.

The agar must be removed from the autoclave as soon as possible then allowed to cool. One weeks supply can be autoclaved at one time then each days requirements steamed in a saucepan of boiling water for 30 minutes then cooled in a 50˚C water bath for 30 minutes before seeding the agar and pouring the plates. It is essential that the agar is not left at high temperatures for a long time as this changes the consistency of the agar.

Using a quasi-Latin square as a template, 64 wells were cut into the agar with a flamed, cooled 8 mm cork borer and removed with an inoculating needle. The template was prepared on black card 230 x 230 mm. A 25 mm grid was drawn on the card, 27.5 mm away from the sides, and the wells were centred on the intersections of the grid. The intersections were numbered using a white china pencil just above the intersection using a quasi-Latin square, which enabled the samples to be placed randomly on the plate.

2.12.4. Catalase solution

A 2 mg/mL solution of catalase from bovine liver (Sigma C9322 2800 units/mg) in distilled water was prepared fresh each day.

2.12.5. Sample preparation

A primary honey solution was prepared by adding 10 g of well-mixed honey to 10 mL of distilled water in universals and placed at 37˚C for 30 minutes to aid mixing. To prepare secondary solutions, 1 mL of the primary honey solution was added to 1 mL of distilled water in a bijou for total activity testing and 1 mL of the primary honey solution was added to 1 mL of catalase solution for non peroxide activity testing. The density of honey, which is 1.35g/mL, is allowed for in the final calculation.

2.12.6. Application of Samples and Standards

Each sample or standard was tested in quadruplicate by adding 100 µl to each of 4 wells with the same allocated number on the assay plate.

2.12.7. Plate incubation

After application of samples and standards the plates were incubated on individual racks *i.e.* not stacked on top of one another, for 18 hours at 37 °C.

2.12.8. Zone measurement

The plates were placed back over the black quasi-Latin square to measure the diameter of the zones of inhibition with digital calipers (Mitutoyo 500.321).using the points of the prongs used to measure inside diameters of tubes.

2.12.9. Preparation of phenol standards

Standards of 2%, 3%, 4%, 5%, 6%, and 7%, were prepared from a 10% w/v solution of phenol BDH A.R. in water. These solutions were kept at 4˚C for one month before making fresh standards and brought to room temperature in the dark before use. Each standard was placed in two wells to test in duplicate.

2.12.10. Calculation of antibacterial activity of honey

The mean diameter of the clear zone around each phenol standard was calculated and squared. A standard graph was plotted of % phenol against the square of the mean diameter of the clear zone. A best-fit straight line was fitted using Cricket Graph software and the equation of this line was used to calculate the activity of each diluted honey sample from the square of the mean measurement of the diameter of the clear zone. To allow for the dilution and density of honey this figure was multiplied by a factor of 4.69 and the activity was then expressed as the equivalent phenol concentration (% w/v).

(Note : The factor of 4.69 is based on a mean honey density of 1.35g/mL)

2.13. Statistical Analysis (Chapter Seven)

Correlations between the levels of eleven significant components of the diethyl ether extracts of manuka honey (phenylacetic acid, 2-methoxyacetophenone, 2-methoxybenzoic, phenyllactic, octanedioic, *cis*-cinnamic, *trans*-cinnamic, nonanedioic, 4-methoxyphenyllactic, decanedioic acids and methyl syringate) and Unique Manuka Factor (UMF) (non-peroxide antibacterial activity) values were evaluated using multiple regression analysis and backwards stepwise regression analysis. Analyses were performed using S-Plus 6 for Windows software (Insightful Corporation Seattle, Washington, U.S.A) and Microsoft Excel 2002. Results are reported in Chapter 6.

2.13.1. Multiple Regression Analysis

Backwards stepwise multiple regression analysis (the term was first used by Pearson, 1908) were performed in order to determine if correlations existed between the levels of selected extractable organic compounds and UMF values, and to ascertain if an equation could be identified which predicted the UMF value of a manuka honey. The objective of these analyses was to identify the equation with highest R^2 value (best-fit) and lowest standard error. Weakly correlated substances were progressively eliminated from data sets, as correlation analyses were refined.

Multiple regression procedure produces a linear equation of the form:

$$\mathbf{Y = a + b_1*X_1 + b_2*X_2 + ... + b_n*X_n}$$

Where: Y = predicted UMF value (% of phenolic equivalent) (dependent variable)

X_n = concentration of an organic compound (mg/kg) (independent variable)

b_n = the regression coefficient, representing the amount the UMF changes when the level of organic compound changes

a = is the constant, where the regression line intercepts the y-axis, representing the amount the UMF (dependent y) will be when all the concentrations of organic compounds (independent variables) are 0.

Interpretation of the Correlation Coefficient (R)

The degree to which two or more organic compounds (X) (*e.g.* phenyllactic acid etc.) are related to the UMF value (Y) is expressed in the correlation coefficient R. In multiple regressions, R has values between 0 and 1. If a b_n coefficient is positive, the degree of correlation increases as the concentration of the species increases, whereas if the b_n coefficient is negative the degree of correlation increases as the concentration of the species decreases. If a b_n coefficient is equal to 0 there is no relationship between the variables.

2.13.2. Minimum Detection Limit (MDL)

For statistical analysis, minimum detection limits (MDL) must be used in order to avoid zero values in the data set. In this investigation the MDL value was taken to be half the practical minimum quantification level (MQL). For relatively clean analytical samples MQL is typically defined to be 2-3 times background noise (Jones *et. al*., 2005) for weak samples the greater the volume injected the greater the MQL value. For complex honey mixtures, a practical MQL based on resolvability and reliability of integration above background of minor (sometimes partly overlapped) peaks was considered rather than a theoretical MQL since it was often difficult to resolve some of the small peaks out of the broad complex background.

The MQL value was defined to be the minimum level of a target species that could be routinely resolved from other minor components in the extract. A further

consideration for inclusion in the set of selected compounds was that the component should be present in at least 50% of the samples

Practical MQL's of selected components (phenylacetic acid, 2-methoxyacetophenone, 2-methoxybenzoic, octanedioic, *cis*-cinnamic, *trans*-cinnamic, nonanedioic, 4-methoxyphenyllactic and decanedioic acids) were individually evaluated. Typically MQL's were in the range 0.2 -0.34 mg/kg (Table 2.6). These values were strongly influenced by the complexity of the GC/MS profiles in the vicinity of each of the target peaks. MQL values were not assigned for phenyllactic acid and methyl syringate, since these two compounds were present at moderate to elevated levels in the manuka honey extracts.

Table 2.6. Practical minimum quantification limit (MQL) and minimum detection limit (MDL) detected for nine methylated organic compounds present in manuka honey extracts.

	compound	MQL (mg/kg)	MDL (mg/kg)
1	phenylacetic acid	0.02	0.01
2	2-methoxyacetophenone	0.24	0.12
3	2-methoxybenzoic acid	0.10	0.05
4	octanedioic acid	0.04	0.02
5	*cis*-cinnamic acid	0.10	0.05
6	*trans*-cinnamic acid	0.08	0.04
7	nonanedioic acid	0.10	0.05
8	4-methoxyphenyllactic acid	0.34	0.17
9	decanedioic acid	0.02	0.01

Minimum Detection Limit for UMF Value

The minimum detection limit for UMF value, as determined by other researchers and commercial laboratories, was not known, and it was not considered appropriate to substitute half of an arbitrary non-zero value where no non-peroxide activity was observed. Therefore, statistical analysis was performed only for the samples for which positive UMF values were observed. Samples which showed only trace (< 5) or no UMF activity were excluded from statistical analyses. Predicted UMF values in the range 0-5 correspond to no measurable UMF activity.

2.13.3. Multiple Regression Analysis for the Active Manuka Honey Samples

Multiple regression analyses (including backwards stepwise multiple regression analyses) were performed for 3 sets of active manuka honey samples, derived from the following sources:

1. 20 active manuka honey samples collected from North Island and South Island sites supplied by Professor P. C. Molan, Honey Research Unit, The University of Waikato, Hamilton,

2. 17 freshly harvested (2002 season) active manuka honey samples collected from the East Cape and Northland regions (North Island) supplied by Mr Jon Stephens, Honey Research Unit, The University of Waikato, Hamilton,

3. 23 Commercial-grade active manuka honey samples (2003 season) provided by Comvita New Zealand Ltd., Wilson Road South, Te Puke.

Statistical analyses were performed on the following combinations of samples and UMF data:

(i) set 1 samples with UMF data supplied by Ms Kerry Allen, Honey Research Unit,
(ii) set 2 samples with UMF data supplied by Mr Jon Stephens, Honey Research Unit,
(iii) set 3 samples with commercially determined UMF values (assayed by Gribbles Ltd. for Comvita New Zealand Ltd.),
(iv) set 3 samples with UMF data supplied by Ms. Carol Goss, Chemistry Department, The University of Waikato,
(v) set 1, 2, 3 samples with UMF as in (i), (ii) and (iii) (University of Waikato and commercially determined UMF values).

The UMF values were regressed against the concentrations of 11 dominant or consistently present oxygenated (non-hydrocarbon) compounds (phenylacetic acid, 2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, octanedioic acid, *cis*-cinnamic acid, *trans*-cinnamic acid, nonanedioic acid, 4-methoxyphenyllactic

acid, decanedioic acid and methyl syringate). Backwards stepwise multiple regression analysis was performed to obtain the best-fit regression equations in the expectation that it might be possible predict the UMF value of the honey sample from one or more of these equations.

Chapter Three

Extractable Organic Substances from New Zealand Kamahi (*Weinmannia racemosa)* Honey

Chapter Three

Extractable Organic Substances from New Zealand Kamahi (*Weinmannia racemosa)* Honey

3.1. Introduction

While Tan (1989) has reported limited data for some methylated diethyl ether extracts of some 1986 season kamahi honey samples, little information is available in respect of the identification of dominant constituents of the extracts other than the identification of kamahines A, B and C (Broom, 1992; 1995) and melirecemoic acid (Ede *et al*., 1993) (see Section 1.3.3).

The principal objective of the work reported in this chapter was to define the typical GC/MS profile and levels of extractable compounds present in 10 commercial unifloral grade, 2002 season kamahi honey samples, supplied by Airborne Honey Export Limited, Leeston, Canterbury, New Zealand. Emphasis was placed on the identification of possible floral marker compounds, in addition to kamahines A, B and C and melirecemoic acid.

The ten honey samples utilised in this investigation were classified as unifloral grade kamahi honeys, based on a combination of organoleptic and pollen data (Table 3.1) determined by Airborne Honey Limited (Leeston, Canterbury, New Zealand) as part of their quality assurance programme.

Table 3.1. Pollen (% contribution and grains per 10 g of honey), colour score (mm), moisture (%) and sugars (fructose%, glucose% and maltose%) for the ten 2002 season kamahi honey samples[a].

sample number	kamahi pollen%	pollen grains per 10 g honey	colour mm	moisture %	HMF[c] mg/kg	fructose %	glucose %	maltose %
1 (15194)[b]	84.2	139,950	36	17.5	3.2	36.6	34.0	3.2
2 (15222)	73.5	243,250	30	17.1	0.2	35.7	31.9	4.8
3 (15253)	69.1	169,950	54	17.9	1.8	38.5	33.1	2.2
4 (15214)	79.4	133,300	28	18.5	1.3	38.1	32.9	4.5
5 (15447)	79.0	139,900	36	17.5	3.2	37.2	32.2	3.6
6 (15215)	86.3	149,950	30	17.9	0.0	37.3	33.0	3.6
7 (15202)	56.0	211,600	36	17.5	0.4	37.7	34.1	3.4
8 (15281)	80.6	356,600	32	17.4	0.0	36.9	31.9	4.1
9 (15336)	78.9	113,250	36	18.0	0.8	38.4	33.3	3.5
10 (15411)	62.0	89,950	22	17.4	2.0	39.5	38.0	1.2
average	76.0	184194	35	18.0	1.2	37.6	33.4	3.6
stdev	9.8	78084	8.4	0.4	1.2	1.1	1.8	1.1
CV (%)	13	42	24	2.3	100	2.9	5.4	29

[a]data supplied by Airborne Honey Ltd., Leeston, Canterbury, New Zealand; [b]Inventory number; [c]Hydroxymethylfurfuraldehyde.

3.2. Experimental

3.2.1. Extraction of Honey Samples

The ten unifloral grade kamahi samples provided by Airborne Honey Limited (Leeston, Canterbury, New Zealand) were extracted and methylated with an ethereal solution of diazomethane using the extraction procedure described in Section 2.2.1. (Chapter 2).

3.2.2. Extraction and Separation of the Bulk Kamahi Honey Sample

Kamahi honey (62.5 g) was liquid-liquid extracted for 72 h with diethyl ether as described for analytical samples, other than that a 750 mL extractor was used (Chapter Two). The resulting extract was concentrated using a rotary evaporator, derivatised with an ethereal solution of diazomethane, and applied to a silica radial plate which was developed with 50 mL portions of petroleum ether:diethyl ether and ethanol:diethyl ether mixtures (Table 3.2). Nineteen 25 mL fractions were collected and concentrated by slow evaporation in a fume-hood to *ca* 1 mL and analysed by GC/MS.

Table 3.2. The solvent gradient used for radial chromatrography of the bulk methylated kamahi extract.

fraction number	solvent (%) petroleum ether:diethyl ether	total volume (mL)
1, 2	0:100	50
3, 4	10:90	50
5, 6	20:80	50
7, 8	40:60	50
9, 10, 11	60:40	75
12, 13	80:20	50
14, 15	100:0	50
16, 17	80:20[a]	50
18, 19	60:40[b]	50

[a]diethyl ether:ethanol (80:20); [b]diethyl ether:ethanol (60:40).

GC/MS analyses showed that fractions 17 and 18 were comprised dominantly of a compound which exhibited strong *m/z* 43, 55, 67, 71 and 82 ions. Fractions 17 and 18 were combined, and subjected to one and two-dimensional NMR analysis with $CDCl_3$ as solvent.

3.2.3. Ethylated and Acetylated Sub-Samples

Prior to methylation of the bulk kamahi extract (see Section 3.2.2) two sub-samples of the diethyl ether extracts were ethylated with an ethereal diazoethane solution prepared as described in Section 2.3.1. Thereafter one of the ethylated sub-samples was acetylated using a mixture of pyridine and acetic anhydride (1:1).

3.3. Results and Discussion

3.3.1. Analytical Samples

The levels of compounds identified in the methylated diethyl ether extracts of the ten 2002 season kamahi honey samples analysed in this investigation are given in Table 3.3. The average concentrations, standard deviations and coefficients of variance of the methylated extractable organic substances detected in the ten samples are given in Table 3.4.

The GC/MS profile of a typical methylated diethyl ether extract is shown in Figure 3.1. The detection of a methyl ester in the methylated extracts was considered to indicate the presence of the parent acid in crude extracts, with the exception of methyl syringate. Comparison of the GC/MS profiles determined for methylated and ethylated sub-samples substantiated this contention. Peak identifications were, in general, based on a combination of MS data, comparisons of retention times and Kovat's indices for authentic standards and/or for compounds known to be present in honey extracts previously examined at the University of Waikato (Tan 1989; Wilkins *et al*., 1993; Broom, 1998).

Peaks eluting after tetracosanoic acid (peak 38) were found to be higher chain length hydrocarbons or fatty acids (detected as the corresponding methyl

esters). Since these compounds are well known constituents of beeswax (Tan *et al*., 1988; Bonaga *et al*., 1986; Graddon *et al*., 1979; Tulloch and Hoffmann, 1972) details of their characterization and levels are not presented here.

The most distinctive peaks, at least from the point of view of floral source characterization, were peaks 3, 27, and 29 (Figure 3.1). Peaks 27 and 29 were identified as meliracemoic acid (Ede *et al*., 1993) and a mixture of kamahines A-C (Broom *et al*., 1992; 1994) respectively. A sufficient quantity of peak 3 was isolated from the bulk extract (Section 3.2.2) for structure determination using one- and two-dimensional NMR analyses. This data showed peak 3 to be 2,6-dimethylocta-3,7-diene-2,6-diol (Wilkins *et al*., 1993).

Two pairs of isomers of oxygenated monoterpenes were tentatively identified as 2,6-dimethylocta-7-ene-2,3,6-triol isomers (peaks 15 and 16) and 2,6-dimethylocta-3,7-diene-1,2,6-triol isomers (peaks 7 and 9). Along with dehydroabietic acid (a resinous woody compound) these compounds were identified for the first time in kamahi honey extracts.

Table 3.3. Concentration (mg/kg) of compounds detected in methylated diethyl ether extracts of ten 2002 season New Zealand kamahi honey samples. Acids were quantified as the corresponding methyl esters.

peak	Kovat's		sample number									
no.	no.	compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	1216	2,6,6-trimethyl-2-cyclohexene-1,4-dione	2.1	2.6	6.9	8.8	8.0	5.3	2.6	4.2	3.7	5.2
2	1227	unknown (*m/z* 43, 59, 88, 101, 117)[a]	3.9	5.8	13	7.8	5.7	7.0	6.4	8.5	4.1	12
3	1234	2,6-dimethylocta-3,7-diene-2,6-diol	25	27	26	49	31	37	26	34	35	16
4	1272	unknown (*m/z* 43, 59, 71, 88, 103)	2.9	2.3	2.9	-	-	-	1.3	1.9	1.6	-
5	1278	unknown (*m/z* 44, 59, 72, 99, 142)	2.6	3.0	4.1	2.0	-	2.6	1.4	-	-	6.7
6	1282	2-methoxyacetophenone	-	-	-	-	6.9	2.6	8.4	5.5	-	-
7	1286	2,6-dimethylocta-3,7-diene-1,2,6- triol (isomer-A)[b]	3.1	5.6	2.2	3.1	-	-	1.2	7.0	-	-
8	1289	unknown (*m/z* 43, 55, 93, 111, 139)	-	3.6	4.0	3.3	-	2.9	3.0	2.2	2.3	-
9	1295	2,6-dimethylocta-3,7-diene-1,2,6-triol (isomer-B)[b]	1.5	3.0	1.2	1.4	-	-	-	4.4	-	-
10	1301	2,6-dimethylocta-2,7-diene-1,6-diol	1.9	1.7	1.8	0.9	2.4	-	2.9	1.4	1.1	-
11	1334	phenyllactic acid	-	9.4	6.5	-	46	4.4	108	31	29	5.5
12	1338	4-methoxybenzoic acid	-	4.4	13	11	-	8.8	9.7	4.4	-	7.6
14	1344	2-propanone,1-[4-methoxyphenyl]	-	5.5	4.2	-	20	-	12	13	1.8	-
15	1352	2,6-dimethyloct-7-en-2,3,6-triol (isomer-A)[b]	9.7	7.5	3.7	3.4	6.1	2.8	3.2	6.6	2.0	1.3
16	1355	2,6-dimethyloct-7-en-2,3,6-triol (isomer-B)[b]	16	14	10	7.5	8.7	5.2	5.0	9.8	4.3	1.8
17	1364	unknown (*m/z* 43, 55, 71, 111, 139, 171)	12	12	9.0	7.6	6.3	0.7	5.6	11	5.7	-
18	1377	unknown (*m/z* 43, 55, 71, 85, 98)	8.5	12	8.3	3.2	-	-	2.4	8.0	0.4	-
19	1420	unknown (*m/z* 44, 55, 79, 97, 111, 123, 138, 168)	5.0	5.6	2.9	-	-	-	2.0	7.0	-	-
20	1429	unknown (*m/z* 43, 57, 77, 91, 109, 137, 165)	2.5	1.8	1.4	1.0	2.8	-	1.2	2.1	1.3	4.8

Table 3.3. continued (compounds 21-38).

peak no.	Kovat's no.	compound	sample number 1	2	3	4	5	6	7	8	9	10
21	1438	unknown (*m/z* 43, 53, 71, 83, 111)	0.9	4.1	-	-	-	-	1.0	4.5	-	-
22	1484	dodecanoic acid	4.1	1.7	3.4	3.9	6.0	3.0	3.0	-	1.2	7.5
23	1504	nonanedioic acid	8.0	3.5	7.8	1.3	9.5	4.5	7.2	5.6	6.0	13
24	1580	4-methoxyphenyllactic acid	-	5.8	4.4	-	65	6.7	207	42	14	6.4
25	1610	decanedioic acid	6.2	4.9	8.2	3.5	8.9	4.6	7.4	5.9	5.1	10
26	1699	tetradecanoic acid	2.3	1.3	1.7	1.9	1.7	1.2	1.2	1.3	0.8	2.9
27	1873	meliracemoic acid	10	15	21	17	15	21	12	12	9.5	9.3
28	1929	hexadecanoic acid	61	22	45	44	51	34	38	16	19	49
29	1929	kamahines A, B and C	66	65	144	65	58	79	100	43	63	47
30	2028	unknown (*m/z* 43, 55, 74, 88, 95, 109, 127, 155, 179)	11	14	12	8.5	17	20	-	22	19	7.1
31	2093	abscisic acid	2.1	-	2.6	-	5.0	2.5	-	-	-	-
33	2154	stearic acid	66	17	23	44	58	39	37	9.3	11	56
34	2330	unknown (*m/z* 43, 55, 67, 81, 95, 111, 121, 169)	4.0	4.2	1.5	2.6	-	1.6	3.3	4.6	2.8	4.4
35	2349	dehydroabietic acid	1.7	1.0	2.2	0.9	-	0.8	0.7	1.1	0.8	1.5
36	2364	eicosanoic acid	4.8	1.4	1.7	3.2	3.3	2.6	2.2	0.8	0.9	3.4
37	2558	docosanoic acid	1.9	3.1	1.3	1.0	0.8	1.3	0.9	0.7	1.2	2.0
38	2732	tetracosanoic acid	6.7	16	12	3.6	2.1	4.1	2.2	2.3	0.6	1.7

[a]prominent ions observed in the mass spectra of unknown compounds are given in brackets; [b]tentative identification.

Table 3.4. Average concentrations (mg/kg), standard deviations (stdev), and coefficient of variance (CV%) determined for compounds identified in the methylated diethyl ether extracts of ten 2002 season kamahi honey samples. Acids were quantified as the corresponding methyl esters.

peak no	Kovat's no	compound	average conc. mg/kg	stdev mg/kg	n	CV (%)
1	1216	2,6,6-trimethyl-2-cyclohexene-1,4-dione	4.9	2.4	10	48
2	1227	unknown (43, 59, 88, 101, 117)[a]	7.4	3.0	10	41
3	1234	2,6-dimethylocta-3,7-diene-2,6-diol	31	9.0	10	28
4	1272	unknown (43, 59, 71, 88, 103)	2.2	0.7	6	31
5	1278	unknown (44, 59, 72, 99, 142)	3.2	1.7	7	54
6	1282	2-methoxyacetophenone	5.8	2.4	4	42
7	1286	2,6-dimethylocta-3,7-diene-1,2,6-triol (isomer A)[b]	3.7	2.2	6	59
8	1289	unknown (43, 55, 93, 111, 139)	3.1	0.7	7	22
9	1295	2,6-dimethylocta-3,7-diene-1,2,6-triol (isomer B)[b]	2.3	1.4	5	60
10	1301	2,6-dimethylocta-2,7-diene-1,6-diol	1.8	0.7	8	37
11	1334	phenyllactic acid	30	35	8	116
12	1338	4-methoxybenzoic acid	8.3	3.1	7	37
14	1344	2-propanone,1-[4-methoxyphenyl]	9.5	6.9	6	73
15	1352	2,6-dimethyloct-7-ene-2,3,6-triol (isomer A)[b]	4.6	2.7	10	59
16	1355	2,6-dimethyloct-7-ene-2,3,6-triol (isomer B)[b]	8.3	4.5	10	54
17	1364	unknown (43, 55, 71, 111, 139, 171)	7.8	3.7	9	48
18	1377	unknown (43, 55, 71, 85, 98)	6.1	4.2	7	68
19	1420	unknown (44, 55, 79, 97, 111, 123, 138, 168)	4.6	2.1	5	46
20	1429	unknown (43, 53, 71, 83, 111)	2.1	1.2	9	56
21	1438	unknown (43, 53, 71, 83, 111)	2.6	1.9	4	74
22	1484	dodecanoic acid	3.7	2.0	9	55
23	1504	nonanedioic acid	6.6	3.2	10	49
24	1580	4-methoxyphenyllactic acid	44	70	8	159
25	1610	decanedioic acid	6.5	2.1	10	30
26	1699	tetradecanoic acid	1.6	0.6	10	38
27	1873	meliracemoic acid	14	4.2	10	30
28	1929	hexadecanoic acid	38	15	10	39
29	1929	kamahines A, B and C	73	30	10	41
30	2028	unknown (43, 55, 74, 83, 95, 109, 127, 155, 179)	14	5	9	37
31	2093	abscisic acid	3.1	1.3	4	43
32	2112	heptadecanoic acid	2.6	1.0	8	38
33	2154	stearic acid	36	20	10	56
34	2330	unknown (43, 55, 67, 81, 95, 111, 121, 169)	3.2	1.2	9	36
35	2349	dehydroabietic acid	1.2	0.5	9	42
36	2364	eicosanoic acid	2.4	1.3	10	52
37	2558	docosanoic acid	1.4	0.7	10	51
38	2732	tetracosanoic acid	5.2	5.1	10	99

[a]prominent ions observed in the mass spectra of unknown compounds are given in brackets; [b]tentative identification.

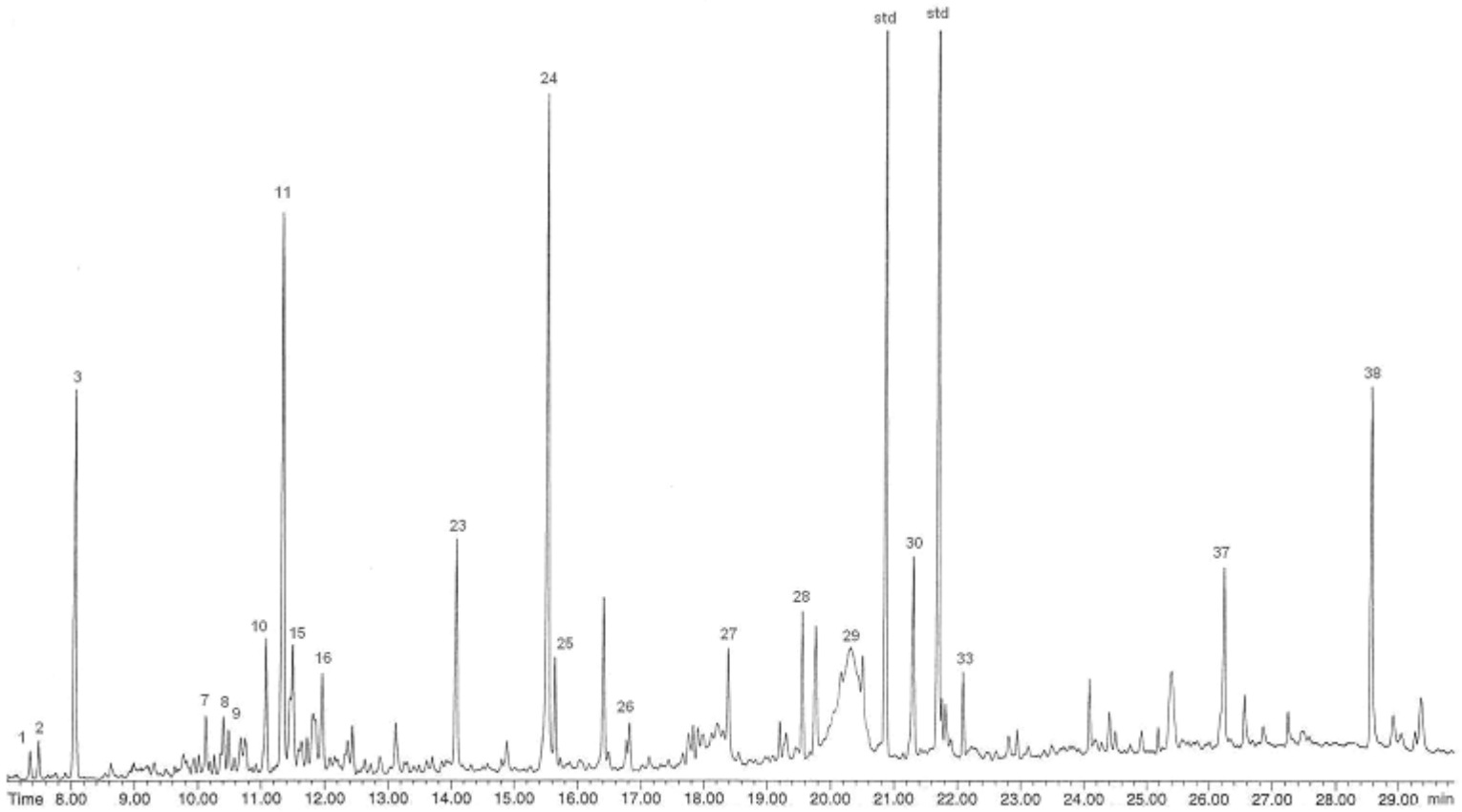


Figure 3.1. GC/MS profile of a representative methylated kamahi honey diethyl ether extract. Peak identifications are given in Table 3.3.

3.3.2. Kamahines A-C (Peak 29)

Kamahines A, B and C (Figure 3.2), the levels of which ranged from 43 to 144 mg/kg were a dominant constituent of the methylated diethyl ether extracts of the ten 2002 season kamahi honey samples. Hitherto Broom *et al*. (1994) have reported that in solvents such as chloroform, interconversion of kamahines A-C *via* reversible ring opening and closure of the hemiacetal entity present in these compounds leads to a equilibrium mixture of kamahines A, B, and C in a *ca* 1:2:1 ratio. The presence in the GC/MS profiles of the methylated kamahi honey extracts of a board, late eluting peak (peak 29), (Figure 3.1) is consistent with the proposal that interconversion between isomeric forms of kamahines A-C also occurs in the vapour phase.

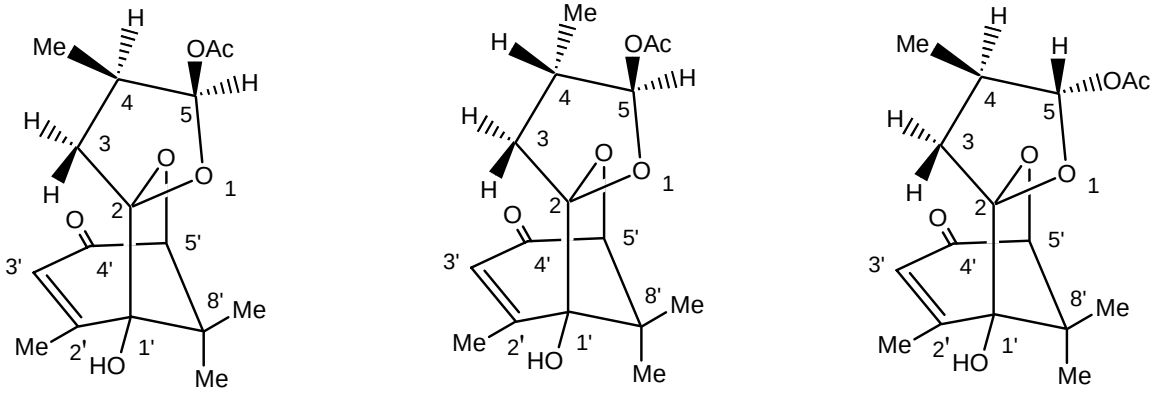


Figure 3.2. Chemical structures of kamahines A, B and C (Broom *et al.*, 1994).

In his pioneering study, Tan (1989) reported levels of 10.8 -156 mg/kg of these compounds in the methylated diethyl ether extracts of eleven 1986 season kamahi honey samples which at the time were described as unknown degraded carotenoid-like substances, while Hyink (1998) reported the levels of these three isomers in the 1996 season kamahi honey extracts ranged from trace to 71 mg/kg. The levels of kamahines A, B and C in the extracts of 2002 season (average level 73 mg/kg) kamahi honey were similar to 1986 season kamahi honey extracts (average level 85 mg/kg) (Tan, 1989) and much higher than the samples collected during the 1996 season (average level 37 mg/kg) (Hyink, 1998).

Acetylation of Kamahines A, B and C

The presence in GC/MS profiles of a broad peak attributable to inter-converting kamahine A, B and C isomers, while diagnostic for the presence of kamahines, inhibits the detection and quantification of other minor component concealed under this broad peak. It was reasoned that if extracts were acetylated prior to GC/MS analyses, interconversion would be prevented, and three well-defined, sharp peaks would be observed for the kamahines isomers. This proved to be the case. GC/MS analysis of an acetylated sub-sample of a methylated kamahines extract afforded three well-defined peaks attributable to non-equilibrating acetylated variants of kamahines A, B and C (designated as peaks Ac-1, Ac-2 and Ac-3) (Figure 3.3).

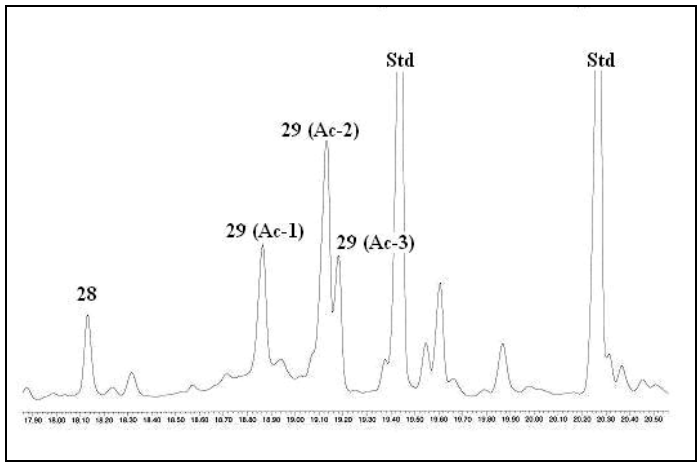


Figure 3.3. Partial GC/MS profile of an acetylated sub-sample of a methylated kamahi honey extract showing the peaks attributable to the three non-equilibrating acetylated kamahines isomers (Ac-1, Ac-2 and Ac-3) (Std = 17:0 Me and 17:0 Et respectively).

The mass spectra of an equilibrating mixture of kamahines A-C and of one of the acetylated kamahine isomers (Ac-2) are shown in Figure 3.4. The mass spectrum of kamahines A-C was characterised by strong *m/z* 43, 55, 109, 137 and 152 ions. Broom (1998) has proposed a fragmentation pathway leading to *m/z* 109, 124, 137 and 152 ions. The origin of the *m/z* 170 ion (Figure 3.4 (a)) is uncertain. The mass spectra of the acetylated isomers (Ac-1 Ac-2 and Ac-3) were similar to the mass spectrum of the kamahines A, B and C mixture, other than for the observation of low intensity *m/z* 194, 221, 251 $(M\text{-}OAc)^+$ and 268 $(M\text{-}CH_2CO)^{\cdot+}$ ions along with a weak molecular ion

at *m/z* 310 (M^+). The *m/z* 170 fragment ion was not observed in the mass spectrum of the acetylated compounds.

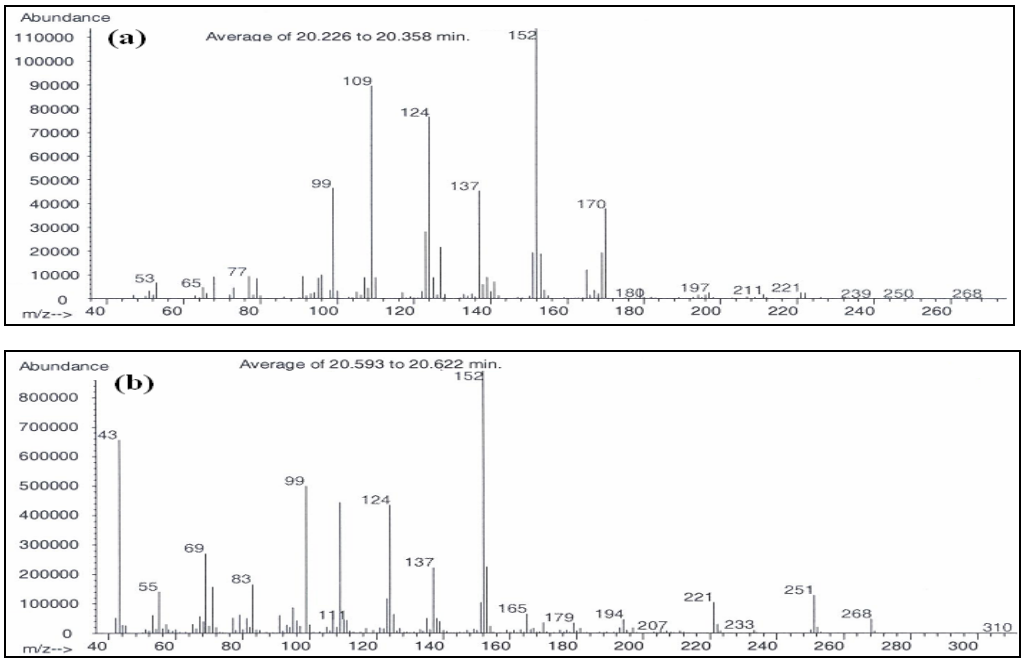


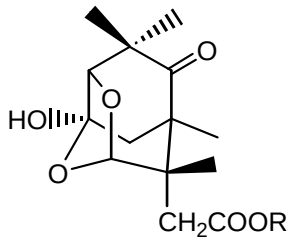
Figure 3.4. Mass spectra of (**a**) an equilibrating mixture of kamhines A-C, and (**b**) an acetylated isomer (Ac-2).

3.3.3 Meliracemoic Acid (Peak 27)

Ede *et al.* (1993) have previously reported the isolation and structural elucidation of meliracemoic acid (a 2,5-methano-hexahydro-1,3-benzodioxolane analogue). Meliracemoic acid is believed to be a rearrangement product of abscisic acid. Broom (1998) reported that structural similarities between kamahines A-C and meliracemoic acid (both are 14-carbon degraded carotenoid-like compounds) suggest that they may arise *via* rearrangement of a common precursor and that this may be a consequence of biosynthetic processes in the plant, rather than oxidative processes within the honey.

Relatively high levels of meliracemoic acid methyl ester (peak 27) [**1**], ranging from 9.3 to 21 mg/kg (stdev 4.2 mg/kg), were detected in all of the methylated diethyl ether extracts of the 2002 season kamahi honey samples. The average level of meliracemoic acid methyl ester (14 mg/kg) was somewhat higher than that found in

1986 season (6.9 mg/kg) (Tan, 1989) and 1996 season (11 mg/kg) (Hyink, 1998) kamahi honey extracts. The results presented in Table 3.4 substantiate the proposal that like kamahines A-C, meliracemoic acid is a distinctive floral marker for kamahi honeys.



[**1**]

Meliracemoic acid methyl ester [**1**] possessed the mass spectrum depicted in Figure 3.5. The mass spectrum of this compound included a moderate intensity molecular ion at *m/z* 268 (M^+) and strong fragment ions at *m/z* 43, 83, 98, 125 and 126.

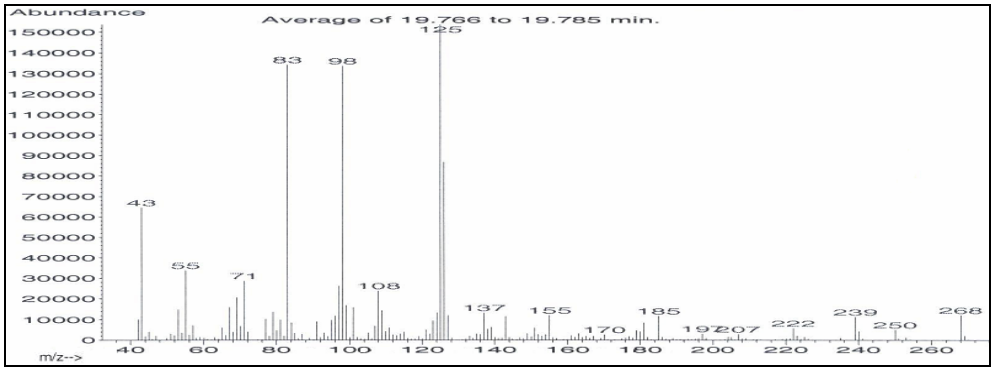


Figure 3.5. Mass spectrum of meliracemoic acid methyl ester.

3.3.4. 2,6-Dimethylocta-3,7-diene-2,6-diol (Peak 3)

Separation of a bulk extract (see Section 3.2.2) by radial chromatography on silica gel afforded a quantity of 2,6-dimethylocta-3,7-diene-2,6-diol (Peak 3) sufficient for structure determination using one- and two-dimensional NMR methods. A complete assignment of the ^{1}H NMR resonances of this compound (Table 3.5) was determined from detailed analyses of one- and two-dimensional ^{1}H, COSY and

TOCSY NMR data. The quantity of peak 3 was not sufficient to record its ^{13}C and DEPT135 NMR spectra.

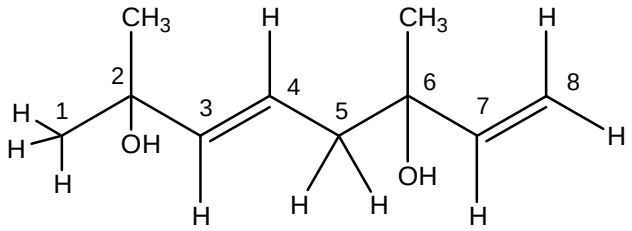


Figure 3.6. Chemical structure of 2,6-dimethylocta-3,7-diene-2,6-diol.

Table 3.5. ^{1}H NMR data for 2,6-dimethylocta-3,7-diene-2,6-diol.

Proton	**δ ^{1}H ($CDCl_3$), *J* Hz**
2-C<u>H</u>$_3$	1.32 s
H-3(C=C<u>H</u>)	5.70 d, ^{3}J 15.8 Hz
H-4 (C=C<u>H</u>)	5.62 dt, ^{3}J 15.8, 6.9 Hz
H-5′ (C<u>*H*</u>H)	2.25 dd ^{2}J 14.2, ^{3}J 7.8 Hz
H-5" (CH<u>*H*</u>)	2.28 dd ^{2}J 14.2, ^{3}J 6.3 Hz
6-C<u>H</u>$_3$	1.28 s
H-7 (C=C<u>*H*</u>)	5.92 dd, ^{3}J 17.2, 10.8 Hz
H-8′ (*cis*) (=C<u>*H*</u>H)	5.06 dd, ^{3}J 10.8 Hz, ^{2}J 1.3 Hz
H-8″ (*trans*) (=CH<u>*H*</u>)	5.20 dd, ^{3}J 17.2 Hz, ^{2}J 1.3 Hz

The ^{1}H NMR spectrum of peak 3 included signals attributable to three aliphatic methyl groups, 1.32 (6H, 2 x Me) and 1.28 ppm (3H, 1 x Me) respectively, two methylene protons, 2.25 (H-5') and 2.28 ppm (H-5"), and five olefinic protons.

The vinylic methylene protons at 5.06 (H-8') and 5.20 (H-8") ppm showed ^{3}J coupling of 10.8 Hz and 17.2 Hz respectively to the olefinic proton at 5.92 (H-7) ppm. This showed H-7 was located *cis* with respect to H-8′ and *trans* with respect to H-8″. The H-8' and H-8" protons showed a mutual ^{2}J couplings of 1.3 Hz. The ^{3}J coupling (15.8 Hz) between the olefinic protons at 5.70 (H-3) and 5.62 ppm (H-4) showed that theses were *trans*-orientated across the olefinic double bond.

The COSY spectrum of peak 3 included correlations between the olefinic protons at 5.62 (H-4) and 5.70 ppm (H-3) and between the vinylic methylene protons at 5.06, 5.20 (H-8' and H-8") and 5.92 ppm (H-7). The olefinic proton at 5.62 ppm (H-4) also showed correlations to the methylene proton at 2.25 (H-5') and 2.28 ppm (H-5").

The ^{1}H NMR data for 2,6-dimethylocta-3,7-diene-2,6-diol presented in Table 3.5 are comparable to those previously determined for a specimen of this compound isolated from the extracts of New Zealand nodding thistle honey (Wilkins *et al*., 1993).

The mass spectrum of 2,6-dimethylocta-3,7-diene-2,6-diol (Figure 3.7) was characterised by strong *m/z* 71 and 82 ions. The highest intensity observed ion occurred at *m/z* 152 $[M\text{-}H_2O]^{\cdot+}$, consistent with and attributable to loss of water molecule for a substance of molecular formula $C_{10}H_{18}O_2$.

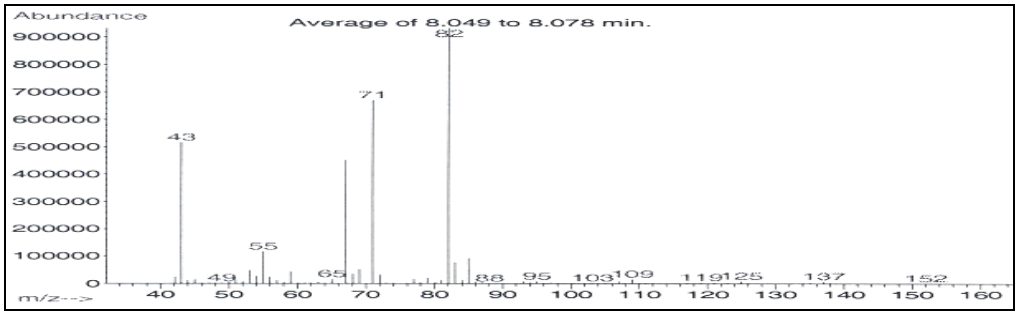


Figure 3.7. Mass spectrum of 2,6-dimethylocta-3,7-diene-2,6-diol (peak 3).

When a sub-sample of the diethyl ether extract was reacted with acetic anhydride/pyridine, acetylation of peak 3 was not observed. This is consistent with the presence of two tertiary hydroxyl groups in this compound and its identification as 2,6-dimethylocta-3,7-diene-2,6-diol. The identification of peak 3 as 2,6-dimethylocta-3,7-diene-2,6-diol was also substantiated by comparison of its Kovat's indices and previously reported mass spectral data (Tan, 1989; Wilkins *et al*., 1993; D'Arcy *et al*., 1997).

Generally high levels of 2,6-dimethylocta-3,7-diene-2,6-diol (peak 3), ranging from 16 to 49 mg/kg (stdev 9.0 mg/kg), were detected in all of the methylated diethyl ether extracts of the 2002 season kamahi honey samples. The average level (31

mg/kg) of this compound in 2002 season kamahi honey extracts was higher than found in 1986 season kamahi honey extracts (21 mg/kg of unknown peak 64), (Tan, 1989) and lower than found in 1996 season kamahi honey extracts (42 mg/kg) (Hyink, 1998). Tan (1989) has also noted the presence of this diol, as unknown peak 64, in the diethyl ether extracts of some 1986-1989 season thyme honeys (average level 14 mg/kg).

D'Arcy *et al*. (1997) have reported the presence of 2,6-dimethylocta-3,7-diene-2,6-diol in Australian blue gum and yellow box honey extracts and Rowland *et al*. (1995) have identified it in leatherwood honey extracts. This diol was first prepared by photosensitised oxidation of linalool (Matsuura and Butsugan, 1968) and later isolated from the essential oil of ho-leaf (Takaoka and Hiroi, 1976). It has also been identified as a component of grape juices, wine (Williams *et al*., 1980a), and tea (Etoh *et al*., 1980).

3.3.5. Hotrienol

A minor peak which eluted a little before peak 1, was identified as dimethylocta-3,7-diene-2,6-diol (hotrienol) (Wilkins *et al*., 1993). Rowland *et al*., (1995) have identified this trienol as a dominant contributor to the odour of Australian leatherwood (*Eucryphia lucida*) honeys, and noted that thermal dehydration of 2,6-dimethylocta-3,7-diene-2,6-diol during passage through a hot GC injector may afford hotrienol (3,7-dimethylocta-1,5,7-trien-3-ol) (Figure 3.8). Since hotrienol levels were typically less than 2 mg/kg, they are not reported in Tables 3.3 and 3.4.

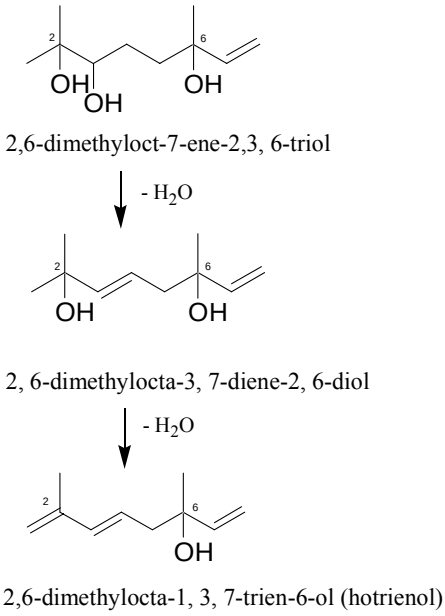
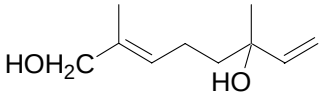


Figure 3.8. Schematic overview of the proposed formation of hotrienol *via* dehydration of 2,6-dimethyloct-7-ene-2,3,6-triol.

The mass spectrum of hotrienol and 2,6-dimethylocta-3,7-diene-2,6-diol were essentially indistinguishable; each of these compounds displayed base peak ions at *m/z* 82 and significant ions at *m/z* 43, 55, 67, 71 along with highest observable ions at *m/z* 152.

3.3.6. 2,6-Dimethylocta-2,7-diene-1,6-diol (Peak 10) [3]

Peak 10 was identified as 2,6-dimethylocta-2,7-diene-1,6-diol [**3**]. This hydroxylated linalool analogue has been previously reported by Tan (1989) as an unknown compound (unknown 121) in nodding thistle honeys. D'Arcy *et al.* (1997) have also reported the presence of this diol compound in the extracts of Australian blue gum and yellow box honeys. This compound was detected in the kamahi honey extracts at levels in the range trace to 2.9 mg/kg with an average level of 1.8 mg/kg (stdev 0.7 mg/kg). The levels of this compound in 8 of the 10, 2002 season kamahi honey samples, were much lower than in nodding thistle honey samples collected during the 1987 season (average level 5.5 mg/kg) (Tan, 1989).



[**3**]

The mass spectrum of 2,6-dimethylocta-2,7-diene-1,6-diol exhibited a base peak at *m/z* 43 and strong fragments ions at *m/z* 55, 67, 71 and 82 along with weaker fragment ions at *m/z* 79, 93, 96, 137 and 152 $[M\text{-}H_2O]^{+}$. Moderate intensity *m/z* 110 and 119 ions were also seen (Figure 3.9a).

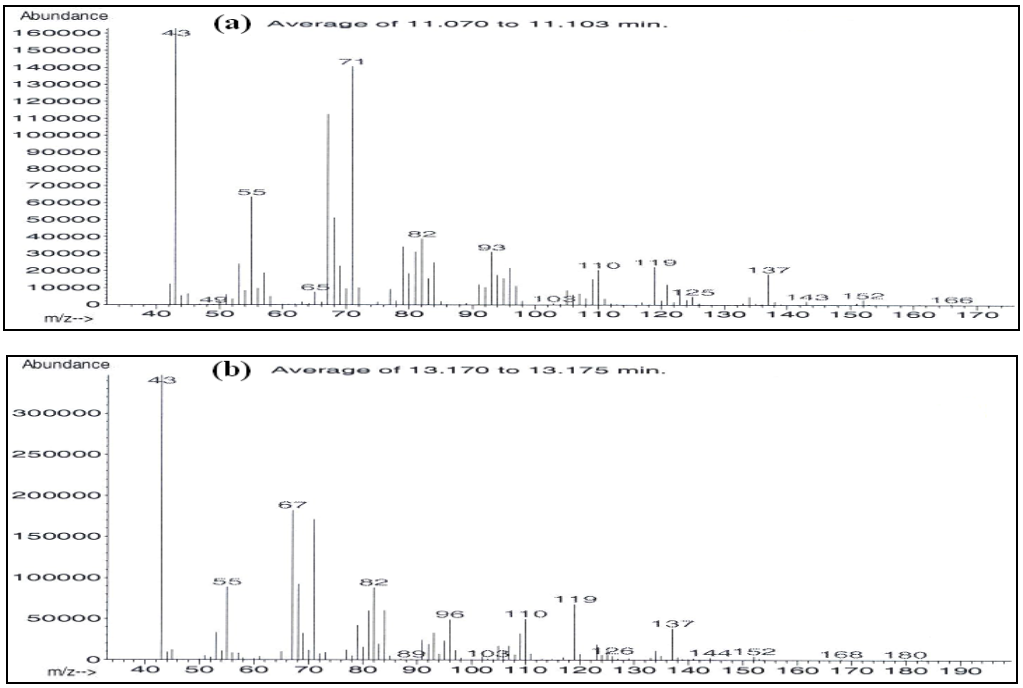


Figure 3.9. Mass spectra of (**a**) 2,6-dimethylocta-2,7-diene-1,6-diol and (**b**) acetylated 2,6-dimethylocta-2,7-diene-1,6-diol.

These ions greatly aided the identification of this compound. The *m/z* 119 ion can be attributed to the $[M\text{-}CH_3\text{-}(2 \times H_2O)]^{+}$ fragment. The origin of the *m/z* 110 ion is uncertain, but it can possibly be attributed to loss of ketene $[CH_2{=}C{=}O]$ or propene $[C_3H_6]$ from the *m/z* 152 ion *via* a rearrangement pathway. Moderate intensity *m/z* 67, 71, 110 and 119 ions are also apparent in the mass spectrum of the acetylated analogue of this compound (Figure 3.9b).

The *m/z* 110 and 119 extracted ion profiles (Figure 3.10) served to revealed the presence of 2,6-dimethylocta-2,7-diene-1,6-diol and its acetylated analogue (1-acetoxy-2,6-dimethylocta-2,7-dien-6-ol) in kamahi, and acetylated kamahi extracts. The acetylated analogue of peak 10 (1-acetoxy-2,6-dimethylocta-2,7-dien-6-ol) eluted *ca* 2 min later than the parent compound (see Figure 3.10).

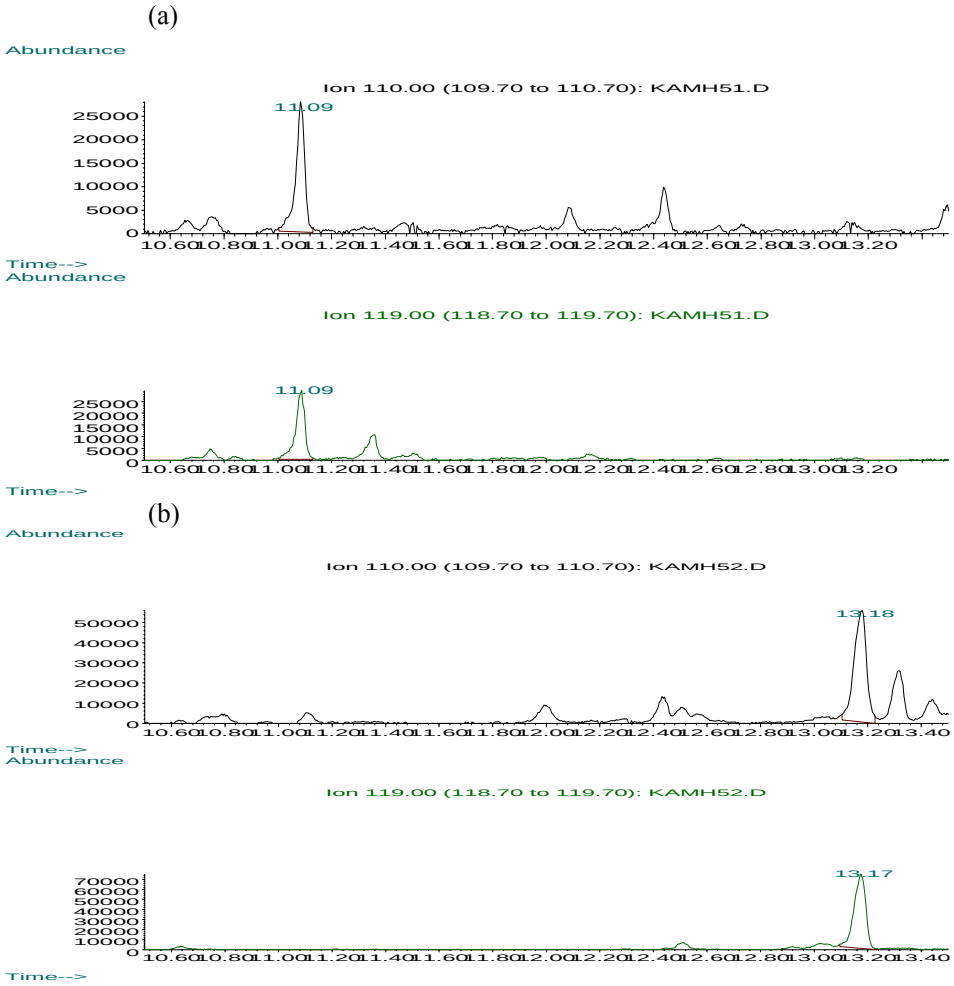
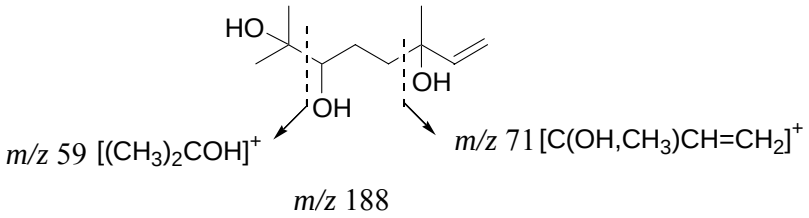


Figure 3.10. *M/z* 110 and 119 extracted ion profiles (10-13.5 min region) determined for (**a**) 2,6-dimethylocta-2,7-diene-1,6-diol (11.09 min) and (**b**) 1-acetoxy-2,6-dimethylocta-2,7-dien-6-ol (13.17 min).

3.3.7. 2,6-Dimethyloct-7-ene-2,3,6-triol (Peaks 15 and 16) [4]

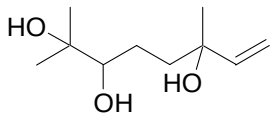
Peaks 15 and 16 exhibited characteristic mass spectral ions at *m/z* 43, 55, 59, 67, 68, 71, 93, 94, 111, 137, 155 and 188 ($M^{\cdot+}$) (Figure 3.11). The strong *m/z* 59 and 71 ions can be attributed to $[(CH_3)_2COH]^+$ and $[C(OH,CH_3)CH{=}CH_2]^+$ fragment ions, respectively, and are indicative of the presence of two tertiary OH groups, as is the case for peak 3 (2,6-dimethylocta-3,7-diene-2,6-diol.), and analogues of this compound.



m/z 188

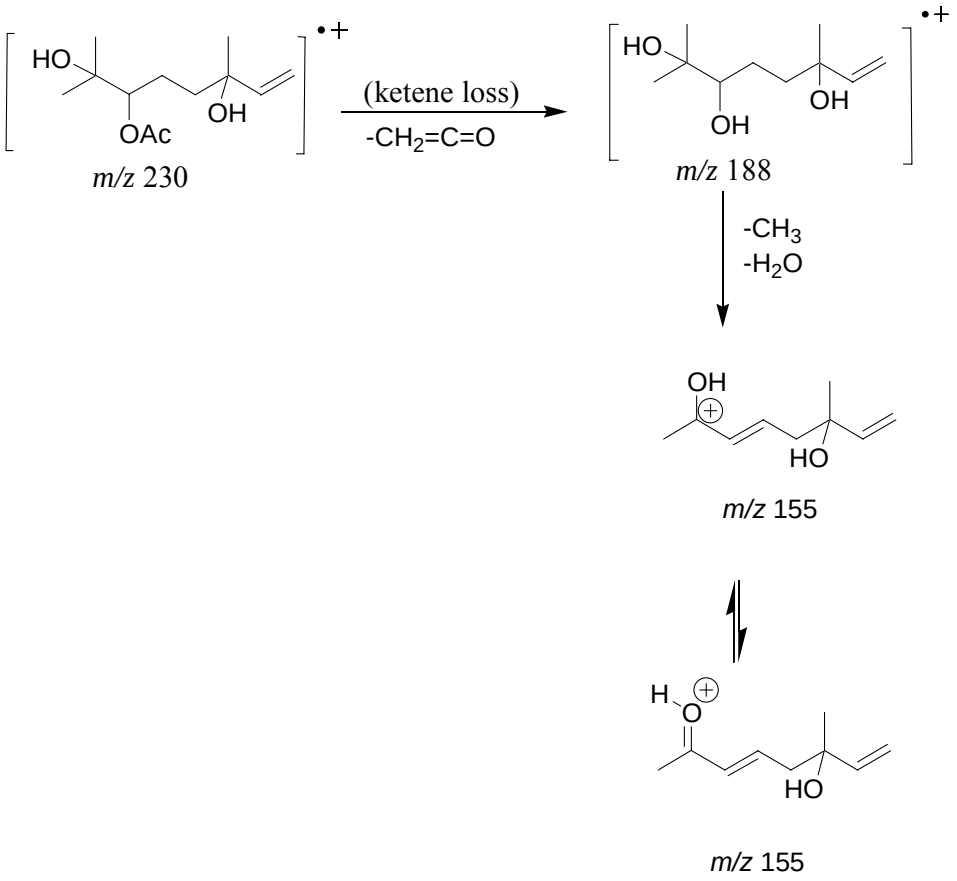
Moderate intensity *m/z* 155 ions can be attributed to $[M - CH_3 - H_2O)]^+$ fragments arising from compounds of molecular formula $C_{10}H_{20}O_3$ (*M* = 188 Daltons). These data are consistent with the proposal that peaks 15 and 16 are diastereoisomeric 2,6-dimethyloct-7-ene-2,3,6-triols [**4**] which are isomeric at C-3 and C-6 (*RR*/SS and *SR*/*RS*) (Williams *et al*., 1980b; Vidari *et al*., 1993). It was not possible to determine which enantiomeric forms of these compounds (*e.g. RR*, *SS*, *RS* and/or *SR*) (4 enantiomers = 2 pairs of GC resolvable diasterisomers) were present in the extracts.

D'Arcy *et al*. (1997) have reported the presence of a pair of peaks (peaks 56 and 57) in Australian blue gum and yellow box honey extracts which exhibited similar MS characteristics to those determined for peaks 15 and 16.



[**4**]

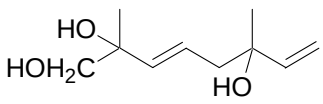
Acetylation of peaks 15 and 16 afforded only monoacetates, as evidenced by ions which appeared at *m/z* 197 (assigned as M- CH_3 - H_2O ions from $C_{12}H_{22}O_4$) (M = 230 Daltons). Pronounced *m/z* 59 and 71 fragments attributable to $[(CH_3)_2COH]^+$ and $[C(OH,CH_3)CH=CH_2]^+$ ions respectively are consistent with the presence of tertiary C-2 and C-6 hydroxyl groups which will not be acetylated with acetic anhydride/pyridine.



The levels of peaks 15 and 16 in the kamahi honey extracts were in the range 1.3 to 9.7 mg/kg (average level 4.6 mg/kg) and 1.8 to 16 mg/kg (average level 8.3 mg/kg) respectively.

3.3.8. 2,6-Dimethylocta-3,7-diene-1,2,6-triol (Peaks 7 and 9) [5]

Relatively low amounts of peaks 7 and 9 (3.7 and 2.3 mg/kg respectively) were detected in the kamahi honey extracts. These peaks were tentatively identified as 2,6-dimethylocta-3,7-diene-1,2,6-triol isomers [**5**].



[**5**]

Peaks 7 and 9 afforded mass spectra which included moderate intensity *m/z* 75 ions, (proposed structure $[CH_3(CH_2OH)COH]^+$) together with *m/z* 43, 55, 67, 68, 81, 93, 111, 137, 155 $(M\text{-}CH_2OH)^+$, and 171 $(M\text{-}CH_3)^+$ ions (Figure **3.11)**.

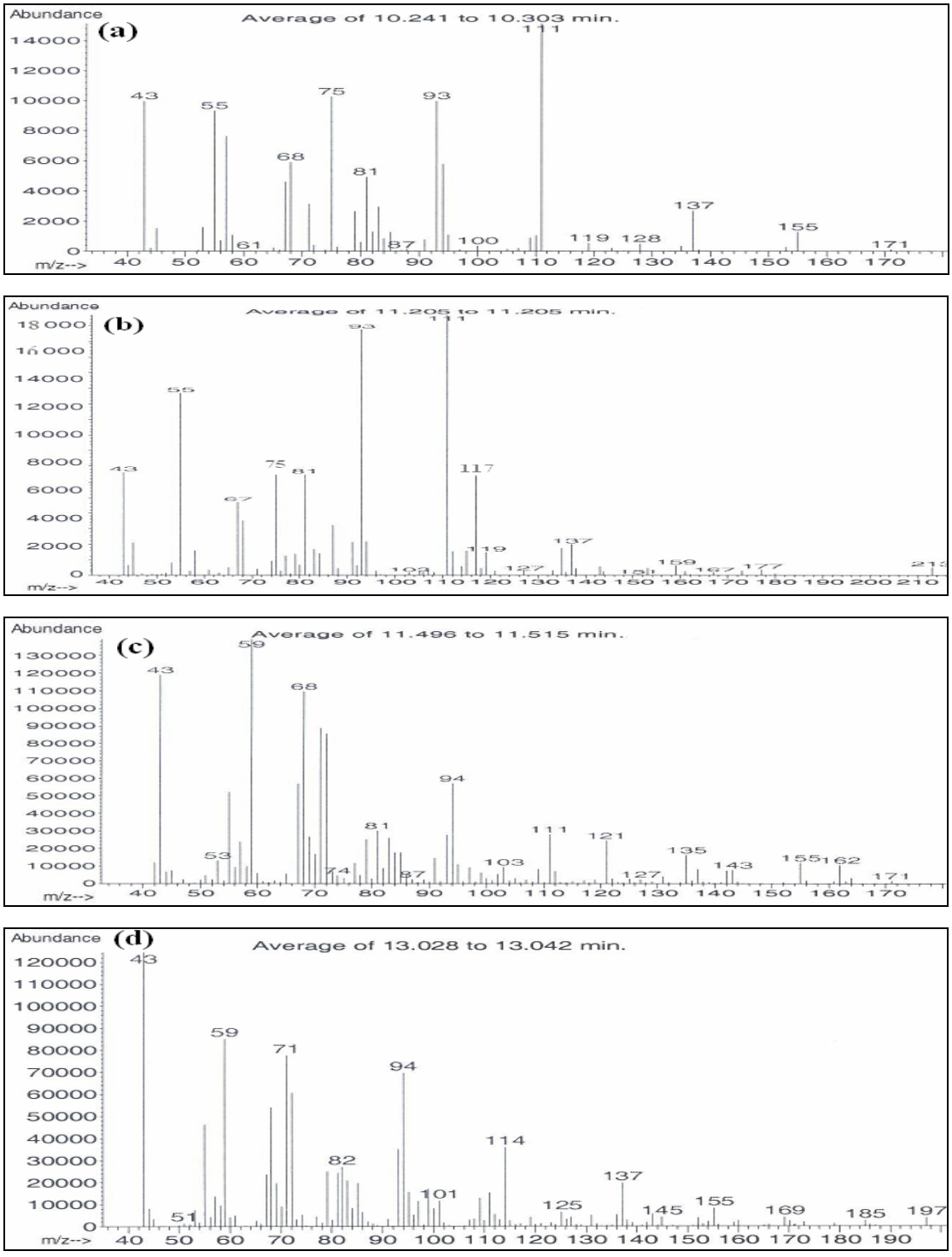


Figure 3.11. Mass spectra of (**a**) peak 9, (**b**) acetylated peak 9, (**c**) peak 15, and (**d**) acetylated peak 15.

The *m/z* 75 ion is particularly distinctive, and can be attributed to a hydroxylated version of the *m/z* 59 ion seem in the mass spectrum of peak 3. Also, the foregoing ions are for the most part, analogous to those observed in the mass spectrum of hotrienol (see above) other than for the mass increase attributable to the additional

C-8 substituent. The mass spectral fragment pattern observed for peaks 7 and 9 is consistent with their being isomeric $C_{10}H_{18}O_3$ triols (2,6-dimethylocta-3,7-diene-1,2,6-triol isomers) [**5**].

The mass spectrum of the acetylated analogues of these peaks included moderate *m/z* 117 and *m/z* 75 ions. The *m/z* 117 ions (proposed structure $[CH_3(CH_2OAc)COH]^+$) are attributable to acetylated variants of the *m/z* 75 ions. Loss of ketene from the *m/z* 117 ions affords the *m/z* 75 ions. The highest observed ion in the mass spectra of acetylated peaks 7 and 9 occurred at *m/z* 213. This ion can be attributed to the loss of a methyl radical from a monoacetate of molecular formula $C_{12}H_{20}O_4$ (Figure **3.12**). The mass spectra of the mono-acetylated analogues, like those of the parent triols, showed pronounced *m/z* 43, 55, 67, 68, 81, 93, 111 and 137 ions.

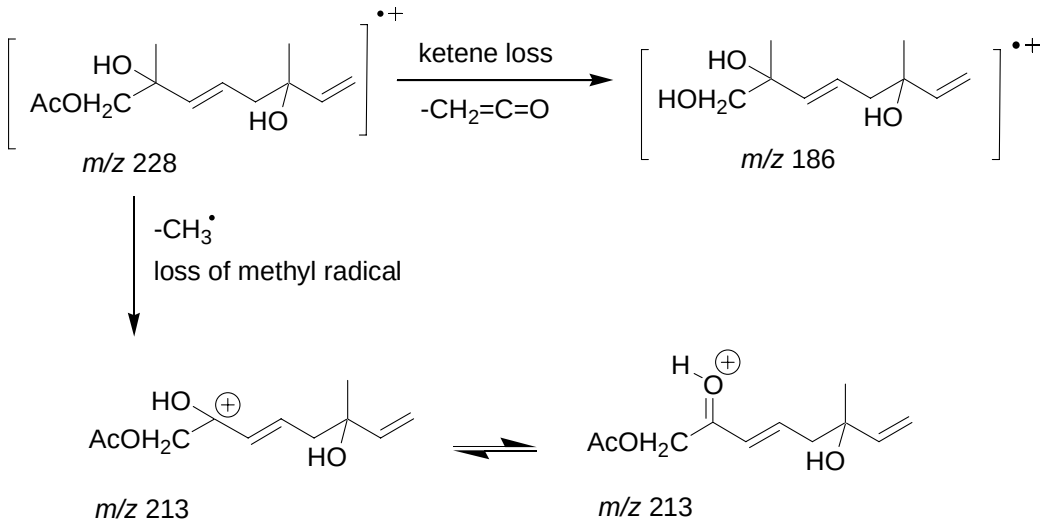


Figure 3.12. Loss of ketene and a mehyl radical from a monoacetylated 2,6-dimethylocta-3,7-diene-1,2,6-triol isomer [**5**] to give the *m/z* 186 and 213 ions respectively.

3.3.9. Peak 30

The mass spectrum of peak 30 showed a strong base peak at *m/z* 83, and significant ions at *m/z* 127, 155 and 179, along with weaker ions at *m/z* 238, 252, 267 and 298 ($M^{+\cdot}$). Acetylation led to the appearance of the highest observed ion at *m/z*

340 ($M^{+\cdot}$). The mass spectra of methylated and acetylated peak 30 are shown in Figure 3.13. This compound is believed to be an unknown analogue of kamahines A, B and C containing one primary or secondary hydroxyl group (as evidenced by the mass increase of the highest observed ion from *m/z* 298 to *m/z* 342). Tan (1989) has previously detected this compound in some pre-1990 kamahi honey samples.

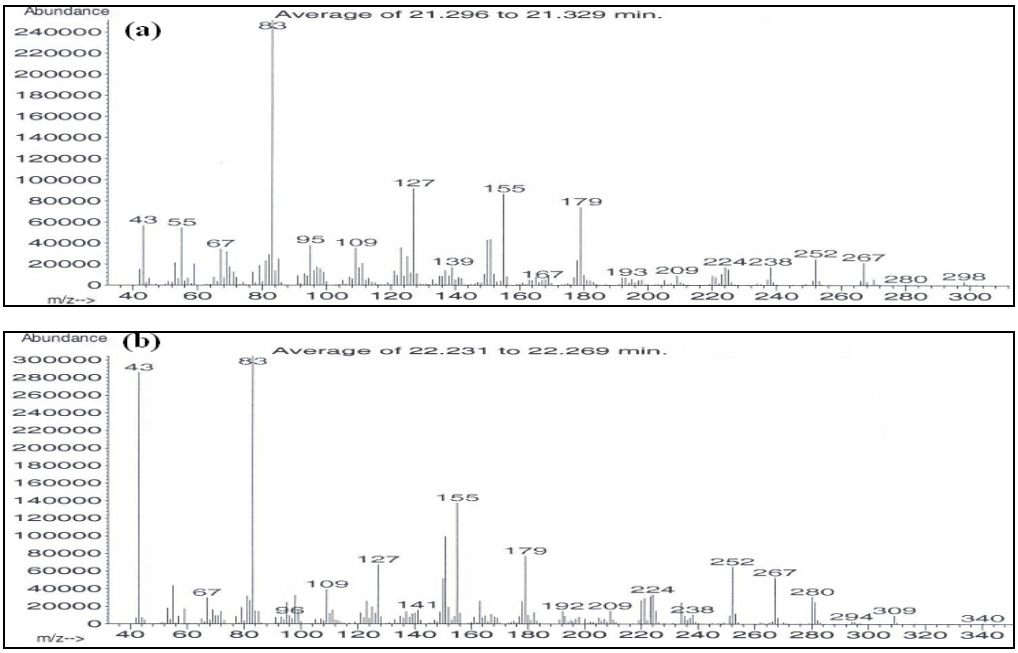


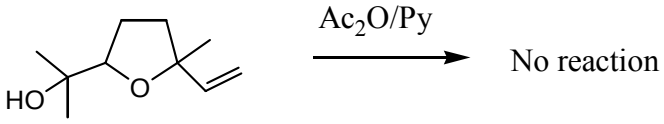
Figure 3.13. Mass spectra of peak 30; (**a**) methylated and (**b**) methylated and acetylated.

A considerable level of peak 30 (average level 14 mg/kg) was detected in all of the kamahi honey extracts. Possibly this compound may serve as a marker floral compound. Tan (1989) reported the average level of 6.7 mg/kg of this compound (as unknown 211) in the 1986 season kamahi honey extracts.

3.3.10. Other Linalool Analogues

In addition to peaks 3, 7, 9, 15, 16, 27, 29 and 30, variable but generally minor, levels of an array of unknown compounds which appeared to be linalool derivatives (possibly lilac aldehyde, lilac alcohol, or linalool analogues) were detected in the extracts. The mass spectra of the unknown compounds included strong *m/z* 43, 71 and 111 ions, reminiscent of those seen in the mass spectra of primary or secondary lilac alcohol, or linalool type compounds (Tan, 1989).

Each of the unknown compounds was believed to possess one or more secondary or primary hydroxyl groups, since acetylation afforded higher molecular weight, and later eluting peaks. The foregoing observations discounted the presence of isomeric lilac analogues possessing an only oxido, or tertiary hydroxyl groups, (for example *cis* and *trans*-furan linalool oxides isomers [**6**] and α,α,5-trimethyl-5-vinyltetrahydrofurfuryl alcohols), since they would not be acetylated using acetic anhydride/pyridine in the absence of dimethylaminopyridine (DMAP) as catalyst.



[**6**]

The series of linalool type compounds detected in the kamahi honey extracts could be the result of either a variety of natural biosynthetic or enzyme-mediated chemical processes, including hydroxylation, epoxidation, oxidation and dehydration processes, mediated by the bee or occurring within the honey, in the plant, or during extraction (see Figure 3.14).

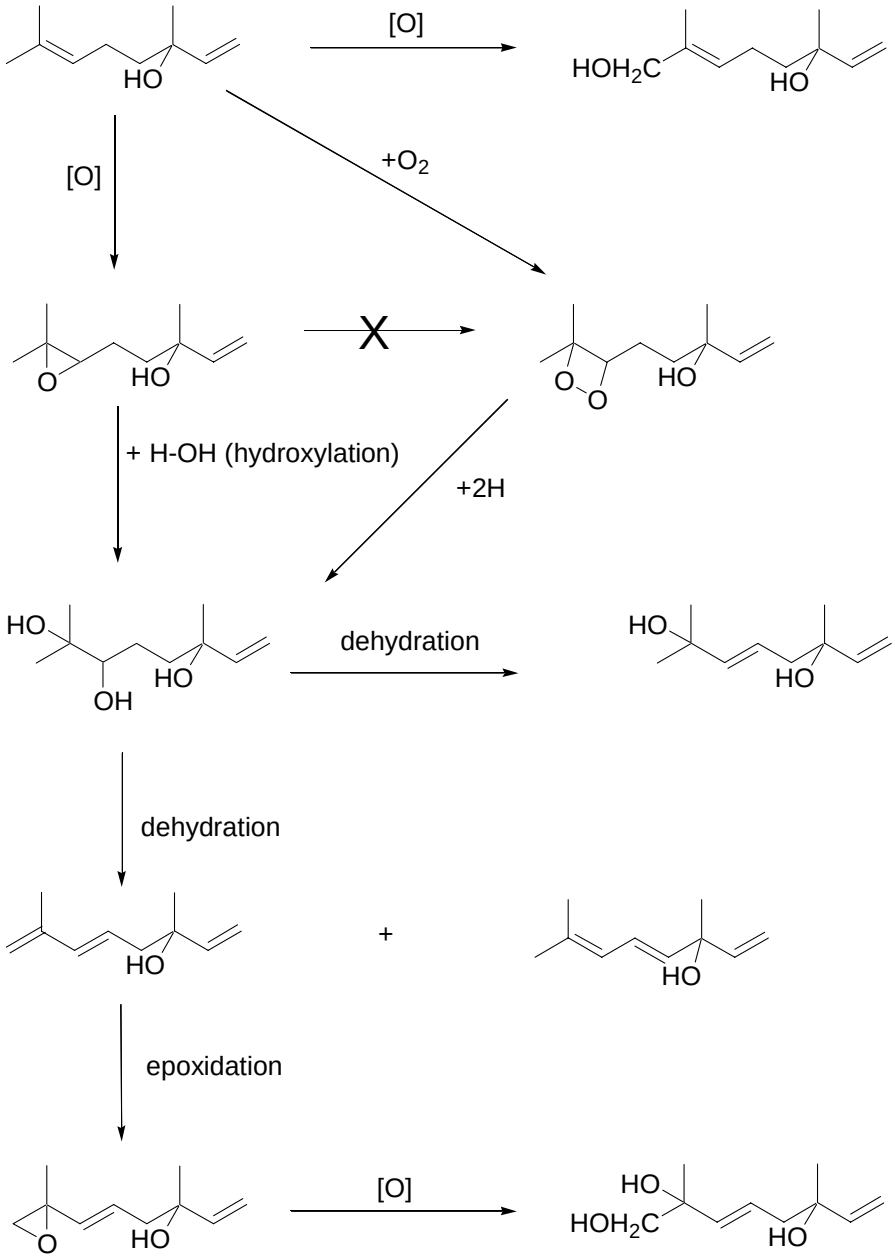


Figure 3.14. Schematic overview of the proposed formation of some linalool derivatives (*via* epoxidation, oxidation, hydroxylation and dehydration process).

3.3.11. Phenyllactic Acid (Peak 11) and 4-Methoxyphenyllactic acid (Peak 24)

Sample 7 had the lowest kamahi pollen contribution (56.0%) and a moderate colour score (36 mm), indicative of a minor input from a dark coloured honey such as manuka (see Table 3.1). The levels of phenyllactic acid (peak 11) and 4-methoxyphenyllactic acid (peak 24) (108 and 207 mg/kg respectively) detected in this sample are consistent with a minor manuka input (Tan *et al*., 1988; Wilkins *et al*., 1993a).

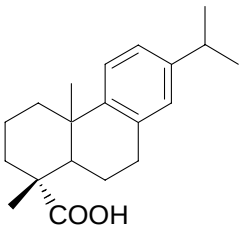
Sample 10 also had a low kamahi pollen contribution (62.0%) and low levels of compounds considered to be kamahi marker compounds (*e.g.* kamahines A-C, meliracemoic acid and 2,6-dimethylocta-3,7-diene-2,6-diol; 47, 9.3 and 16 mg/kg respectively), however this sample exhibited a lesser colour score (22 mm) and lower levels of phenyllactic acid and 4-methoxyphenyllactic acid (5.5 and 6.4 mg/kg respectively) than was the case for sample 7. Sample 3 had the greatest colour score (54 mm), but like sample 10 possessed only low levels of phenyllactic acid and 4-methoxyphenyllactic acid (6.5 and 4.4 mg/kg respectively). This appears to be indicative of a minor input to sample 3 from a dark coloured honey, other than manuka.

Unlike the other extractable organic substances listed in Table 3.4, phenyllactic acid and 4-methoxyphenyllactic acid levels showed high CV's (116% and 159% respectively). This variability can be attributed to appreciable variations in the minor manuka inputs to the kamahi honeys. In all cases the manuka contribution to the samples, as might be inferred from phenyllactic acid and 4-methoxyphenyllactic acid levels, is low and below that which would threaten their unifloral status. Typically > 700 mg/kg of phenyllactic acid and 4-methoxyphenyllactic acid should be present in unifloral grade manuka honey samples (Tan *et al*., 1988; Wilkins *et al*., 1993a).

3.3.12. Other Substances

Other substances detected in the kamahi honey extracts included 2,6,6-trimethyl-2-cyclohexene-1,4-dione (peak 1, average level 4.9 mg/kg), 2-methoxyacetophenone (peak 6, average level 5.8 mg/kg), 4-methoxybenzoic acid (peak 12, average level 8.3 mg/kg), 2-propanone,1-[4-methoxyphenyl] (peak 14, average level 9.5 mg/kg) and abscisic acid (peak 31, average level 3.1 mg/kg). An array of methylated aliphatic acids and diacids including dodecanoic acid (peak 22), nonanedioic acid (peak 23), decanedioic acid (peak 25), tetradecanoic acid (peak 26), hexadecanoic acid (peak 28), heptadecanoic acid (peak 32), stearic acid (peak 33), eicosanoic acid (peak 36), docosanoic acid (peak 37) and tetracosanoic acid (peak 38) were also detected in the kamahi honey extracts.

Unexpectedly, at least when it was first encountered, a low level of dehydroabietic acid (**7**) (peak 35, average level 1.2 mg/kg) was also detected in the extracts of 9 of the 10 kamahi honey samples. Dehydroabietic acid has been found to show acute oral toxicity in rats (LD_{50} of 3000-4000 mg/kg) and no delayed toxicity up to 14 days after administration (Villeneuve *et al*., 1977), thus its presence in the kamahi honey samples at levels in the range trace to 2.2 mg/kg, does not give rise to concern. Dehydroabietic acid (a well known resinous component of woods) may have been, at least in part, absorbed into the honey from wooden (timber) hive boxes.



[**7**]

This is the first report of the presence of dehydroabietic acid in honey extracts. Previously investigations have only reported the identification of methylated extractable organic substances eluting before stearic acid (Tan *et al*., 1988; 1989a; 1989b; 1990; Wilkins *et al*., 1993a; 1993b), however examination of the GC/MS profiles of other honey samples showed dehydroabietic acid (which elutes from GC columns several minutes after stearic acid) to be almost universally present in honey samples at levels comparable to those reported here for the kamahi honeys. Lesser levels of some other resin acids, including abietic acid and pimaric acid, were also detected (but not quantified) in some of the kamahi honey extracts

3.4. Analysis of Bulk Extraction Fractions

Bulk extraction (Section 3.2.2) was carried out in the expectation that some of the more dominant extractives could be isolated in sufficient amount for the structural characterisation using one- and two-dimensional NMR.

The elution order of the compounds on silica gel chromatography plate is almost exclusively dependent on their relative polarities. Thus non-polar compounds were present in early eluting fractions, while polar compounds appeared in later

fractions. Fractions 1, 2 and 3 contained only residual solvent hydrocarbons while the majority of the unknown compounds appeared in fractions 14, 15, 17 and 18.

In addition to the more dominant compounds reported in Table 3.3, lesser amounts of an array of aliphatic compounds, benzene derivatives, degraded carotenoids, methylated hydroxyl fatty acids and unknown oxygenated monoterpenes were detected in some of the chromatotron fractions (see Table 3.6). The identification of minor compounds does not however facilitate the identification of the dominant floral source of the bulked honey sample.

Table 3.6. Dominant compounds detected in fractions 4-18 derived from separation of the methylated diethyl ether extract of the bulked 2002 season kamahi honey sample.

fraction	compound type	compound
4 and 5	fatty acid methyl esters	dodecanoic acid methyl ester (peak 22) tetradecanoic acid methyl ester (peak 26) hexadecanoic acid methyl ester (peak28) eiscosanoic acid methyl ester (peak 36) tetracosanoic acid methyl ester (peak 38)
6	aromatic acid methyl esters with one aryl OMe group	4-methoxybenzoic acid methyl ester (peak 12) unknown[a] (*m/z*: 45, 73, 183, 197 and 256) unknown[a] (*m/z*: 43, 57, 77, 95, 137, 193, 250 and 299)
7	aromatic acid methyl esters with one aryl OMe group	2-methoxyacetophenone (peak 6) 4-methoxybenzoic acid methyl ester (peak 12) 4-methoxyphenylacetic acid methyl ester unknown[a] (*m/z*: 43, 57, 109, 125, 153, 168 and 252)
8	diacid dimethyl esters	nonanedioic acid dimethyl ester (peak 23) decanedioic acid dimethyl ester (peak 25) undecanedioic acid dimethyl ester unknown[a] (*m/z*: 43, 57, 109, 125, 153, 168, and 252)
9	diacid dimethyl esters, aromatic acid methyl esters and ketones	2-methoxybenzoic acid methyl ester, 1-[4-methoxyphenyl]-2-propanone octanedioic acid dimethyl ester nonanedioic acid dimethyl ester (peak 23) decanedioic acid dimethyl ester (peak 25)
10	aromatic acid methyl esters with 2 or more aryl OMe groups, and alcohols	methyl syringate hotrienol unknown[a] (*m/z*: 43, 57, 83, 111 and 153) unknown[a] (*m/z*: 43, 57, 165 and 180)
11	aromatic acid methyl esters with 2 or more aryl OMe groups, and alcohols	methyl syringate 3,4-dimethoxybenzoic acid methyl ester hotrienol unknown[a] (*m/z*: 43, 55, 97, 112, 125, 126 and 157) unknown[a] (*m/z*: 43, 59, 75, and 85) unknown[a] (*m/z*: 43, 57, 153, 165, and 196)

Table 3.6. continued (fractions 12-18).

fraction	compound type	compound
12	hydroxy fatty acid methyl esters	3-hydroxy-decanoic acid methyl ester 2-methoxy phenyllactic acid methyl ester (peak 24) unknown[a] (*m/z*: 43, 55, 97, 112, 125, 126 and 157)
13	oxygenated monoterpenes	2-methoxyphenyllactic acid methyl ester (peak 24) unknown[a] (*m/z*: 43, 55, 67, 95, 111, 169) (peak 34) unknown[a] (*m/z*: 43, 68, 134, 111 and 170) unknown[a] (*m/z*: 43, 55, 67, 111, 139 and 198)
14	kamahine analogues, diketones and oxygenated monoterpenes	kamahines A, B and C (peak 29) meliracemoic acid methyl ester (peak 27) 2,6,6-trimethyl-2-cyclohexene-1,4-dione (peak 1) phenyllactic acid methyl ester (peak 11) 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one unknown[a] (*m/z*: 43, 59, 72, 104 and 148) unknown[a] (*m/z*: 43, 55, 71, 99, 139, 152 and 168) unknown[a] (*m/z*: 43, 55, 83, 109, 155 and 179) (peak 30)
15	kamahine analogues diketones and oxygenated monoterpenes	kamahines A, B and C (peak 29) melirecemoic acid methyl ester (peak 27) 2,6,6-trimethyl-2-cyclohexene-1,4-dione (peak 1) phenyllactic acid methyl ester, (peak 11) 2-methoxyphenyllactic acid methyl ester (peak 24) 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one unknown[a] (*m/z*: 43, 59, 72, 104 and 148) unknown[a] (*m/z*: 43, 55, 67, 111, 153 and 198) unknown[a] (*m/z*: 43, 55, 74, 83, 95, 109, 127 and 155, 179) (peak 30) unknown[a] (*m/z*: 68, 96, 110, 170 and 183), unknown[a] (*m/z*: 43, 55, 71, 99, 139, 152 and 168).
16	hydroxy acid methyl esters and diketones	phenyllactic acid methyl ester (peak 11) 2-methoxyphenyllactic acid methyl ester (peak 24)
17	diols, hydroxy acid methyl esters and hydroxy ketones	2,6-dimethylocta-3,7-diene-2,6-diol (peak 3) 2,6-dimethylocta-2,7-diene-1,6-diol (peak 10) 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one unknown[a] (*m/z*: 43, 55, 71 and 84) unknown[a] (*m/z*: 43, 59, 71, 88 and 103) (peak 4) unknown[a] (*m/z*: 43, 55, 69, 83, 98, 111, 126 and 168)
18	diols and triols	2,6-dimethylocta-3,7-diene-2,6-diol (peak 3), 2,6-dimethylocta-3,7-diene-1,2,6-triol (peaks 7/9)[b] 2,6-dimethyloct-7-ene-2,3,6-triol (peaks 15/16)[b] unknown[a] (*m/z*: 43, 55, 67, 71, 82, 93, 111 and 155) unknown[a] (*m/z*: 43, 59, 60, 69 and 118) unknown[a] (*m/z*: 43, 57, 69, 85, 99, 139 and 154) unknown[a] (*m/z*: 43, 55, 111, 123 and 168) (peak 19)

[a]prominent ions observed in the mass spectra of unknown compounds are given in brackets; [b]tentative identification.

Fatty acid methyl esters were identified as dominant compounds in fractions 4 and 5. Fraction 6 contained low quantities of aromatic acid methyl esters with one aryl methoxyl group while fraction 7 contained predominantly 4-methoxyphenylacetic acid methyl ester (~ 60%) along with some other aromatic acid methyl esters including 4-methoxybenzoic acid methyl ester.

Fraction 8 contained mainly nonanedioic acid methyl ester (~ 50%), and decanedioic acid methyl ester (~ 20%) and lesser levels of other fatty acid methyl esters. Fraction 9 was comprised of mainly of nonanedioic acid methyl ester (~ 45 %) and octanedioic acid methyl ester (~ 25%), together with some aromatic acid methyl esters (~ 10% of 2-methoxybenzoic acid methyl ester) and ketones (~ 18% of 1-[4-methoxyphenyl]-2-propanone).

A 1:1 mixture of methyl syringate and an unknown compound (*m/z*: 43, 57, 165, 180) were the dominant compounds (~ 90%) of fraction 10, together with a trace level of hotrienol, (dimethylocta-3,7-diene-2,6-diol).

Fraction 11 contained hotrienol, aromatic methyl esters with 2 or more aryl methoxyl groups (methyl syringate and 3,4-dimethoxybenzoic acid methyl ester) and unknown compounds. Comparison of GC/MS data determined for methylated and ethylated sub-samples of the bulk extract showed that methyl syringate was in the original extract [*e.g.* it occurs in kamahi honeys, as is also the case for manuka and erica honeys, as the methyl ester rather than the parent acid (Tan, 1989; Chapter 5)]. An unknown compound (*m/z* 43, 55, 69, 83, 97, 112, 125, 126, 157, 282) and trace levels of some hydroxylated fatty acid methyl esters (*e.g.* 3-hydroxydecanoic acid methyl ester) were present in fraction 12.

Fraction 13 contained predominantly compounds believed to be linalool analogues which showed strong *m/z* 71 ions. The major constituent of fraction 13 showed *m/z* 43, 55, 67, 111, 139, 198 ions.

Fractions 14 and 15 contained mainly kamahines A, B, and C, meliracemoic acid methyl ester and an unknown compound (peak 30) believed to be a kamahine analogue (*m/z* 43, 55, 74, 83, 95, 109, 127, 155, 179). Additionally, modest levels of

phenyllactic acid methyl ester, 2-methoxyphenyllactic acid methyl ester and 2,6,6-trimethyl-2-cyclohexene-1,4-dione, together with low levels of 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one and some other degraded carotenoid-like compounds were present in fractions 14 and 15. Fraction 16 was comprised predominantly of a 1:3 mixture of phenyllactic acid methyl ester and 2-methoxyphenyllactic acid methyl ester.

GC/MS analyses showed that fractions 17 and 18 contained elevated levels of a linalool analogue which was subsequently identified by NMR analyses as 2,6-dimethylocta-3,7-diene-2,6-diol (see Section 3.3.4), together with lesser levels of a number of other minor compounds including phenyllactic acid methyl ester, 2-methoxyphenyllactic acid methyl ester, 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one (*m/z* 43, 147, 162, 204), (Tan *et al*., 1989a; 1989b), dimethylocta-2,7-diene-1,6-diol, 2,6-dimethylocta-3,7-diene-1,2,6-triol, 2,6-dimethyloct-7-ene-2,3,6-triol and some unknown linalool-like compounds.

3.5. Conclusions

The data reported here, when considered alongside those reported by Tan and others, (Ede *et al*., 1993; Broom, 1992; 1995) show that New Zealand kamahi honey is characterised by the presence of 2,6-dimethylocta-3,7-diene-2,6-diol, meliracemoic acid, and kamahines A-C and that these compounds should typically be present in unifloral grade kamahi honey at average levels of 31, 14, and 73 mg/kg respectively. These compounds are proposed as floral marker compounds for New Zealand kamahi honey. Lesser levels of a series of oxygenated monoterpenes, tentatively identified as trihydroxy linalool analogues, are also present in kamahi honey extracts.

Apart from the unique compounds, others substances detected in kamahi honey extracts include 2-methoxyacetophenone (average level 5.8 mg/kg), phenyllactic acid (average level 30 mg/kg), 4-methoxybenzoic acid (average level 8.3 mg/kg), 2-methoxyphenyllactic acid (average level 44 mg/kg), abscisic acid (average level 3.1 mg/kg), dehydroabietic acid (average level 1.2 mg/kg) and common mono and diacids; for example dodecanoic acid (average level 3.7 mg/kg), hexadecanoic acid (average level 38 mg/kg) and nonanedioic acid (average level 6.6 mg/kg).

Some of the characteristic manuka honey compounds (such as phenyllactic acid and 4-methoxyphenyllactic acid) were also observed in the kamahi honey extracts, but they were detected in much lower levels than in manuka honey extracts.

Summary

The principal conclusions of this investigation are:

(i) New Zealand kamahi honey is characterised by the presence of 2,6-dimethylocta-3,7-diene-2,6-diol, meliracemoic acid, and kamahines A-C at average levels of 31, 14, and 73 mg/kg respectively;

(ii) lesser levels of a series of oxygenated monoterpenes, tentatively identified as linalool analogues, are also present in kamahi honey extracts; and

(iii) dehydroabietic acid, a resinous woody compound is reported for the first time in honey samples.

Chapter Four

Extractable Organic Substances from New Zealand (South Island) Beech (*Nothofagus* spp.) Honeydew Honey

Chapter Four

Extractable Organic Substances from New Zealand (South Island) Beech (*Nothofagus* spp.) Honeydew Honey

4.1. Introduction

Honeydew is sweet liquid excreted by hemipterous insects (plant aphids and scale insects) feeding on plants. New Zealand honeydew honey, a relatively dark honey, is one of New Zealand's premium export honeys.

In the South Island of New Zealand, honeydew honey is derived from hives located adjacent to two species of beech tree, Black Beech (*Nothofagus solandri var solandri*) and Red Beech *(N. fusca*) inhabited by two species of honeydew insect *(Ultracoelostoma assimile and U. brittini,* sooty beech scales) which feed on the beech tree sap from phloem cells (Moller and Tilley, 1986). *Ultracoelostoma brittini* tends to typically inhabit the lower trunks and larger branches, while *U. assimile* favour the upper canopy. The black colour of trees and plants with a honeydew source is due to the growth of a black sooty mould (*Capnodium fungus*) on the surplus nectar exuding over the tree. Manuka is also known to be a honeydew source. In this case the principal scale insects are *Eriococcus orariensis* and some *Coelostomidia* spp.

To date, only limited information is available in respect of the identification of dominant organic constituents of the extracts of New Zealand beech honeydew honeys. Hitherto Hyink (1998) has reported some data for eight 1994-97 season samples.

The principal objectives of the work reported in this chapter were to define the typical chemical fingerprints (GC/MS profile) and determine the levels of extractable organic substances present in ten 2002 season New Zealand commercial unifloral grade honeydew honey samples supplied by Airborne Honey Ltd., Leeston, Canterbury, New Zealand, and to prepare for publication a definite

account of the extractable organic compounds from New Zealand beech honeydew honeys.

4.2. Experimental

4.2.1. Extraction of Honey Samples

Ten 2002 season unifloral grade South Island beech honeydew samples were provided by Airborne Honey Ltd., Leeston. These samples were extracted and methylated with an ethereal solution of diazomethane using the extraction procedure described in Sections 2.2.1 and 2.3 (Chapter 2).

4.2.2. Ethylated Sub-samples

Prior to methylation of the diethyl ether extracts of honeydew honey samples, two sub-samples were ethylated with an ethereal diazoethane solution prepared as described in Section 2.3.1.

4.3. Results and Discussion

New Zealand beech honeydew honeys, which are clear dark brown liquids, can readily be distinguished from the other honeys by their organoleptic and physical properties including a water content of less than 17%, conductivity in the range 10-15 mS/cm and Pfund scale colour scores around 87 mm (Airborne Honey, 2004). Honeydew honeys have higher fructose and lower glucose levels, and an array of oligosaccharides at levels greater levels than those found for other plant sourced New Zealand honeys (Astwood *et al*., 1998).

The levels of compounds identified in the methylated diethyl ether extracts of the ten 2002 season beech honeydew honey samples analysed in this investigation are given in Table 4.1. The concentrations of resin acids found in these samples are reported in Table 4.2. The mean concentrations, standard deviations and coefficients of variances of the methylated extractable organic substances detected in extracts of the ten 2002 season and the eight 1994-97 season samples examined by Hyink (1998) are compared in Table 4.3.

The GC/MS profile of a representative methylated honeydew honey diethyl ether extract is presented in Figure 4.1 (peak identifications are given in Table 4.1). Acids and some phenols were detected as their methyl ester, or methyl ether derivatives (Tan *et al*., 1988; 1989b), unless otherwise indicated. Comparison of ethylated and methylated diethyl ether extracts showed that methyl syringate (but not syringic acid) was present in the extracts.

With the exception only of some resin acids, compounds eluting after stearic acid (peak 27) were found to be higher chain length hydrocarbons or fatty acids (detected as the corresponding methyl esters). These compounds have previously been identified as primarily constituents of beeswax (Tulloch and Hoffmann, 1972; Graddon *et al.*, 1979; Bonaga *et al*., 1986; Tan *et al*., 1988). Details of their characterization and concentrations are not presented here.

Table 4.1. Concentrations (mg/kg) of components detected in diethyl ether extracts of ten 2002 season New Zealand beech honeydew honey samples. Acids were quantified as the corresponding methyl esters.

		sample number									
peak	**compound**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	nicotinic acid	1.3	0.5	1.1	-	2.8	0.8	0.5	-	-	4.7
2	phenylacetic acid	14	48	37	75	35	38	32	39	18	26
3	salicylic acid	5.5	8.0	11	23	11	15	9.2	3.6	4.4	9.6
4	nonanoic acid	1.7	3.0	1.9	4.1	2.0	2.5	1.2	1.8	1.4	1.9
5	hexanedioic acid	0.7	-	0.3	1.4	0.9	0.2	0.2	-	-	1.0
6	2-methoxyacetophenone	2.4	-	0.9	5.1	7.8	6.1	1.5	19	6.6	3.0
7	decanoic acid	0.5	0.8	0.7	2.0	0.6	0.8	0.6	-	0.4	0.7
8	2-methoxybenzoic acid	1.6	1.2	2.7	9.7	7.0	6.0	2.4	13	8.1	4.7
9	heptanedioic acid	1.0	0.2	0.6	2.6	1.1	0.3	-	-	0.4	1.1
10	phenyllactic acid	136	4.9	40	313	179	103	51	263	430	127
11	4-methoxybenzoic acid	5.6	3.5	7.4	12	7.8	7.6	4.5	-	-	3.3
13	2-methoxyphenylacetic acid	-	1.4	1.0	3.1	0.8	0.7	0.6	-	-	-
14	4-methoxyphenylacetic acid	4.1	3.7	8.5	23	11	11	4.9	9.5	11	4.1
15	octanedioic acid	3.1	1.1	2.2	11	4.5	1.9	0.6	-	1.2	5.0
16	4-hydroxyphenylacetic acid	-	1.9	4.9	16	5.2	6.4	2.1	-	3.6	7.4
17	dodecanoic acid	0.7	1.3	1.9	1.7	1.0	1.4	0.9	0.6	0.8	0.9
18	nonanedioic acid	8.7	3.3	5.4	11	8.5	4.0	1.7	4.3	9.0	9.0
19	3,4-dimethoxybenzoic acid	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
20	4-methoxyphenyllactic acid	276	2.3	36	371	265	126	94	493	814	133
21	decanedioic acid	4.1	3.2	4.3	9.8	5.4	3.0	2.9	4.7	2.6	7.5
22	methyl syringate	35	0.7	1.6	39	37	33	24	133	123	33
23	myristic acid (14:0)	-	2.0	4.7	-	-	-	-	-	-	-
24	indole-3-acetic acid	0.9	5.6	8.3	9.1	2.6	3.4	1.1	4.5	-	5.0
25	palmitic acid	2.7	-	-	11	4.2	6.5	8.4	5.3	5.3	5.1
26	*trans,cis*-abscisic acid	-	tr	tr	-	tr	tr	2.0	3.0	tr	7.2
27	stearic acid (18:0)	1.2	2.3	3.5	9.5	2.0	2.7	33	2.1	2.6	2.4

tr = trace

Table 4.2. Concentrations (mg/kg) of resin acids detected in the diethyl ether extracts of ten 2002 season New Zealand beech honeydew honey samples. Acids were quantified as the corresponding methyl esters.

sample	pimaric	sandaracopimaric	isopimaric	dehydroabietic
1	tr	tr	tr	2.3
2	tr	tr	tr	1.7
3	0.7	0.2	tr	4.0
4	3.6	0.5	4.9	12
5	tr	tr	tr	3.2
6	tr	tr	tr	2.9
7	tr	tr	tr	2.2
8	tr	tr	tr	3.9
9	tr	tr	tr	1.8
10	tr	tr	tr	2.1

tr = trace

Table 4.3. Mean concentration (mg/kg), standard deviation (stdev), and coefficient of variance (CV%) determined for compounds identified in the diethyl ether extracts of ten 2002 season and eight 1994-1997 season[a] honeydew honey samples. Acids were detected and quantified as the corresponding methyl esters.

		2002 season				1994-1997 season[a]			
	compound	Mean	SD	n	CV%	Mean	SD	n	CV%
1	nicotinic acid	1.7	1.5	7	93	-	-	-	-
2	phenylacetic acid	36	17	10	47	27	7.5	8	28
3	salicylic acid	10	5.7	10	57	11	3.2	8	30
4	nonanoic acid	2.2	0.9	10	40	1.9	0.8	4	44
5	hexanedioic acid	0.7	0.5	7	69	-	-	0	-
6	2-methoxyacetophenone	5.8	5.5	9	94	5.5	1.3	2	25
7	decanoic acid	0.8	0.5	9	60	-	-	0	-
8	2-methoxybenzoic acid	5.6	3.9	10	69	2.8	1.0	4	35
9	heptanedioic acid	0.9	0.8	8	85	-	-	0	-
10	phenyllactic acid	165	134	10	82	30	25	8	85
11	4-methoxybenzoic acid	6.4	2.8	8	44	-	-	-	-
13	2-methoxyphenylacetic acid	1.3	0.9	6	74	-	-	-	-
14	4-methoxyphenylacetic acid	9.0	5.8	10	64	2.6	0.8	4	32
15	octanedioic acid	3.4	3.2	9	95	2.4	1.5	4	63
16	4-hydroxyphenylacetic acid	5.9	4.5	8	76	8.3	4.1	6	50
17	dodecanoic acid	1.1	0.4	10	39	5.7	2.5	8	44
18	nonanedioic acid	6.5	3.1	10	48	4.9	0.9	4	17
19	3,4-dimethoxybenzoic acid	-	-	9[b]	-	1.0	0.2	2	22
20	4-methoxyphenyllactic acid	261	248	10	95	19	22	8	113
21	decanedioic acid	4.8	2.3	10	48	-	-	-	-
22	methyl syringate	46	45	10	99	9.0	7.4	7	82
23	myristic acid (14:0)	3.4	1.9	2	57	1.4	0.4	7	31
24	indole-3-acetic acid	4.5	2.9	10	64	9.0	2.6	6	29
25	palmitic acid	6.1	2.6	8	43	6.3	2.2	8	35
26	*trans,cis*-abscisic acid	4.1	2.8	3	68	5.9	6.2	3	106
27	stearic acid (18:0)	6.1	9.7	10	159	2.5	1.4	8	56

[a]data taken from Hyink (1998); [b]trace in 9 other samples.

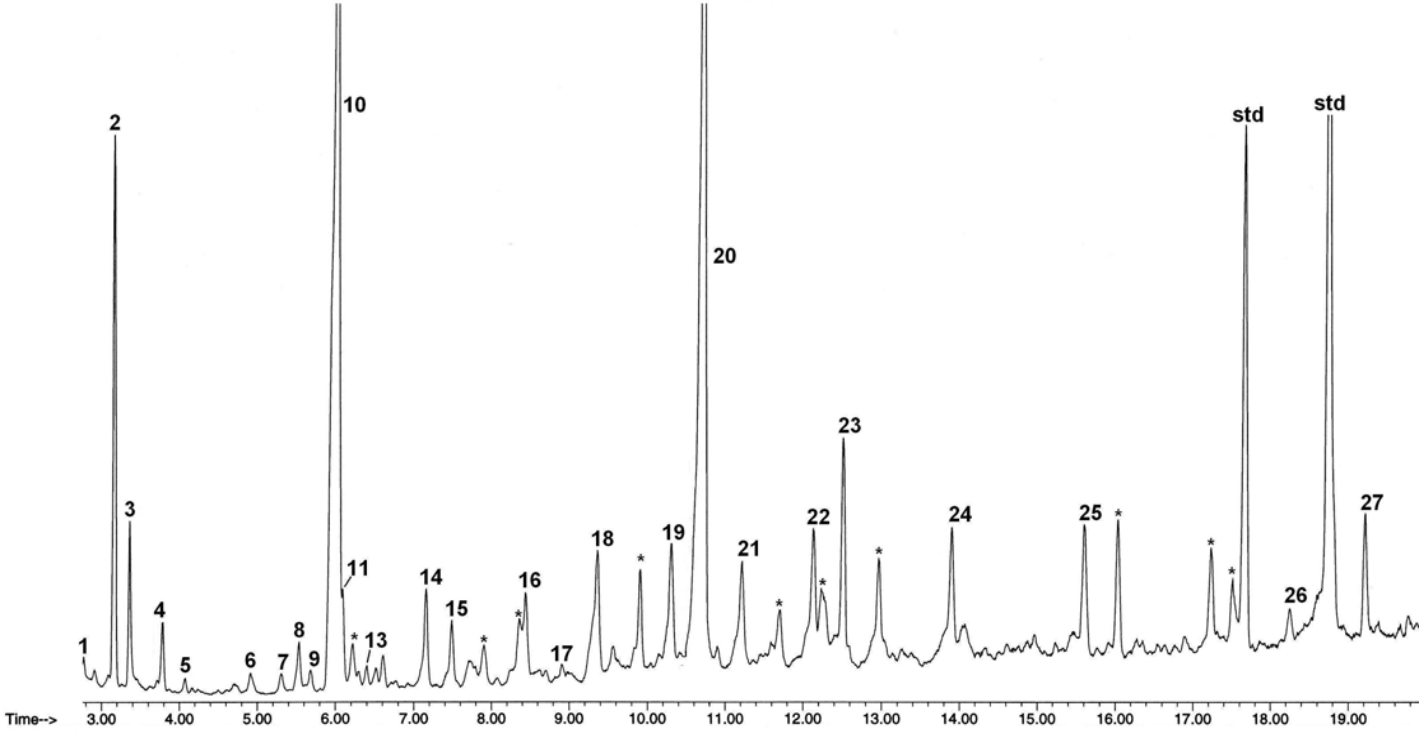
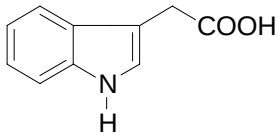


Figure 4.1. GC/MS profile of a representative methylated New Zealand beech honeydew honey diethyl ether extract. Peak identifications are given Table 4.3. Peaks marked with "*"originate from phthalates and/or solvent stabilizers**.**

The principal peaks identified in the methylated diethyl ether honeydew honey extracts were the methyl esters (or methyl ethers) of 4-methoxyphenyllactic acid (2-hydroxy-3-(4'-methoxyphenyl)propanoic acid) (peak 20) (261 mg/kg) and phenyllactic acid (2-hydroxy-3-phenylpropanoic acid) (peak 10) (165 mg/kg). Generally, by relatively high levels of methyl syringate (peak 22) (46 mg/kg), phenylacetic acid (peak 2) (36 mg/kg) and moderate to low levels of salicylic acid (peak 3) (10 mg/kg), 4-hydroxyphenylcetic acid (peak 16) (5.9 mg/kg), indole acetic acid (peak 24) (4.5 mg/kg) and *trans,cis*-abscisic acid (peak 26) (4.1 mg/kg) were found.

4.3.1. Indole Acetic Acid [8]

The only recognizably distinct peak present in the methylated honeydew honey profile, excluding peaks typically found in other New Zealand unifloral honeys (Tan *et al*., 1988, 1989a, 1989b, 1990; Wilkins *et al*., 1993a, 1993b) was the methyl ester of indole acetic acid (peak 24). Relatively low to moderate levels of indole acetic acid methyl ester, ranging from 0.9 to 9.1 mg/kg (stdev 2.9 mg/kg), were detected in the methylated diethyl ether extracts of the 2002 season honeydew honey samples. The level of this compound (mean 4.5 mg/kg) found in the 2002 season honeydew honey samples was lower than the samples collected during 1994-1997 season honeydew honey samples (9.0 mg/kg) (Hyink, 1998).



(8)

Hyink (1998) initially identified indole acetic acid methyl ester by comparison of its mass spectrum with the NIST 98 library mass spectrum and subsequently confirmed his identification by ^{1}H and ^{13}C NMR analyses of a methylated specimen of this compound isolated from the methylated honeydew honey extracts. Indole acetic acid methyl ester possessed the mass spectrum depicted in Figure 4.2 (a) with the most dominant ion at *m/z* 130 and the highest observable ion at 189 $[M]^{\cdot+}$ together with

some weak ions at *m/z* 51, 77 and 103. The strong *m/z* 130 ion can be attributed to loss of a $COOCH_3$ radical from the molecular ion *m/z* 189 $[M]^{\cdot+}$.

The GC/MS profile of an ethylated sub-sample of a honeydew honey extract indicated that this compound was present in the extracts as the acid since the molecular ion (*m/z* 203) of indole acetic acid ethyl ester was observed in the mass spectrum of ethylated compound (Figure 4.2 (b)).

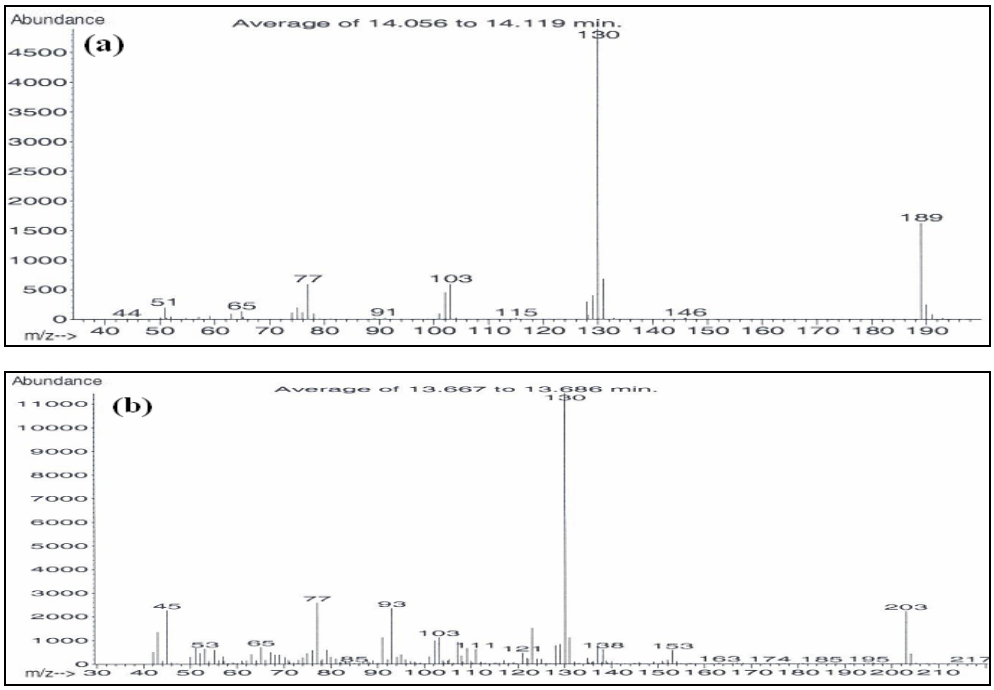
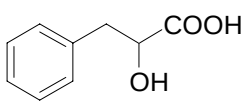


Figure 4.2. The mass spectra of (a) methylated and (b) ethylated indole acetic acid.

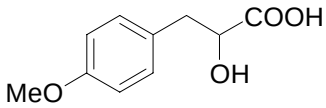
4.3.2. Phenyllactic Acid (Peak 10) [9] and 4-Methoxyphenyllactic Acid (Peak 20) [10]

Appreciable levels of the compounds phenyllactic acid (peak 10) (**9**) (average levels of 165 mg/kg) and 4-methoxyphenyllactic acid (peak 20) (**10**) (average levels of 261 mg/kg) were observed in the extracts of all of the samples. Hitherto these compounds have been identified as major constituents of manuka honey extracts (Tan *et al*., 1988; Wilkins *et al*., 1993b). The mean levels of these compounds in the 1994-1997 season extracts were lower than those found in the 2002 season extracts. Assuming these compounds originate primarily from a minor manuka input to the honeydew honey samples the foregoing observation suggests a lesser manuka nectar (or possibly manuka honeydew) input to the 1994-1997 season samples compared to

the 2002 season samples. The sum of these two components varied between 7.2 and 1244 mg/kg in 2002 season honeydew honey extracts (average level 425 mg/kg) and correlated strongly (R^2 = 0.98) with the total concentration of extractives recovered from each of the honey samples (Figure 4.3). The sum of the concentrations of phenyllactic acid (peak 10) and 4-methoxyphenyllactic acid (peak 20) represented 73% (with a coefficient of variation of 32%) of the total extractable organic substances present in the 2002 season extracts, and 32% (with a coefficient of variation of 52%) of the total extractive organic substances present in the 1994-1997 season samples (Table 4.4).



(**9**)



(**10**)

The average combined levels of phenyllactic acid and 4-methoxyphenyllactic acid found in the honeydew honey extracts (425 and 49 mg/kg in 2002 samples and the 1994-1997 samples respectively) are lower than those found (for example) in some 1989 unifloral grade New Zealand manuka honey extracts (combined average level 893 mg/kg) (Tan *et al*., 1988; Wilkins *et al*., 1993b) (Table 4.4).

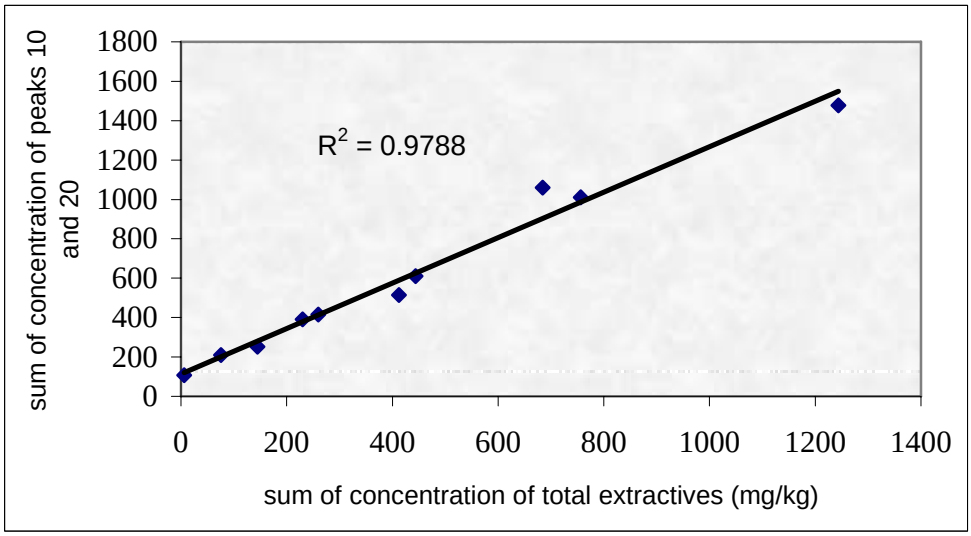


Figure 4.3. Plot of concentration of the sum of phenyllactic acid (peak 10) and 4-methoxyphenyllactic acid (peak 20) *vs* the total concentration of extractable substances recovered from ten 2002 season honeydew honeys.

Table 4.4. Mean concentrations and ranges (mg/kg), % contribution, coefficient of variance (CV%) and R^2 value of the combined concentration of phenyllactic acid (peak10) and 4-methoxyphenyllactic acid (peak 20) concentrations (mg/kg), relative to total extractives recovered from ten 2002 season, eight 94/95-96/97 season honeydew honey samples and fourteen manuka honey samples (1989-90 season)[a].

	honeydew 2002	**honeydew 94/95-96/97**	**manuka[a] 1989**
mean (mg/kg)	425	49	893
range (mg/kg)	7.2-1244	7.8-113	511-1216
% total extractives	73	32	73
CV(%)	32	52	6
correlation to total extractives: R^2	0.99	0.88	0.94

[a]data taken from Wilkins *et al*., (1993b).

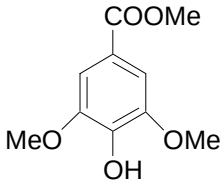
The level of phenyllactic acid detected in New Zealand honeydew honey extracts (4.9-430 mg/kg) is higher than that found in New Zealand ling-heather honey (2-7 mg/kg) (Tan *et al*., 1989b). Comparatively high levels of this compound have been found in European heather honeys (205-585 mg/kg) (Steeg and Montag, 1988).

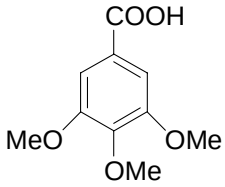
Ehrlich and Jacobsen (1911) have demonstrated that phenyllactic acid can be produced by the action of microganisms on phenylalanine. Hodges and White (1966) reported the presence of (+)-phenyllactic acid in New Zealand honeys and showed that, at least in manuka honey, it was a metabolite of L-phenylalanine. Subsequently Wilkins *et al*., (1993a) have proposed that 4-methoxyphenyllactic acid might be a metabolite of tyrosine which has also undergone methylation of the phenolic hydroxyl group.

4.3.3. Methyl Syringate (Peak 22) [11a]

Significant levels of methyl syringate (**11a**) were observed in the 2002 season samples (46 mg/kg) while moderate levels of this compound were detected in 1994-1997 season samples (9.0 mg/kg). Hitherto Tan *et al*., (1988) have reported methyl syringate to be present in appreciable levels in New Zealand manuka honeys (26-470 mg/kg). Methyl syringate is also abundant in rape and clover honeys (Joerg and Sonntag, 1993).

The level of methyl syringate found in honeydew honey samples is much higher than found in New Zealand ling-heather honey (1.8 mg/kg; Tan *et al*., 1989b), nodding thistle (< 1.5 mg/kg; Wilkins, *et al*., 1993b) and clover honey (nil or trace; Tan *et al*., 1988). The 3,5-dimethoxy-4-hydroxy-substituted aryl moiety of syringic acid is a common structural unit of hard-wood lignin. This leads to the proposal that this type of aromatic component might originate from sap of the tree rather than from nectar (Russell *et al*., 1990).





(**11a**) (Mr = 226 Daltons) (**11b**) (Mr = 226 Daltons)

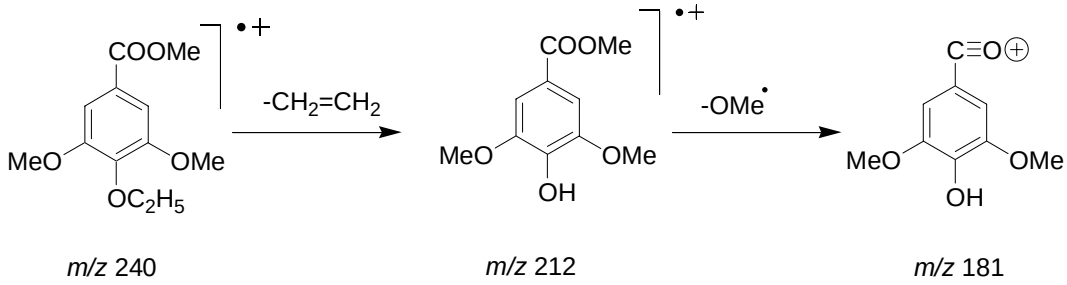
Unlike the other aromatic acids, syringic acid is present in honeys as the corresponding methyl ester (COOMe). This is also the case for many other honey types including New Zealand manuka, kanuka, clover (Tan *et al*., 1989a and 1989b), erica, European heather and Norwegian erica honeys.

Tan (1989a) methylated honey extracts using a rapid technique in which excess diazomethane was removed after 1.5 min. This procedure differentiates between the presence of either or both of syrinigic acid and methyl syringate in the original extracts, since rapid methylation would be expected to methylate the carboxyl group of **11b**, but not the hindered 4-OH (phenolic) group of **11a.**

In the present study, samples were derivatised with excess diazomethane, or diazoethane for an extended period (16-24 h). Model reactions using syringic acid showed the extended reaction time resulted in methylation, or ethylation, of both the carboxylic group and the 4-OH (phenolic) group.

Extended methylation afforded a product which showed an M^+ ion at *m/z* 226, whereas extended ethylation afforded a product which showed a M^+ ion at *m/z* 240 (rather than at *m/z* 254). Thus ethylation introduces only a single ethyl group, and it

follows either a 4-OMe or 1-COOMe group is present in the substance recovered from honey samples The mass spectrum of the ethylated product showed strong *m/z* 212 and 181 ions which can be attributed to the loss of ethylene from the 4-OEt group, followed by loss of a methoxy radical from the COOMe group, as depicted in Figure 4.4.



m/z 240 *m/z* 212 *m/z* 181

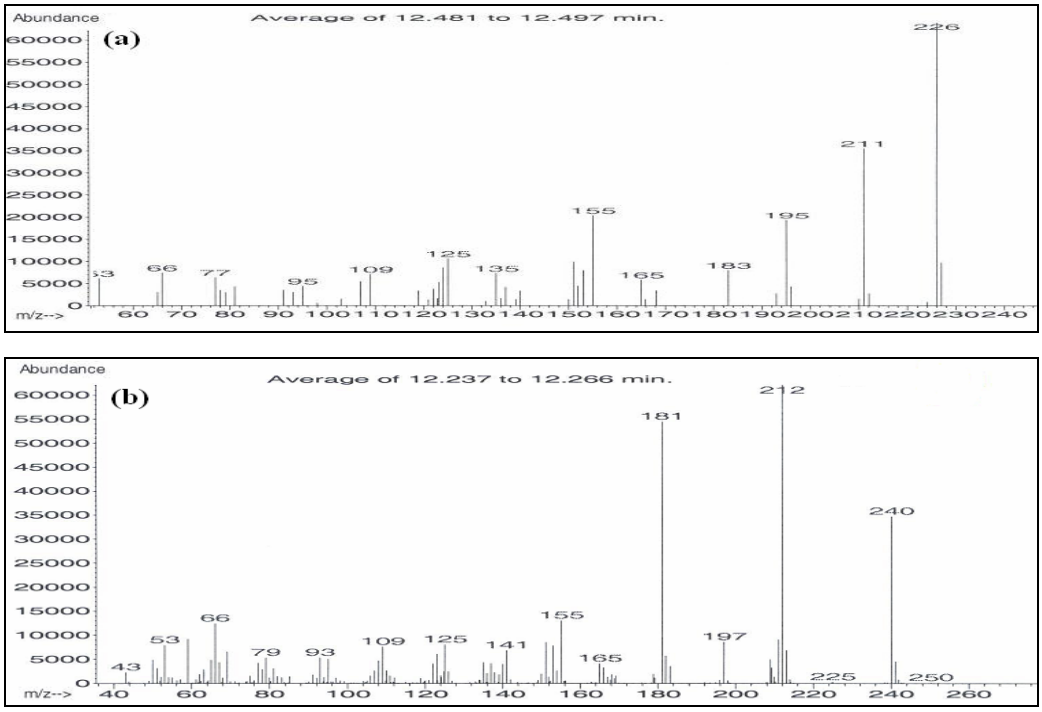


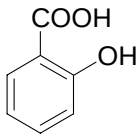
Figure 4.4. The mass spectra of methylated (a) and ethylated (b) derivatives of methyl syringate.

4.3.4. Salicylic Acid (Peak 3) (12a) and 2-Methoxybenzoic Acid (Peak 8) (12b)

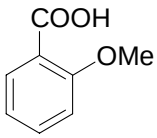
Moderate to low levels of salicylic acid methyl ester (peak 3) (**12a**) (10 mg/kg) and 2-methoxybenzoic acid methyl ester (peak 8) **(12b)** (5.6 mg/kg) were detected in most of the honeydew honey extracts. Hyink (1998) reported similar quantities of these compounds (11 and 2.8 mg/kg respectively) in 1994-1997 season honeydew honey extracts. The levels of salicylic acid (peak 3) found in the honeydew

honey extracts is much higher than those found in manuka honey extracts (1.8 mg/kg, Tan, 1989a; 1.7 mg/kg, Wilkins *et al*., 1993a), while the levels of peak 8 in the honeydew honey extracts is lower than in manuka honey extracts (17 mg/kg, Tan, 1989a; 9.5 mg/kg, Wilkins *et al*., 1993a).

Ethylated extracts showed that the presence of salicylic acid ethyl ester and 2-methoxybenzoic acid ethyl ester, indicating that the unmethylated honeydew honey extracts contained both 2-hydroxybenzoic acid (salicylic acid) **(12a)** and 2-methoxybenzoic acid **(12b)**. While prolonged treatment of a hydroxybenzoic acid isomer with diazomethane generally leads to the formation of the corresponding methoxy methyl ester (in which both the phenolic OH and acid groups are methylated), hydrogen bonding between the phenolic group and the acid group (as is the case in salicylic acid) inhibits methylation of the phenolic 2-hydroxy group.



(**12a**) (2-hydroxybenzoic acid)



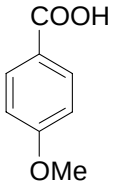
(**12b**) (2-methoxybenzoic acid)

4.3.5. 4-Methoxybenzoic Acid (Peak 11) (13a)

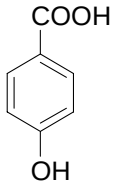
4-Methoxybenzoic acid methyl ester (peak 11) was detected in methylated extracts of the 2002 samples, but not the 1994-1997 samples (Hyink, 1998). This shows that either or both of 4-hydroxybenzoic acid and 4-methoxybenzoic acid may be present in the unmethylated 2002 season extracts, since prolonged exposure of a phenolic hydroxyl group using diazomethane can result in the formation of the corresponding methoxy-derivatives. Since both of 4-methoxybenzoic acid ethyl ester and 4-ethoxybenzoic acid ethyl ester were detected in ethylated extracts it follows that 4-methoxybenzoic acid **(13a**) and 4-hydroxybenzoic acid **(13b**) were present in the diethyl ether extracts.

The level of the 4-ethoxybenzoic acid ethyl ester was typically 2 times higher than the level of 4-methoxybenzoic acid ethyl ester detected in sub-samples of ethylated honeydew honey extracts. This showed that the level of 4-methoxybenzoic

acid in the original extracts was about half of the level of 4-hydroxybenzoic acid. In a previous study, Wilkins *et al*. (1995a) found that these compounds were present in the New Zealand vipers bugloss honey extracts. The sum of the concentrations of these two compounds (6.4 mg/kg) found in the honeydew honey extracts were much higher than that found in New Zealand vipers bugloss honey samples (1.2 mg/kg) (Wilkins *et al*., 1995a).



(**13a**) (4-methoxybenzoic acid)



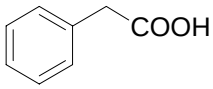
(**13b**) (4-hydroxybenzoic acid)

4.3.6. Other Aromatic Compounds

Significant levels of phenylacetic acid (peak 2), 2-methoxyacetophenone (peak 6), 4-methoxyphenylacetic acid (peak 14) and 4-hydroxyphenylacetic acid (peak 16) were also detected in methylated honeydew honey extracts as the corresponding methylated derivatives.

Phenylacetic Acid (Peak 2)

Relatively high levels of phenylacetic acid (**14**) were present in the honeydew honey extracts. Levels ranged from 18-75 mg/kg (stdev 17 mg/kg). The mean concentration of phenylacetic acid detected in the 2002 season honeydew honey samples (36 mg/kg) is higher than that found in the 1994-1997 season honeydew honey samples (27 mg/kg) (Hyink, 1998). This compound has previously been reported in New Zealand manuka and ling-heather honey extracts (Tan *et al*., 1989a; Wilkins *et al*., 1993a). The level of phenylacetic acid found in the beech honeydew honey sample is much higher than that found in manuka honey samples (typically 3 mg/kg; Wilkins *et al*., 1993a), but lower than that found in New Zealand ling-heather honey samples (189 mg/kg) (Tan *et al*., 1989a).



(**14**)

The mass spectra of methylated and ethylated sub samples of honeydew honey extracts included a peak which exhibited molecular ions at *m/z* 150 and 164 respectively (Figure 4.5). These ions revealed the presence of phenylacetic acid in the honeydew honey extracts.

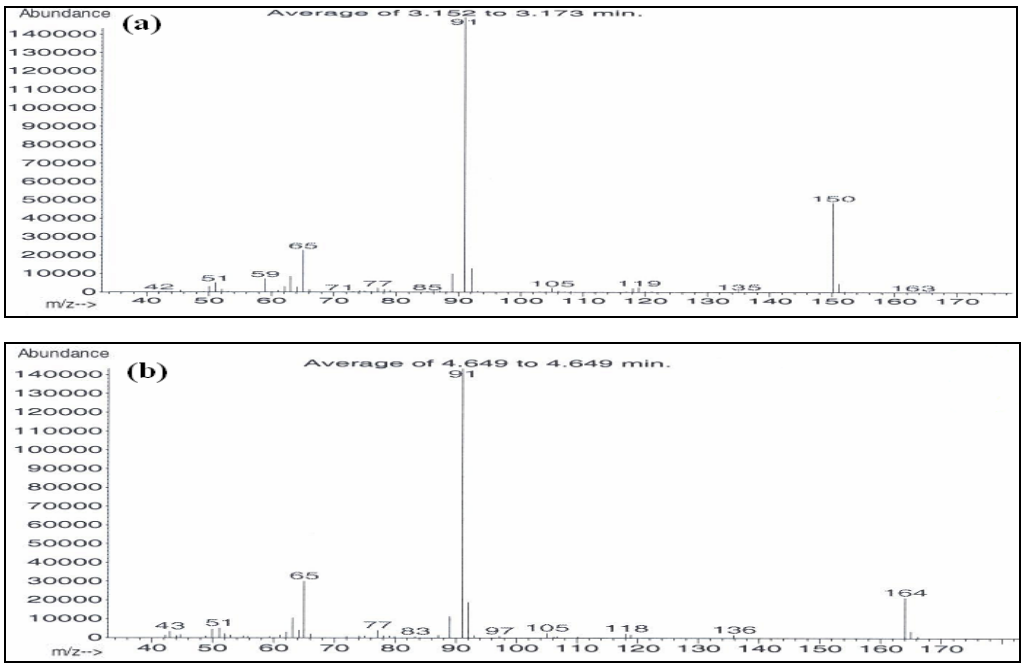


Figure 4.5. The mass spectra of methylated (a) and ethylated (b) derivatives of phenylacetic acid.

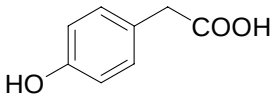
2-Methoxyacetophenone (Peak 6)

A low level of 2-methoxyacetophenone (peak 6) (5.8 mg/kg) was detected in most of the honeydew honey extracts. Hyink (1998) found similar levels of this compound (5.5 mg/kg) in 1994-1997 season honeydew honey extracts. Greater levels of this compound (18-23 mg/kg) occur in manuka honeys (Tan 1989a; Wilkins *et al.*, 1993a).

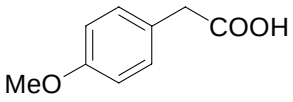
4-Hydroxyphenylacetic Acid (Peak 16) and 4-Methoxyphenylacetic Acid (Peak 14)

Moderate to low levels of 4-hydroxyphenylacetic acid (peak 16) (**15a**) and 4-methoxyphenylacetic acid (peak 14) (**15b**) were detected in most of the honeydew honey extracts (5.9 and 9.0 mg/kg respectively). Hyink (1998) have reported that peaks 16 and 14 were also present in 1994-1997 season honeydew honey extracts (8.3 and 2.6 mg/kg respectively).

The levels of these two compounds in the beech honeydew honey extracts were comparatively higher than that the levels found in the other types of New Zealand honey extracts. For example, these two compounds have been found in trace levels in New Zealand manuka honey extracts (Tan 1989a) and in white clover honey extracts (Tan *et al*., 1989b). 4-Methoxyphenylacetic acid (peak 14) could be a metabolite product of 4-hydroxyphenylacetic acid (peak 16).



(**15a**)

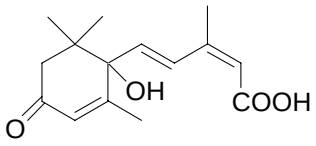


(**15b**)

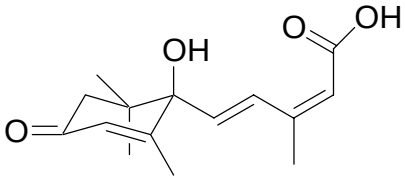
Minor levels of some other aromatic compounds (detected as their methylated derivatives) such as benzoic acid (3.7 mg/kg in 1994-1997 samples, Hyink, 1998), nicotinic acid (peak 1) (1.7 mg/kg in 2002 samples), 2-methoxyphenylacetic acid (peak 13) (1.3 mg/kg in 2002 samples), and 3,4-dimethoxybenzoic acid (peak 19) (trace in 2002 samples and 1.0 mg/kg in 1994-1997 samples) were also detected in the honeydew honey extracts. The concentrations of these compounds are similar to those found for manuka honey extracts (Wilkins *et al*., 1993a). These aromatic carboxylic acids may be secondary plant metabolites (Steeg and Montag, 1988).

4.3.7. Abscisic Acid Isomers [16]

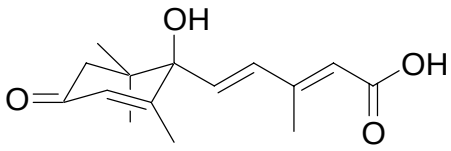
trans,cis-Abscisic acid (peak 26) [**16a**] and *trans,trans*-abscisic acid [**16b**] were detected in trace levels. The *trans,cis*-abscisic acid isomer (peak 26) was always present in a greater amount than the *trans,trans*-abscisic isomer. Tan *et al*. (1990) have previously reported appreciable amounts of abscisic acid isomers in the extracts of a New Zealand willow honey sample. High levels (> 80 mg/kg) of abscisic acid isomers have been detected in the extracts of Spanish and Portuguese erica (heather) (*Erica* spp.) honey samples (Ferreres *et al*., 1996), while much lower levels (0.1 – 5.0 mg/kg) were also detected in some New Zealand ling-heather and thyme honey samples (Tan *et al*., 1989b and 1990).



(16)



(**16a**)



(**16b**)

Figure 4.6. Structures of *trans,cis* (**16a**) and *trans,trans* (**16b**) abscisic acid isomers.

4.3.8. Aliphatic Acids and Diacids

A number of methylated aliphatic acids and diacids including nonanoic acid (peak 4), hexanedioic (peak 5), decanoic acid (peak 7), heptanedioic acid (peak 9), octanedioic acid (peak 15), dodecanoic acid (peak 17), nonanedioic acid (peak 18), decanedioic acid (peak 21), myristic acid (peak 23), palmitic acid (peak 25) and stearic acid (peak 27) were also detected in methylated honeydew honey extracts.

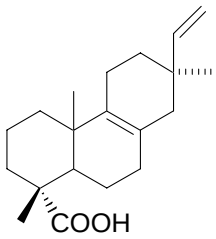
Octanedioic, nonanedioic and decanedioic acids have been recognized as part of the pheromone system of the honey bee *Apis mellifera* (Tan *et al*., 1988). Collow *et al*. (1964) and Lercker *et al*. (1981) reported presence of nonanedioic and decanedioic acids in the extracts of queen larvae or royal jelly. Gochnauer and Shearer (1981) reported octanedioic acid is present in the extracts of worker larvae.

Honeydew honey possesses a moderate level of aliphatic diacids (mainly 14:0 to 28:0 fatty acids). Since these compounds have previously been identified in a variety of other New Zealand unifloral grade honeys including manuka, ling-heather, clover, thyme, willow, viper bugloss and nodding thistle (Tan *et al*., 1988, 1989a, 1990; Wilkins *et al*., 1993), their detection in honeydew honey samples does not assist in the identification of the floral source of a honey sample.

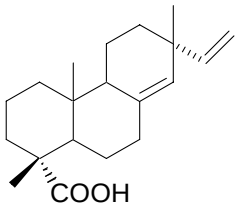
4.3.9. Resin Acids

Low levels of later-eluted resin acids including dehydroabietic (**7**), pimaric (**17a**), sandaracopimaric (**17b**), and isopimaric acids (**17c**), were identified in the 2002 season honeydew honey samples (Table 4.2). The average level of dehydroabietic acid detected in 2002 season honeydew honey extracts was 3.6 mg/kg. This finding, which can be compared with detection of trace levels of resin acids in some kamahi honeys, is the second occasion that resin acids have been identified in New Zealand honey extracts.

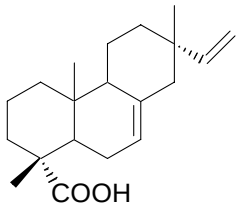
The resin acid contents of previously investigated New Zealand honeys were however not determined, since at the time peaks eluting after stearic acid, and other later eluting fatty acids and wax hydrocarbons were not quantified. The investigations reported in this thesis suggest that low levels of resin acids (woody resinous substances) are likely to be universally present in honeys.



(**17a**)



(**17b**)



(**17c**)

4.4. Conclusions

Honeydew honey sample GC/MS profiles are dominated by peaks arising from phenyllactic acid (peak 10), 4-methoxyphenyllactic acid (peak 20), methyl syringate (peak 22) and phenylacetic acid (peak 2). While these compounds are also present in manuka honeys extracts, their levels were lower that those found in manuka honeys apart from phenylacetic acid (peak 2). The level of phenylacetic acid (peak 2) in honeydew honey extracts is much higher than manuka (~ 3mg/kg) (Wilkins *et al*., 1993a), but lower than New Zealand heather honey (*Calluna* spp.) (75-168 mg/kg) (Guyot *et al*., 1999).

The only peak that appears to be a unique floral marker substance for honeydew honey is indole-3-acetic acid (peak 24). Although the level of this compound was low, it was generally detectable (typically in the range 0.9-10 mg/kg).

The levels of extractable organic substances from New Zealand beech honeydew honey found in this study are similar to those previously identified in beech honeydew honey extracts (Hyink, 1998). The combined gas chromatography-mass spectrometry (GC/MS) profiles of New Zealand (South Island) beech (*Nothofagus spp*.) honeydew honeys can readily distinguish them from other honey types.

Chapter Five

Extractable Organic Substances from New Zealand Erica (*Erica lusitanica*) Honey

Chapter Five

Extractable Organic Substances from New Zealand Erica (*Erica lusitanica*) Honey

5.1. Introduction

Commonly the term 'heather honey' is used to describe a honey arising from the *Ericacea* family. In some countries, especially Portugal, heather honey may be produced from *Erica* spp. (*Ericacae*), whereas in Spain and France heather honey originates from either *Calluna* or *Erica* species (Soler *et al*., 1995). In New Zealand heather honey is only derived from *Calluna vulgaris* (ling-heather) (Tan *et al*., 1995).

Erica honey is rarely produced in New Zealand and its commercial availability is limited. *Erica* spp. flower very early in the spring and sometimes even in late winter. The flowering period is very short (*ca* 3 weeks). New Zealand erica honey is periodically obtained from sites in the Catlins district (coastal South Otago), and the Takaka Hill district (Nelson-Marlborough).

Figure 5.1. *Erica* spp. flower (courtesy of http://fernkloof.com).

Although an account of the extractives of New Zealand *calluna* heather honey (ling-heather) has been published (Tan *et al*., 1989b), little is known about the extractable compounds present in New Zealand erica honey. No account of the typical levels of extractable organic substances in erica honey extracts has been presented, however Hyink (1998) has reported data for a single Nelson-Marlborough collection of New Zealand erica honey. This sample afforded two isomers of a new substance which were named as ericinic acid and isoericinic acid. Hyink (1998) also reported the structural elucidation and relative stereochemistry of the methyl esters of ericinic acid and isoericinic acids. These acids appeared to be rearranged analogues of abscisic acid. No conclusion could be taken as to whether or not these compounds might be floral marker substances for honey derived from erica spp.

A further 7 Nelson-Marlborough and South Otago erica honey samples from 1994-2002 flowering seasons were analysed in the present study. The objectives of this work were to:

- -define the typical chemical fingerprint (GC/MS profiles) of New Zealand produced erica honeys

- -determine the levels of extractable organic substances present in New Zealand erica honeys and compare these data with those reported for Portuguese erica honey (Ferreres *et al*., 1996a)

- -prepare a publication reporting the presence of floral marker compounds in New Zealand erica honeys and

- -isolate ericinic acid and isoericinic acid and carry out a structural characterization of these acids (rather than the corresponding methyl esters).

5.2. Experimental

5.2.1. Honey Samples

Seven erica honey samples from the 1994-2002 flowering seasons were extracted, methylated and analysed as described in Sections 2.2.1, 2.3 and 2.4 respectively. Samples 1 and 2 (2000 season) were provided by Milburn Apiaries, Milton, sample 3 (1994 season) was supplied by Lyn McKenzie, Marlborough, sample 4 was supplied by Kotare Apiaries, Nelson, and samples 5, 6 and 7 were supplied by Nelson Apiaries. Samples 1, 2, 3 and 4 appeared to be high quality erica honey samples whereas samples 5, 6 and 7 contained wax and comb residues and it was not clear if they could be considered to be unifloral grade erica honeys.

5.2.2. Erica Flower Sample Extraction Procedure

Five 2002-2003 season samples with white flowers and 5 samples with pink flowers, collected from a local garden were provided by Professor Wilkins (The University of Waikato) and extracted using an adoption of the micro-scale technique developed by Brophy *et al*. (1989). The flowers (8-15) were soaked in methanol:dichloromethane (1:1) (~ 2 mL) in glass vials (18-20 h) at room temperature after which 2 mL portions of the extracts were transferred to GC vials and methylated with an ethereal solution of diazomethane using the procedure described in Section 2.3 (Chapter 2).

5.2.3. GC/MS Analyses of Flower Extracts

GC/MS of substances present in the methylated flower extracts were performed using TIC and SIM and the following GC oven programme: 20 sec isothermal at 45 ^{o}C, then increased to 100 ^{o}C at 30 ^{o}C/min., then raised at 6 ^{o}C/min to the final temperature of 295 ^{o}C which was held for 15 min. Qualitative SIM analyses were performed for ericinic acid and abscisic acid using *m/z* 134, 190 and 278 ions and *m/z* 162 and 278 ions respectively.

5.2.4. Bulk Extraction

New Zealand erica honey for bulk extraction was obtained from the Nelson- Marlborough district (1994 season). An erica honey sample (200 g) was liquid-liquid extracted for 72 h with diethyl ether as described for analytical samples using a 750 mL extractor (Section 2.2.1). The bulk honey extract was applied to a radial silica plate which was developed with 50-200 mL portions of petroleum ether:diethyl ether and ethanol:diethyl ether mixtures (Table 5.1). 30 x 25 mL fractions were collected and concentrated by slow evaporation in a fume hood to *ca* 1 mL and analysed by GC/MS.

Table 5.1. The solvent gradient used for radial chromatography of the bulk underivatized erica honey extract.

fraction number	solvent (%) petroleum ether:diethyl ether	total volume (mL)
1, 2	85:15	50
3, 4	70:30	50
5-12	40:60	200
13-14	10:90	50
15-18	0:100	100
19-24	75:25[a]	200
25-30	75:25[a] (+ 0.5% HCOOH)	200

[a]diethyl ether:ethanol (75:25).

Fractions were analyzed by GC/MS and the target acids (ericinic, isoericinic and abscisic acid isomers) were identified as major components of fractions 28 and 29. Fractions 28 and 29 were combined and evaporated using a rotary evaporator (~ 40 ºC). TLC analyses were performed using three solvent systems (A = 60:40 (diethyl ether:petroleum ether + 0.5% HCOOH)), (B = 99.5% diethyl ether + 0.5% HCOOH) and (C = 90:10 (diethyl ether:ethanol + 0.5% HCOOH)) with abscisic acid as a standard, followed by visualization under UV light at 254 nm, or development with 10% sulfuric acid showed 2-3 major spots. The combined material from fractions 28 and 29 were dissolved in a mixture of methanol:water-5% formic acid (3:7) and separated using HPLC.

5.2.5. HPLC Conditions

HPLC separation was carried out as described by Ferreres *et al*. (1996a) using a Waters ILD on-line degasser, a Waters 515 HPLC pump and a Waters 996 photodiode-array UV detector interfaced to a MILLENNIUM 32 data system. Chromatograms were recorded at 240 and 400 nm. The column was a reversed-phase column LiCrochart C-18 (125 × 8 mm I.D., particle size 5 μm). Water-formic acid (19:1, v:v) and methanol mixtures were used as mobile phases. Elution was performed at a solvent flow rate of 1 mL/min, with a linear gradient elution starting with 30% methanol and 70% water-formic acid (15 min), rising to 40% methanol at 20 min, 45% methanol at 30 min, 60% methanol at 50 min, and 80% methanol at 52 min (8 min hold).

Prior to HPLC separation of fractions 28 and 29 (Section 5.2.4), a 200 μL portion of an abscisic acid (1 mg/mL) standard (in methanol:water-5% formic acid (3:7) was injected into the LC system in order to observe the retention time of the abscisic acid (25-27 min). After that, 200 μL portions of the combined fractions 28+29 solution in methanol:water-5% HCOOH (3:7) were injected into the LC system and 3 fractions (1A: 5-8 min; 2A: 19-24 min. and 3A: 24-27 min) were collected. GC/MS of methylated sub-samples of fractions 2A and 3A showed that fraction 2A was comprised of ~ 66% ericinic acid and ~ 33% abscisic acid isomers and that fraction 3A was comprised of ~ 75% abscisic acid isomers and 25% ericinic acid.

Subsequently fractions 2A and 3A were recombined, re-concentrated (rotary evaporator ~ 40 ºC) and subjected to a further cycle of HPLC separation (fine separation). In this cycle, five fractions (1B: 19-20 min; 2B: 20-22 min; 3B: 22-24 min; 4B: 24-26 min. and 5B: 26-40 min) were collected and the target acidic components were in fractions 2B, 3B and 4B. GC/MS and NMR analyses (Figure 5.2), including 400 MHz ^{1}H, ^{13}C, COSY, NOESY spectral data determined in D_6-DMSO showed that fraction 2B was comprised of ~ 95% *trans,trans*-abscisic acid, fraction 3B was comprised of ~ 85% ericinic acid (~ 10% *trans,cis*-abscisic acid)

and fraction 4B was comprised of ~ 85% *trans,cis*-abscisic acid (~ 10% ericinic acid).

Methylated Acids

After NMR analysis, D_6-DMSO was removed by freeze and vaccum drying and recovered material was methylated with diazomethane (Section 2.3). Each of the methylated samples were redissolved in 2 mL of dichloromethane and fractionated by radial chromatography on silica gel. Radial plates were developed with 50 mL portions of petroleum ether/diethyl ether mixtures (80:20, 60:40, 40:60, 20:80, 0:100) and a 50 mL portion of diethyl ether/ethanol mixture (80:20). Twelve fractions (25 mL each) were collected from each sample (2B: 1-12; 3B: 1'-12' and 4B: 1"-12") and analysed by GC/MS.

Ericinic acid methyl ester and isoericinic acid methyl ester were identified (GC/MS, NMR) as the dominant (essentially pure) components of fractions 8', 8", 9' and 9" while fractions 10, 10' and 10" were comprised predominantly of *trans,trans* and *trans,cis*-abscisic acid methyl esters respectively. NMR samples were prepared by rotary evaporation (~ 40 ºC) of solvent, followed by air drying of the crystalline material thus obtained. Crystalline material was taken up in $CDCl_3$ for NMR analysis.

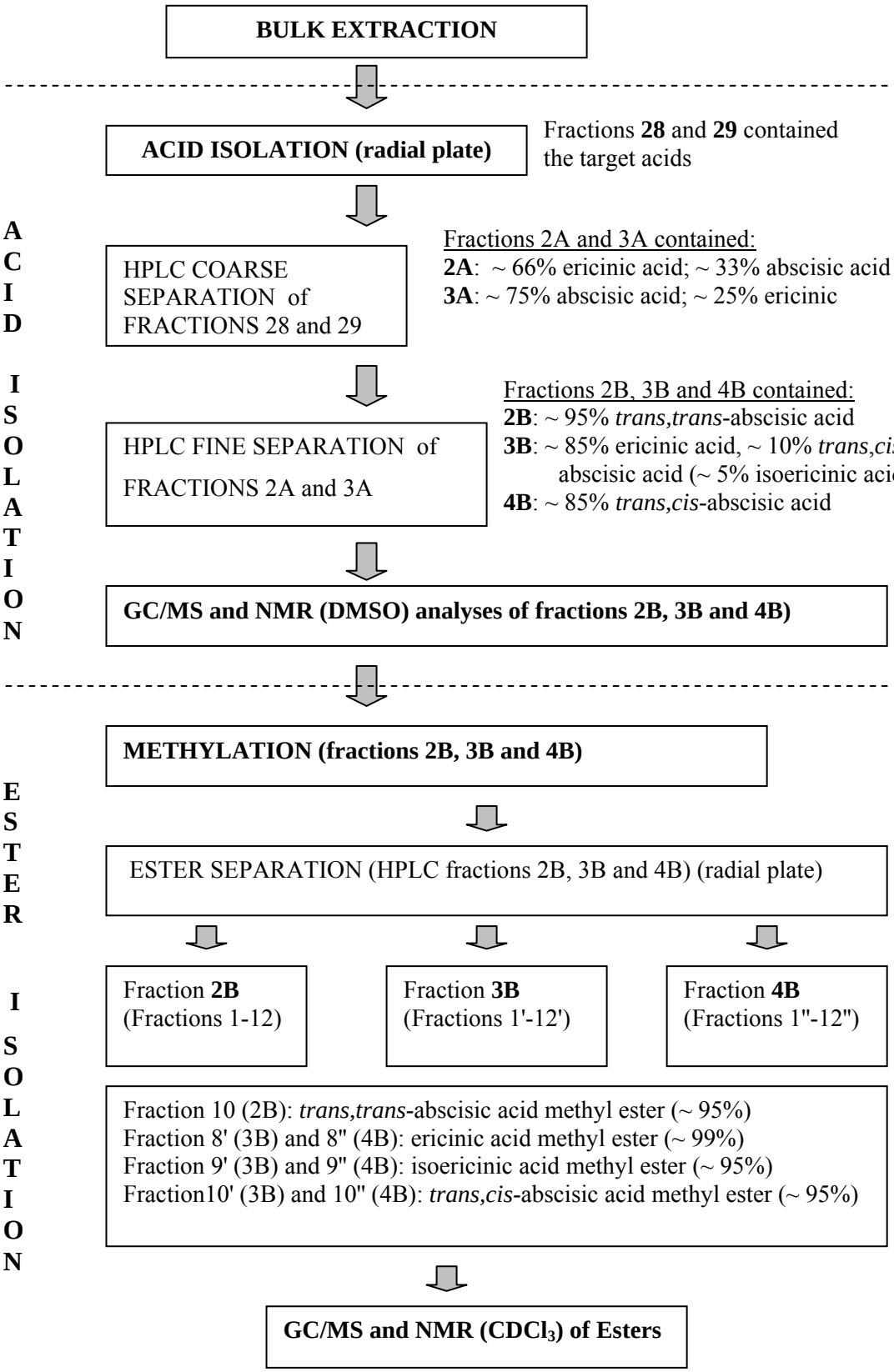


Figure 5.2. Schematic overview of isolation of ericinic acid, isoericinic and abscisic acid isomers from the bulk erica honey extract.

5.3. Results and Discussion: Analytical Samples

The levels of compounds identified in the methylated diethyl ether extracts of the seven 1994-2002 seasons erica honey samples analyzed in this investigation are presented in Table 5.2. The levels of resin acids detected in these honey samples are reported in Table 5.3. The GC/MS profile of a typical methylated diethyl ether extract of erica honey is presented in Figure 5.3 (peak identifications are given in Table 5.2). The detection of a methyl ester in the methylated extracts was considered to indicate the presence of the parent acid, with the exception of methyl syringate (see Section 4.3).

As described in Section 4.3, peaks eluting after tetracosanoic acid (peak 30) were found to be higher chain length hydrocarbons or fatty acids (detected as the corresponding methyl esters) and details of their characterization and concentrations are not presented here.

Samples 1-4 had a range of extractives (see Table 5.2) which corresponded to those reported by Hynik (1998) for the single sample which he examined. However the levels of individual compounds in the four samples examined in this investigation were generally more than twice those found by Hyink.

The level of benzoic acid detected in the four samples was more than 10 greater than that reported by Hyink (1998). The level of benzoic acid is such that it may cause marketplace difficulties since sodium benzoate is well-known as a food preservative and honeys are marketed as “no added preservative”. Some years ago, a consignment of manuka honey was temporarily withdrawn from an overseas market due to significant levels of benzoic acid being found during an analysis designed to detect added preservative.

Table 5.2. Concentrations (mg/kg) of components detected in diethyl ether extracts of seven (1994-2002 seasons) New Zealand erica honey samples. Acids were quantified as the corresponding methyl esters.

peak no.	compound	sample number 1	2	3	4	5	6	7	(Hyink)[a]
1	benzoic acid	10801	5771	2219	9009	12	-	-	405
2	octanoic acid	10	5.0	6.1	11	-	-	-	-
3	2,6,6-trimethyl-2-cyclohexene-1,4-dione	41	12	10	24	-	-	-	-
4	phenylacetic acid	28	9.0	11	16	11	-	9.3	12
5	salicylic acid	14	-	6.0	11	-	-	6.8	2.1
6	nonanoic acid	22	9.7	10	19	-	-	2.8	-
7	4-methoxyphenaldehyde	48	148	40	255	127	51	-	14
8	unknown[b] (*m/z* 83, 98, 140, 154, 167, 182)	31	9.2	8.8	17	-	-	-	-
9	decanoic acid	7.8	4.7	4.6	7.8	-	-	2.7	-
10	heptanedioic acid	13	2.8	-	4.8	-	-	-	-
11	4-hydroxyphenaldehyde	84	-	-	9.9	6.0	14	5.0	67
12	phenyllactic acid	217	115	342	506	135	132	123	91
13	4-methoxybenzoic acid	964	588	526	1077	20	146	46	92
14	phenylpropanoic acid	32	7.9	-	15	-	-	-	13
15	4-methoxyphenylacetic acid	23	5.7	2.7	9.1	-	23	21	3.3
16	octanedioic acid	48	10	8.1	21	23	19	25	-
17	dodecanoic acid	22	6.9	-	4.9	-	-	-	-
18	nonanedioic acid	109	35	23	68	40	43	42	-
19	4-methoxyphenyllactic acid	172	237	28	67	219	156	-	-
20	decanedioic acid	-	66	49	139	28	37	23	9.4
21	methyl syringate	147	110	110	231	303	133	94	4.3
22	ericinic acid	518	285	176	472	86	43	6.3	121
23	palmitic acid (16:0)	163	50	45	44	36	50	24	7.8
24	isoericinic acid	58	23	13	42	-	-	-	14
25	*trans,cis*-abscisic acid	471	139	137	459	161	128	13	104
26	stearic acid (18:0)	147	27	21	22	16	15	23	-
27	*trans,trans*-abscisic acid	364	115	108	309	130	92	17	82
28	docosanoic acid	74	19	-	16	8.0	26	-	-
29	unknown[b] (*m/z* 43, 187, 213, 253, 269, 328)	44	15	11	27	-	-	-	-
30	tetracosanoic acid	206	85	68	85	107	318	-	-

[a]data taken from Hyink (1998); [b]prominent ions observed in the mass spectra of unknown compounds are given in brackets.

Table 5.3. Concentrations (mg/kg) of resin acids detected in methylated diethyl ether extracts of seven (1994 -2002 seasons) New Zealand erica honey samples. Acids were quantified as the corresponding methyl esters.

sample	concentration (mg/kg)			
	pimaric	sandaracopimaric	isopimaric	dehydroabietic
1	0.3	0.3	0.3	2.9
2	0.2	0.2	0.2	2.5
3	0.2	0.1	0.6	2.9
4	0.4	0.3	0.5	1.7
5	0.3	0.3	0.2	1.6
6	0.6	0.4	0.2	0.8
7	3.8	1.2	8.1	9.6

The lesser levels of extractable compounds found in the samples 5 6 and 7 are consistent with the presence of significant levels of wax and comb residues, and the judgement that they were not unifloral grade samples. Accordingly summary statistical calculations were performed only for samples 1-4. The mean concentrations, standard deviations and coefficients of variance of the methylated extractable organic substances detected in the four samples considered to be unifloral grade are given in Table 5.4.

The most distinctive peaks, at least from the point of view of floral source characterization, were peaks 1, 13, 22, 24, 25 and 27 (Figure 5.3). Peaks 22 and 24 were identified as ericinic acid methyl ester and isoericinic acid methyl ester, respectively (Hyink, 1998) while peaks 25 and 27 were identified as abscisic acid isomers (Ferreres *et al*., 1996a).

Table 5.4. Mean concentration (mg/kg), standard deviation (stdev), and coefficient of variance (CV%) determined for compounds identified in the diethyl ether extracts of four (1994-2002 seasons) erica honey samples considered to be unifloral grade. Acids were quantified as the corresponding methyl esters.

peak no.	compound	mean conc. mg/kg	stdev mg/kg	n	CV (%)
1	benzoic acid	6950	3779	4	54
2	octanoic acid	8.0	2.9	4	36
3	2,6,6-trimethyl-2-cyclohexene-1,4-dione	22	14	4	65
4	phenylacetic acid	16	8.5	4	53
5	salicylic acid	10	4.0	3	39
6	nonanoic acid	15	6.3	4	41
7	4-methoxybenzldehyde,	123	101	4	82
8	unknown[a] (*m/z* 83, 98, 140, 154, 167, 182)	16.5	10.4	4	63
9	decanoic acid	6.2	1.8	4	29
10	heptanedioic acid	6.9	5.4	3	79
11	4-hydroxybenzldehyde	47	52	2	112
12	phenyllactic acid	295	169	4	57
13	4-methoxybenzoic acid,	789	273	4	35
14	phenylpropanoic acid	18	12	3	68
15	4-methoxyphenylacetic acid,	10	9.0	4	89
16	octanedioic acid	22	18	4	84
17	dodecanoic acid	11	9.3	3	83
18	nonanedioic acid	59	39	4	66
19	4-methoxyphenyllactic acid	126	96	4	76
20	decanedioic acid	85	48	3	56
21	methyl syringate	150	57	4	38
22	ericinic acid	363	160	4	44
23	palmitic acid (16:0)	76	58	4	77
24	isoericinic acid	34	20	4	59
25	*trans,cis*-abscisic acid	302	189	4	63
26	stearic acid (18:0)	54	62	4	114
27	*trans,trans*-abscisic acid	224	132	4	59
28	docosanoic acid	36	33	3	90
29	unknown[a] (*m/z* 43,187, 213, 253, 269, 328)	24	15	4	61
30	tetracosanoic acid	111	64	4	58
	resin acids				
1	pimaric acid	0.3	0.1	4	40
2	sandaracopimaric acid	0.2	0.1	4	35
3	isopimaric acid	0.4	0.2	4	47
4	dehydroabietic acid	2.5	0.6	4	23

[a]prominent ions observed in the mass spectra of unknown compounds are given in brackets.

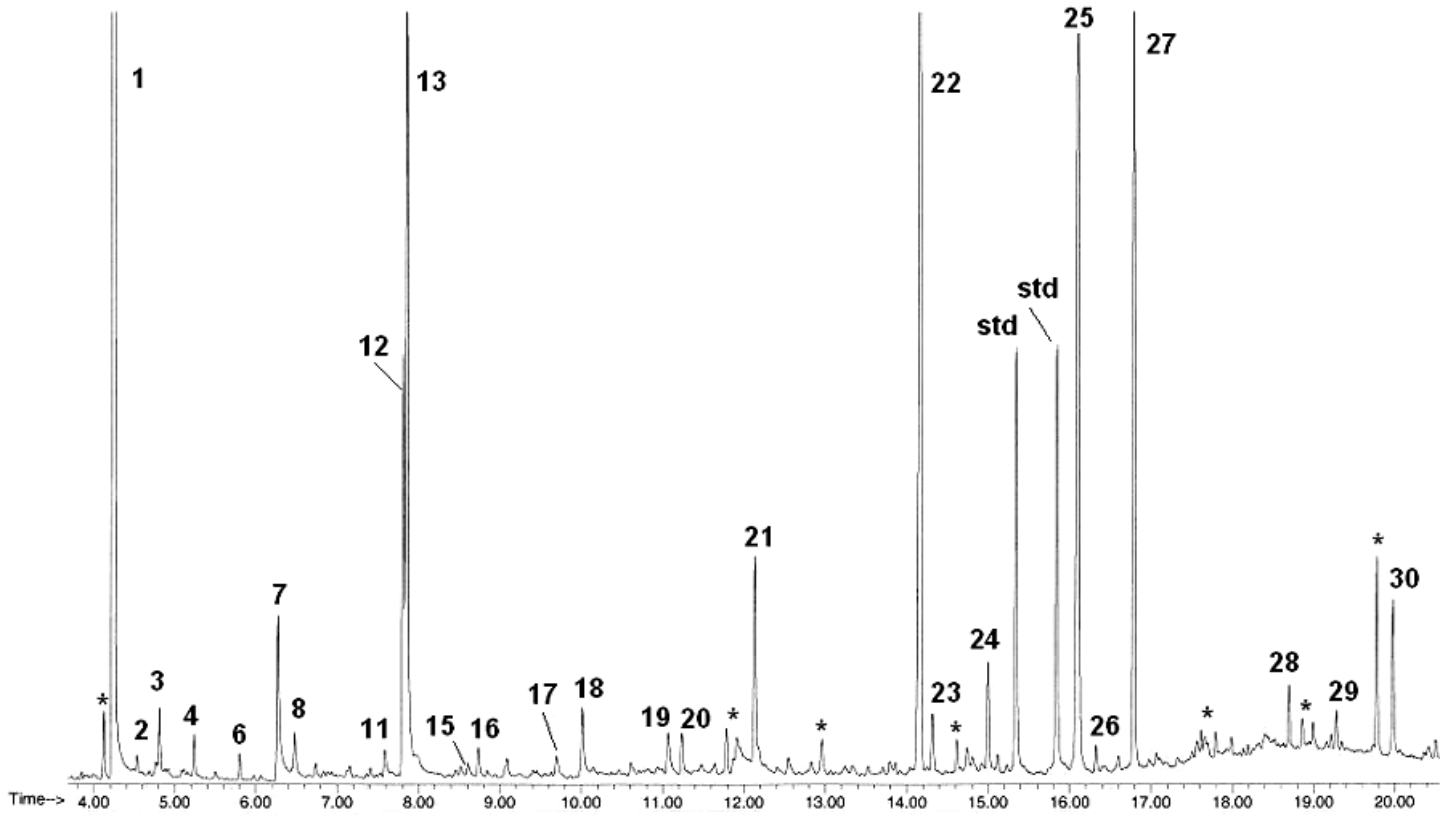
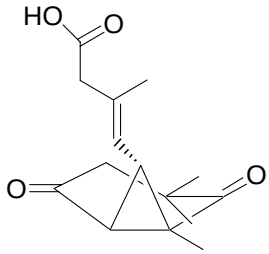


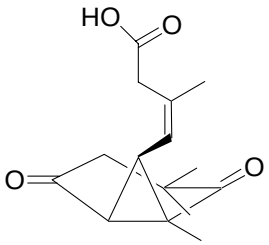
Figure 5.3. GC/MS profile of a representative methylated New Zealand erica honey diethyl ether extract. Peak identifications are given in Table 5.2. (Peaks marked with "*" originated from phthalates and/or solvent stabilizers).

5.3.1. Ericinic Acid Isomers (Peaks 22 and 24) [18a and 18b]

Generally high levels of ericinic acid methyl ester (peak 22), ranging from 121-518 mg/kg (stdev 160 mg/kg), were detected in the methylated diethyl ether extracts of the four unifloral grade and three other erica honey samples. The average level of ericinic acid (363 mg/kg) in methylated diethyl ether extracts of the 4 unifloral grade samples was significantly higher than that found in the methylated extracts of a 1994 season sample (121 mg/kg) (Hyink, 1998). Tan (1989b) has previously noted the presence of very low levels (< 1 mg/kg) of ericinic acid methyl ester, as an unknown compound, in the methylated diethyl ether extracts of some New Zealand ling-heather (*Calluna* spp.) honey samples.



[**18a**] ericinic acid



[**18b**] isoericinic acid

Isoericinic acid methyl ester was detected in the 4 unifloral 1994-2002 season erica honey extracts (average level 34 mg/kg). Hyink (1998) has previously proposed that isoericinic acid is the C-7 epimer of ericinic acid.

Since an elevated level of ericinic acid methyl ester (average level 363 mg/kg) and a considerable level of isoericinic acid methyl ester (average level 34 mg/kg) were detected in each of the four unifloral grade erica honey extracts, these compound can be proposed as floral marker compounds for New Zealand erica honey.

5.3.2. Abscisic Acid Isomers (Peaks 25 and 27) [16a and 16b]

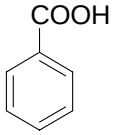
Abscisic acid isomers were identified as the dominant components in fractions 2B and 4B (Section 5.2.5) by GC/MS and NMR analysis. The ^{1}H and ^{13}C NMR chemical shifts of isomeric abscisic acids corresponded closely with chemical shifts for the *trans,cis* and *trans,trans*-abscisic acid isomers reported by Ferreres *et al*. (1996a and 1996b). Ferreres *et al*. (1996a) have previously reported that *trans,cis* and

trans,trans-abscisic acids were the major extractable organic constituents (range 25 – 166 mg/kg) of Portuguese *Erica* spp. honey which were described as 'heather' honeys. However, other authors consider only honeys derived from *Calluna* spp. to be 'heather' honeys.

It is apparent that 'heather' honeys derived from *Erica* spp. possess an array of extractable organic substances which differ significantly from those derived from *Calluna* spp. In particular, the concentrations of abscisic acid isomers found in Portuguese erica honey extracts are much higher than those found in New Zealand ling-heather (*Calluna* spp.) honey extracts, and also other New Zealand unifloral honeys (*e.g.* willow, thyme and honeydew honeys) (Tan *et al*., 1989b, 1990) (see Section 4.3.7).

5.3.3. Benzoic Acid (Peak 1) [19]

The major constituent of the diethyl ether extract of New Zealand erica (*Erica* spp.) honey samples was benzoic acid (peak 1) [**19**] (average level 6950 mg/kg). The mean level of benzoic acid recovered from New Zealand erica honey was substantially greater than those detected in six erica honey samples (*Erica arborea*) obtained from France, Greece and Italy (average level 15 mg/kg) (Guyot *et al*., 1999).



[**19**]

The levels of benzoic acid reported in Table 5.4 are much higher than those found in other New Zealand unifloral honey types. For examples, the average levels of this compound found in 1989 season manuka, 1982-1986 season clover and 1986 season nodding thistle honey extracts were 7.8 mg/kg (Tan *et al.,* 1993), 3.7 mg/kg (Tan *et al*., 1988) and 0.4 mg/kg (Wilkins *et al*., 1993) respectively.

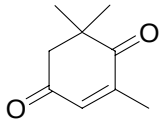
5.3.4. 4-Methoxybenzoic Acid (Peak 13) [13a]

Elevated levels of 4-methoxybenzoic acid (526-1077 mg/kg) were present in all of the methylated diethyl ether erica honey extracts. The mean concentration (average level 789 mg/kg) of 4-methoxybenzoic acid detected in the erica honey extracts was more than a 100-fold greater than that found in New Zealand honeydew honeys (6.4 mg/kg) (see Section 4.3.5) and in vipers bugloss honeys (1.2 mg/kg) (Wilkins *et al*., 1995a). The presence of 4-methoxybenzoic acid in other New Zealand honey types has not been reported (Tan 1989; Tan *et al*., 1988, 1989a, 1989b and 1989b; Wilkins *et al*., 1993a, 1993b, 1995b; Hyink, 1998).

5.3.5. Phenylacetic Acid (Peak 4) [14]

Relatively low levels of phenylacetic acid (peak 4) [**14**] (average level 16 mg/kg) were detected in the four New Zealand unifloral grade erica honey samples. Christine *et al*. (1999) did not observe this compound in erica (*Erica arborea*) samples from France, Greece and Italy. On the other hand Speer and Montag (1984) have reported that European calluna heather honeys can be distinguished from other honeys by their high phenylacetic acid and benzoic acid contents.

5.3.6. 2,6,6-Trimethyl-2-cyclohexene-1,4-dione (Peak 3) [20] (3,5,5-Trimethylcyclohex-2-ene-1,4-dione (4-Oxoisophorone)

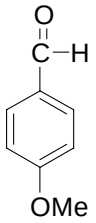


[**20**]

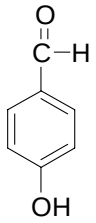
Significant levels (average level 22 mg/kg) of 2,6,6-trimethyl-2-cyclohexene-1,4-dione (peak 3) were detected in the four unifloral grade erica honey samples. Tan *et al*. (1989b) reported that this compound was also present in trace level in the methylated extracts of New Zealand ling-heather honey extracts. 2,6,6-Trimethyl-2-cyclohexene-1,4-dione is an example of a widely distributed class of plant compounds often referred to as degraded carotenoids.

Low levels (average level 4.9 mg/kg) of 2,6,6-trimethyl-2-cyclohexene-1,4-dione were also detected in the methylated extracts of New Zealand kamahi honey samples (Section 3.3.12) (Chapter 3). The level of this compound in erica honey is much greater than that found in ling-heather and kamahi honey samples. Rowland *et al.* (1995) has found this compound in the range of trace to 4.2 mg/kg in the methylated organic extracts of Australian Leatherwood (*Eucryphia lucida*) honey while D'Arcy *et al*. (1997) has found this compound in trace level (average level 0.1 mg/kg) in the unmethylated extracts of Australian Yellow Box honey extracts.

5.3.7. 4-Methoxyphenaldehyde (Peak 7) [21] and 4-Hydroxyphenaldehyde (Peak 11) [22]



[**21**]



[**22**]

Appreciable levels of 4-methoxyphenaldehyde (peak 7) (avearge 123 mg/kg) and 4-hydroxyphenaldehyde (peak 11) (average level 47 mg/kg) were detected in 4 methylated diethyl ether extracts of the 4 unifloral grade erica honey samples. The average level of 4-methoxyphenaldehyde in erica honey extracts (123 mg/kg) in the present study was much higher than found in the 1986-1987 season ling-heather (*Calluna* spp.) (2.1 mg/kg) (Tan *et al*., 1989b), in 1986-1987 season nodding thistle (0.78 mg/kg) (Wilkins *et al*., 1993b) and in 1989-1990 season manuka (3.4 mg/kg) (Tan *et al*., 1993a) honey extracts.

Wilkins *et al*. (1995) have reported the presence of both of these compounds (4-methoxyphenaldehyde and 4-hydroxyphenaldehyde) in vipers bugloss honey (average levels 0.9 and 1.7 mg/kg respectively) honey extracts.

5.3.8. Phenyllactic Acid (Peak 12) [9], 4-Methoxyphenyllactic Acid (Peak 19) [10] and Methyl Syringate (Peak 21) [11a]

The concentrations of phenyllactic acid (peak 12), 4-methoxyphenyllactic acid (peak 19) and methyl syringate (peak 21) in the 4 unifloral grade erica honey samples ranged from 115-506, 28-237 and 110-231 mg/kg respectively. These aromatic compounds are the dominant constituents of the methylated extracts of New Zealand manuka honeys (see Section 4.3.2 and 4.3.3) (Chapter 4) (Tan *et al*., 1988; Wilkins *et al.*, 1993a).

Due to the short erica flowering season, and the likely availability during of nectar from other floral sources during flowering period, inputs to the honey from other floral sources may lead to the detection of substances originating from secondary floral sources. The presence of significant levels of phenyllactic acid, 4-methoxyphenyllactic acid and methyl syringate (123-135 mg/kg, trace-219 mg/kg and 94-303 mg/kg respectively) (Table 5.2) in the extractive profiles of the 4 erica honey samples may be indicative, at least in part, of a moderate manuka input to the honey samples.

The ratio of the sum of the levels of ericinic and isoericinic acids, to the sum of the phenyllactic acid, 4-methoxyphenyllactic acid and methyl syringate levels ranged between 1:1 to 3:1 for the 4 unifloral grade samples (samples 1- 4), compared to 1:2 to 1:6 for the 3 low quality samples (samples 5-7), possibly indicating a greater manuka input to the low quality samples (Table 5.5 and Figure 5.4). The ratio of these compounds in the sample of which Hyink examined was 3:1.

Table 5.5. Sums of the concentrations of selected manuka related compounds (phenyllactic acid, 4-methoxyphenyllactic acid and methyl syringate) and selected erica compounds (*e.g.* ericinic and abscisic acid isomers) (mg/kg), and the ratio of these compounds, in 8 erica honey samples.

sample	∑ericinic and abscisic acid isomers[a]	∑manuka compounds[b]	ratio: ∑ericinic and abscisic[a] / ∑manuka compounds[b]
1	1411	536	3:1
2	562	462	1:1
3	434	480	1:1
4	1282	804	2:1
5	377	657	1:2
6	263	421	1:2
7	36	217	1:6
Hyink[c]	321	95	3:1

[a]sum of ericinic acid, isoericinic acid, *trans,cis* and *trans,trans*-abscisic acid concentrations;
[b]sum of phenyllactic acid, 4-methoxyphenyllactic acid and methyl syringate concentrations; [c] data taken from Hyink (1998).

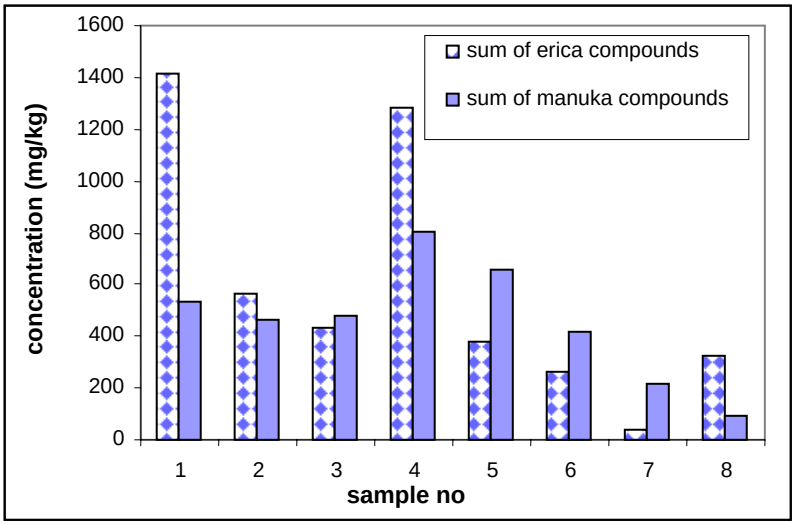


Figure 5.4. Graphical depiction of the sum of the concentrations of selected manuka related compounds (phenyllactic acid, 4-methoxyphenyllactic acid and methyl syringate) and erica compounds (ericinic, isoericinic and abscisic acid isomers) (mg/kg) in 8 erica honey samples.

5.3.9. Other Aromatic Compounds

Salicylic acid (2-hydroxybenzoic acid) (peak 5) (average level 10 mg/kg), β-hydroxyphenylpropanoic acid (peak 14) (average level 18 mg/kg) and 4-methoxy phenylacetic acid, (peak 15) (average level 10 mg/kg) were detected in the all of the erica extracts.

5.3.10. Resin Acids

Low levels of pimaric acid (average level 0.28 mg/kg), sandaracopimaric acid (average level 0.23 mg/kg), isopimaric acid (average level 0.41 mg/kg) and dehydroabietic acid (average level 2.5 mg/kg) were detected as their methyl esters in the erica honey extracts. The levels of these resin acids detected in erica honey extracts were comparatively higher than those observed in kamahi and honeydew honey extracts (Sections 3.3.12 and 4.3.9 respectively).

5.3.11. Fatty Acids

Erica honey extractives included an array of methylated aliphatic acids and diacids. Octanoic acid (peak 2), nonanoic acid (peak 6), decanoic acid (peak 9), heptanedioic acid (peak 10), octanedioic acid (peak 16), dodecanoic acid (peak 17), nonanedioic acid (peak 18), decanedioic acid (peak 20), palmitic acid (peak 23), stearic acid (peak 26), docosanoic acid (peak 28) and tetracosanoic acid (peak 30) were detected as their methyl esters in seven erica honey extracts. These compounds have previously been identified in the extracts of other unifloral honeys (*e.g.* manuka, ling-heather and willow honeys) (Tan *et al*., 1988, 1989b; Wilkins *et al*., 1993a, 1993b, 1995a; 1995b).

5.4. Results and Discussion: Bulk Extraction

Bulk extraction (Section 5.2.4) was carried out in the expectation that some of the more dominant extractives could be isolated in sufficient amount for structural characterisation using one- and two-dimensional NMR. A sufficient quantity of peak 22 (underivatized ericinic acid) was isolated from the bulk extract for structure confirmation using one- and two-dimensional NMR analyses.

5.4.1. Isolation of Ericinic Acid (Peaks 22 and 24) [18a and 18b]

Separation of a bulk extract (Section 5.2.4), by radial chromatography on silica gel and HPLC on reverse phase C-18 column, afforded a quantity of ericinic acid (peak 22) (Figure 5.3) sufficient for structure confirmation using one- and two-dimensional NMR methods. Ericinic acid was identified (GC/MS) as a component of fraction 3B which was comprised predominantly of an approximately 85:10:5 mixture of ericinic acid (peak 22), *trans,cis*-abscisic acid (peak 25) and isoericinic acid (peak 24) respectively (Figure 5.2). The NMR spectrum of fraction 4B which was comprised predominantly with *trans,cis*-abscisic acid, was subtracted from the NMR spectrum determined for fraction 3B in order to eliminate unwanted NMR signals including *trans,cis*-abscisic acid signals.

Only a very low level of isoericinic acid (parent acid) (peak 24) (Figure 5.3) was detected (GC/MS) in fraction 3B (Figure 5.2). It proved difficult to separate isoericinic acid from abscisic acid isomers (mainly *trans,cis*-abscisic acid in fraction 3B), therefore NMR data were not obtained for isoericinic acid.

5.4.2. NMR Structural Elucidation of Ericinic Acid

Ericinic acid (peak 22) was fully characterized by one- and two-dimensional ^{1}H and ^{13}C NMR spectroscopy. ^{1}H and ^{13}C NMR data are listed in Tables 5.5 and 5.6. The ^{1}H and ^{13}C NMR chemical shifts determined for ericinic acid correspond closely with those reported by Hyink (1998) for methyl ericinate.

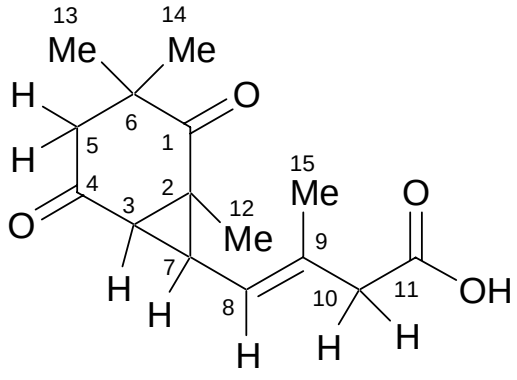


Figure 5.5. Structure of ericinic acid isomers with atom numbering scheme.

Table 5.6. ^{1}H and ^{13}C NMR data for ericinic acid determined in DMSO (atom numberings as in Figure 5.5).

Carbon	$\delta^{13}C$ (ppm) *J* Hz	$\delta^{1}H$ (ppm) *J* Hz
1	210.8 s (<u>C</u>O)	-
2	38.9 s (<u>C</u>)	-
3	40.3 d (<u>C</u>H-CO)	H-3 (C<u>H</u>-CO) 2.36 m
4	203.8 s (CO)	
5'	49.6 t (<u>C</u>H_2-CO)	H-5' (C<u>H_2</u>-CO) 2.19 d, ^{2}J 16.1 Hz
5"		H-5" (C<u>H_2</u>-CO) 2.48 d, ^{2}J 16.1 Hz
6	46.7 s (<u>C</u>)	
7	32.0 d, (<u>C</u>H)	H-7 (C<u>H</u>) 2.25 d, ^{3}J 9.2 Hz
8	119.9 d (<u>C</u>H=C)	H-8 (C<u>H</u>=C) 5.11 broad d, ^{3}J = 4.3 Hz
9	137.9 s (=<u>C</u>)	-
10	43.6 t (<u>C</u>H_2-COOH)	H-10 (<u>C</u>H_2-COOH) 2.95 broad s
11	172.1 s (<u>C</u>OOH)	-
12	22.6 q (<u>C</u>H_3)	H-12 (C<u>H_3</u>) 1.30 s
13	25.9 q (<u>C</u>H_3)	H-13 (C<u>H_3</u>) 1.05 s
14	23.2 q (<u>C</u>H_3)	H-14 (C<u>H_3</u>) 0.96 s
15	17.4 q (<u>C</u>H_3)	H-15 (C<u>H_3</u>) 1.65 s

The ^{1}H NMR of ericinic acid recorded in DMSO included resonances assignable to 4 methyl groups, an olefinic methyl group (CH_3-C=), and three aliphatic methyl groups (3 x Me). Three of the aliphatic methyl groups (0.96, 1.05 and 1.30 ppm) and the olefinic methyl (1.65 ppm) were attached to quaternary carbons.

The signals at 2.19 and 2.36 ppm were attributed to methylene protons and a methine proton adjacent to carbonyl group, respectively. A doublet of doublets at 2.25 ppm suggested the presence of a methine proton interspersed between two other methine groups. The broad doublet at 5.11 ppm was consistent with presence of an olefinic proton, adjacent to a single methine proton.

The ^{13}C NMR spectrum (recorded in DMSO) revealed the presence of 15 carbon atoms. Multiplicity editing using the DEPT sequence indicated the presence of four methyl groups, 2 methylene, three methine and six quaternary carbons.

Downfield singlet carbons at 203.8 and 210.8 ppm were assigned to keto groups, while a singlet carbon at 172.1 ppm indicated the presence of an acid carbonyl carbon. Signals at 119.9 (d) and 137.9 (s) ppm were consistent with the presence of two olefinic carbons.

HMBC spectral data revealed ^{2}J ^{13}C-C-^{1}H and ^{3}J ^{13}C-C-C-^{1}H connectivity and occasionally also weak ^{4}J ^{13}C-C-C-C-^{1}H couplings. Structurally significant HMBC correlations observed for ericinic acid are listed in Table 5.7.

The long-range ^{1}H-^{13}C correlations observed in the HMBC spectrum supported the structure of ericinic acid. In particular the geminal methyl group proton signals at 0.96 (H-14) and 1.05 (H-13) ppm exhibited ^{2}J correlations to a singlet carbon at 46.7 (C-6) ppm and ^{3}J correlations to carbons at 210.8 (C-1), 49.6 (C-5) ppm. The ^{3}J correlations between the H-13 and H-14 methyl protons to the carbon signal at 210.8 (C-1) ppm indicated that the methyl groups were located adjacent to a carbonyl group. The H-14 protons (0.96 ppm) also exhibited a weak ^{4}J correlation to the carbonyl signal which occurred at 203.8 (C-4) ppm.

Table 5.7. HMBC correlations (^{2}J, ^{3}J and ^{4}J) observed for ericinic acid (atom numberings as in Figure 5.5).

Proton	**δ^{1}H**	**HMBC (δ^{13}C)[a]**
H-3 (C<u>H</u>-CO)	2.36	203.8 (C-4), 38.9 (C-2)
H-5 (C<u>H$_2$</u>-CO)	2.19	203.8 (C-4), 46.7 (C6), 210.8 (C1), 25.9 (C13)
H-7 (C<u>H</u>)	2.25	210.8 (C1), 203.8 (C4), 22.6 (C12), 49.6 (C5)
H-8 (C<u>H</u>=C)	5.11	32.0 (C7), 43.6 (C10), 17.4 (C15), 38.9 (C2)
H-10 (C<u>H$_2$</u>)	2.96	137.9 (C9), 119.9 (C8), 172.1 (C11), 17.4 (C15)
H-12 (C<u>H$_3$</u>)	1.30	32.0 (C7), 38.9 (C2), 40.3 (C3), 210.8 (C1)
H-13 (C<u>H$_3$</u>)	1.05	210.8 (C1), 23.2 (C14), 49.6 (C5), 46.7 (C6)
H-14 (C<u>H$_3$</u>)	0.96	46.7 (C6), 210.8 (C1), 25.9 (C13), 49.6 (C5), 203.8 (C4)
H-15 (C<u>H$_3$</u>)	1.65	137.9 (C9), 119.9 (C8), 43.6 (C10), 32.0 (C7), 172.1 (C11)[b]

[a]listed in order of cross peak intensity , [b]very weak peaks.

The proton signal centred at 2.19 (H-5) ppm exhibited ^{2}J correlations to the carbon signals which occurred at 203.8 (C-4), 46.7 (C-6) ppm. This indicated that the methylene protons were adjacent to the carbonyl carbon. The proton signals centred at 2.19 (H-5) ppm also exhibited ^{3}J correlation to carbons at 25.9 (C-13) ppm.

The methylene protons, adjacent to the olefinic double bond at 2.96 (H-10) ppm showed strong correlations to the two olefinic carbons at 137.9 (^{2}J) (C-9) and 119.9 (^{3}J) (C-8) ppm, and to the acid carbonyl carbon which occurred at 172.1 (C-11) ppm. The olefinic methyl group proton signal at 1.65 ppm (H-15) showed ^{2}J or ^{3}J correlations to the carbon signals at 137.9 and 119.9 ppm (C-9 and C-8 respectively) together with weaker ^{4}J couplings to the carbon signals at 172.1 (C-11) and 32.0 (C-7) ppm.

The foregoing observations support the structure previously proposed for ericinic acid by Hyink (1998).

5.4.3. Mass Spectral Data

The mass spectra of ericinic acid methyl ester (peak 22) and isoericinic acid methyl ester (peak 24) (Figure 5.6) were characterised by strong *m/z* 91, 107, 134, and 162 ions along with highest observed ions at *m/z* 278 $[M]^{\cdot+}$ which established the molecular formula $C_{16}H_{22}O_4$. These isomers exhibited a mass spectral fragmentation pattern similar to that observed for abscisic acid methyl ester, but had a base peak at *m/z* 134, compared to a base peak at *m/z* 190 for abscisic acid methyl ester, and much stronger M^{+} ion at *m/z* 278 than was the case for abscisic acid methyl ester (Figure 5.6).

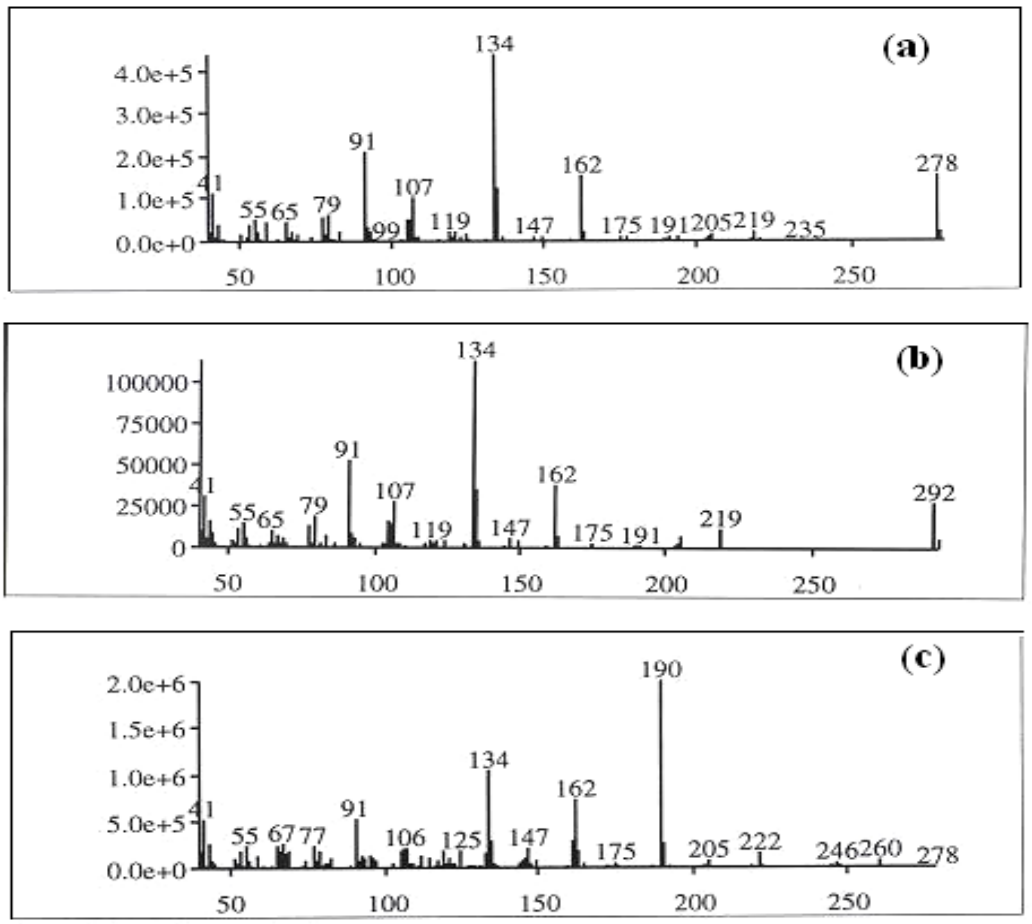


Figure 5.6. Mass spectra of ericinic acid (**a**) methyl ester (peak 22), (**b**) ethyl ester and (**c**) *trans,trans*-abscisic acid methyl ester.

The GC retention time and the mass spectra of methylated and ethylated sub samples of erica honey extracts were similar, other than for the occurrence of the $M^{+\bullet}$ ion of the ethylated analogue at *m/z* 292 compared to *m/z* 278 in the methylated analogue (Figure 5.6).

5.4.4. Methylated Analogues

The GC/MS analysis showed that HPLC fractions 8' and 8'' were comprised of essentially pure (~ 99%) ericinic acid methyl ester while HPLC fractions 9' and 9'' were comprised predominantly (~ 95%) of isoericinic methyl ester (Figure 5.2). The ^{1}H and ^{13}C NMR data determined for these methyl esters corresponded exactly with data previously reported for these compounds by Hyink (1998).

5.4.5. Polarity of Abscisic Acid and Ericinic Acid Isomers

TLC and radial plate data showed ericinic acid methyl ester and isoericinic acid methyl ester to be less polar (*e.g.* faster eluting) than abscisic acid methyl ester isomers, when chromatographed on silica gel (radial plate). The GC/MS retention times of ericinic acid methyl ester and isoericinic acid on a 5% phenyl and 95% dimethylpolysiloxane column were also shorter than those of abscisic acid isomers (GC/MS profile Figure 5.3). Ericinic acid isomers are believed to be rearrangement products of abscisic acid isomers (Hyink, 1998) which lack the tertiary hydroxyl group of abscisic acid isomers. The absence of this hydroxyl group accounts for the lesser polarity of ericinic acid isomers on silica gel, and their faster elution from non-polar GC columns.

Partial overlap of the abscisic acid and ericinic acid isomer peaks was observed when using a C-18 reverse phase HPLC column. Thus *trans,cis*-abscisic acid (fraction **4B**) eluted later than ericinic acid (fraction **3B**), however *trans,trans*-abscisic acid (fraction **2B**) eluted earlier than ericinic acid (Figure 5.2).

5.5. Comparison of New Zealand Erica and Ling-Heather Honeys

As noted previously (see section 5.1), some workers consider ‘heather’ honey to be derivable from both *Erica* and *Calluna* spp., whereas others consider heather honey to arise only from *Calluna* spp. It is apparent from the results reported by Ferreres *et al*. (1996a), Tan *et al*. (1989b) and in this chapter (Table 5.4) that the extractable organic profiles of erica and calluna derived ‘heather’ honeys differ significantly. A comparison of selected (either dominant or distinctive) constituents found in New Zealand erica and ling-heather (*Calluna vulgaris*) honeys is reported in Table 5.8.

Table 5.8. Ranges (mg/kg) of selected compounds which can be utilized to differentiate the New Zealand unifloral grade erica (*Erica* spp.) from ling-heather (*Calluna vulgaris*)[a] honeys.

peak no	compounds	erica honeys (mg/kg)	calluna honeys[a] (mg/kg)
1	benzoic acid	405-10801	63-114
2	octanoic acid	trace-11	0.9-2.7
4	phenylacetic acid	9.0-28	153-214
5	salicylic acid	tr-14	0.2-0.4
7	4-methoxyphenaldehyde	14-255	0.9-2.9
12	phenyllactic acid	91-506	2.2-6.8
13	4-methoxybenzoic acid	92-1077	-
17	dodecanoic acid	tr-22	0.7-0.9
21	methyl syringate	4.3-231	1.6-1.9
22	ericinic acid	121-518	-
23	palmitic acid	7.8-163	4.5-6.9
24	isoericinic acid	14-58	-
25	*trans*,*cis*-abscisic acid	104-471	1.6-5.7
27	*trans*,*trans*-abscisic acid	82-364	trace

[a]data taken from Tan *et al*., 1989b.

As noted in previously (Section 5.3) elevated levels of ericinic, isoericinic, *trans*,*cis*-abscisic and *trans*,*trans*-abscisic acids (average levels 363, 34, 302 and 224 mg/kg respectively) were found in erica honey extracts. In previous studies, Tan *et al*. (1989b) and Hyink (1998) reported that New Zealand ling-heather honey (*Calluna* spp.) only contained trace levels of ericinic acid (as an unknown compound), *trans*,*cis*-abscisic acid and *trans*,*trans*-abscisic acid. This suggests that ericinic acid isomers and abscisic acid isomers could be useful for determining the floral origin of erica honeys, rather than as marker compounds for the calluna heather (ling-heather) honeys.

Benzoic acid was the dominant constituent of the 4 unifloral grade erica samples. The average level of benzoic acid in erica honey extract (average level 6950

mg/kg) detected in the present study was up to 100 times greater than that observed in the 1986-1987 season ling-heather honey extracts (average level 82 mg/kg) (Tan *et al*., 1989b).

An elevated level of 4-methoxybenzoic acid (average level 789 mg/kg) was detected in erica honey extracts, whereas it has not been reported to be a constituent of the extract of ling-heather honey (*Calluna vulgaris*) (Tan, 1989; Tan *et al*., 1989b; Hyink, 1998).

The levels of 4-methoxyphenaldehyde, phenyllactic acid, methyl syringate and salicylic acid recovered from erica honeys (average level 123, 295, 150 and 10 mg/kg respectively) were much greater than those observed from 1986-1987 season calluna honeys (average levels 2.1, 4.3, 1.8 and 0.3 mg/kg respectively) (Tan *et al*., 1989b).

The levels of the fatty acids detected in erica honeys were also higher than that those reported in New Zealand calluna heather (ling-heather) honey (Tan *et al*., 1989b).

The level of the phenylacetic acid found in New Zealand erica honey extracts (average level 16 mg/kg) was not as predominant as was the case for ling-heather (*Calluna* spp.) honey extracts (*e.g.* average level 189 mg/kg in 1986 season honey samples) (Tan *et al*, 1989b). Additionally the ratio of benzoic acid to phenylacetic acid in the erica samples was much greater that than of *ca* 2.25 determined for ling-heather honey extracts (Hyink, 1998).

5.6. Comparison of New Zealand and European Erica (Heather) Honeys

As noted in Section 5.3.2, Ferreres *et al*. (1996a) found abscisic acid isomers (levels in the range 25-166 mg/kg) were the main constituents in Portuguese ‘heather’ honeys derived from *Erica* spp. and they suggested these compounds were the main floral markers for the Portuguese heather (*Erica* spp.) honeys. In this investigation

high levels of abscisic acid isomers were also found in New Zealand *Erica* spp. honeys (82-471 mg/kg).

Intriguingly, while moderate levels of abscisic acid isomers are present in both New Zealand and Portuguese *Erica* spp. honeys. Ferreres *et al*. (1996a and 1996b) have not reported the presence of ericinic acid isomers in Portuguese erica spp. honeys. Whether ericinic acid isomers were not present in the Portuguese *Erica* spp. honey samples, or present, but not identified, cannot be determined from the data reported.

5.7. Plant Material (Erica Flower Extractions)

Since most of the extractable organic substances of honey are believed to be derived from the floral extractives, preliminary (qualitative) investigation of erica flower material was carried out.

A number of compounds present in methylated diethyl ether extracts of erica honeys were also detected in the methylated methanol/dichloromethane extracts of white and pink erica flowers (Table 5.9).

trans,cis-Abscisic acid and *trans,trans*-abscisic acid isomers were identified in both pink and white erica flower extracts. Ericinic acid was observed in only white flower extracts and the presence of benzoic acid in white flower extracts to be appeared much higher than in pink flower extracts. This observation shows that ericinic acid (and probably also isoericinic acid - which was not identifiable at the signal/noise level of the GC/MS analyses) are plant-sourced substances (and by implication also nectar-sourced substances), rather than 'bee-rearrangement' products of abscisic acid.

The other compounds observed in flower extracts included aromatic acids such as phenylacetic acid, 4-methoxybenzoic acid, syringic acid, 4-methoxyphenaldehyde and aliphatic carboxylic acids such as palmitic acid and stearic acid (18:0). These compounds were also observed in the erica honey extracts. In addition to these compounds, a complex mixture of high molecular weight

components which were probably triterpenes and triterpene acids (*e.g.* oleanolic acid, ursolic acid) were also observed in the flower extracts. None of these triterpene-like components were identified in the honey extracts. Further evaluation of plant materials including nectar and flower extracts needs to be carried out to ascertain seasonal variation in the levels of substances and the generality of the findings reported in Table 5.9.

Table 5.9. A qualitative comparison of substances found in erica honey extracts, and in methanol/dichloromethane extracts of erica flowers. Acids were detected as the corresponding methyl esters.

peak no	compound	erica honey extracts	pink flower extracts	white flower extracts
1	benzoic acid	yes	yes	yes
2	phenylacetic acid	yes	yes	yes
7	4-methoxyphenaldehyde	yes	-	yes
13	4-methoxybenzoic acid	yes	yes	yes
15	4-methoxyphenylacetic acid	yes	yes	yes
-	tetradecanoic acid (14:0)[a]	yes	yes	-
-	3,4-dimethoxybenzoic acid[a]	yes	-	yes
21	syringic acid[b]	yes	yes	yes
23	palmitic acid (16:0)	yes	yes	yes
22	ericinic acid	yes	-	yes
25	*trans,cis*-abscisic acid	yes	yes	yes
26	stearic acid (18:0)	yes	-	yes
27	*trans,trans*-abscisic acid	yes	yes	yes

[a]present in < 1 mg/kg in erica honey extracts (peak numbers have not been included in Table 5.2);

[b]methyl syringate was present in the honey extracts.

5.8. Conclusion

The GC/MS profile of seven erica honey samples investigated in the present study proved to be similar to that obtained by Hyink (1998) for a single 1994 season erica honey sample.

The available evidence indicates that moderate levels of ericinic acid, isoericinic acid isomers (average level 363 and 34 mg/kg respectively), *trans,cis*, *trans,trans*-abscisic acid isomers (average level 302 and 224 mg/kg respectively) and benzoic acid (average level 6950 mg/kg) are diagnostic for erica honeys. Ericinic acid was found in erica flower extracts in methanol/dichloromethane. Ericinic acid and isoericinic acid isomers have not been detected in any of the other monofloral honeys analysed to date in this laboratory.

Amongst the other dominant extractives of erica honeys, elevated levels of 4-methoxybenzoic acid (average level 789 mg/kg), 4-methoxyphenaldehyde (average level 123 mg/kg), phenyllactic acid (average level 295 mg/kg) and methyl syringate (average level 150 mg/kg) were detected in all of the erica honey samples. Some of these compounds (*e.g.* phenyllactic acid and methyl syringate) may be sourced from secondary floral sources (possibly manuka).

The total levels of extractable organic substances recovered in the diethyl ether extracts of New Zealand erica honeys are similar to the levels of diethyl ether extractable organic compounds found in New Zealand ling-heather honeys, lower than that found in manuka honeys and higher than clover honeys.

The GC/MS profiles of New Zealand erica honey differ considerably from those of the New Zealand ling-heather honey studied by Tan (1989), Valentine (1992) and Sun (1995). The GC/MS profiles of New Zealand erica honey can readily distinguish them from other New Zealand unifloral honeys.

Chapter Six

Extractable Organic Substances from Norwegian Erica (*Erica* spp.) and Heather (*Calluna*) Honey Samples

Chapter Six

Extractable Organic Substances from Norwegian Erica (*Erica* spp.) and Heather (*Calluna*) Honey Samples

6.1. Introduction

As noted in Chapter 5, there is some confusion concerning the classification of heather honeys since they are often considered to be derived from either or both of *Calluna* and *Erica* species (See Section 5.1). Ferreres *et al*. (1996a) has proposed floral marker compounds for 'heather' honey based on extractives identified in *Erica arborea* honeys, whereas a differing array of extractives are present in New Zealand and European *Calluna* spp. honeys which are often referred to as ling-heather honeys (Tan *et al*., 1989b; Speer and Montag, 1984; Steeg and Montag, 1988a).

A notable aspect of the results reported in Chapter 5 was the detection of ericinic acid and isoericinic acid (envisaged to be rearrangement products of absicsic acids) in the extracts of New Zealand *Erica lusitanica* honey, compared to the apparent absence of this substance in extracts of European *Erica arborea* honeys. On the other hand abscisic acids, while moderate to dominant components of the extracts of *Erica* spp. honey, were only minor or trace constituents of extracts of calluna honeys.

It was therefore of interest to determine if ericinic and isoericinic acids were present in extracts of European erica honeys sourced from regions of Europe other than Portugal. A major constraint in seeking to address this issue are quarantine regulations which prohibit the important of foreign honeys into New Zealand, however it is permissible to import certified 'non-biological' extracts recovered from the honey samples.

During the course of the investigations reported in this thesis an opportunity arose to analyse the extracts of a number of Norwegian erica and mixed erica-heather honey extracts. Samples were extracted in the National Veterinary Institute, Oslo, and

after air drying and verification of the non-biological nature of the extractive mixtures, extracts were forwarded to the University of Waikato for chemical analysis

Thirteen samples of mixed erica-heather honey extracts, sourced from individual beekeepers in the Stavanger district, Southern Norway, were supplied by the Norges Honning Authority. Seven samples were considered to be derived from predominantly erica sources with lesser inputs of *calluna* (erica-rich) honeys while 6 samples which were considered to be derived from predominantly calluna sources (calluna-rich) honeys. A further 5 calluna-rich heather honey samples were purchased from retail outlets in Oslo. All samples were 1999 or 2000 season samples.

Norwegian Honey

Relatively few unifloral honeys are produced in Norway, since the flowering season during which nectar is available is short, and many plants flower at the same time. Norwegian honeys are generally seasonal blends (*e.g.* summer honeys and autumn honeys). One of the few Norwegian honeys (honnings) that is produced in more or less unifloral grade is 'heather' honey from the Stavanger district in the county of Rogaland, south-western Norway. Two forms of 'heather' honeys can be recognised, based on a combination of organoleptic properties, including taste and colour. Some areas of the Rogaland country afford predominantly erica derived honeys, whereas others afford calluna dominant honeys.

The principal objectives of the work reported in this Chapter were:

- -to define the typical chemical fingerprints (GC/MS profiles) of Norwegian 1999-2000 season 7 erica-rich (erica dominant) and 11 calluna-rich (calluna dominant) honey samples, and

- -to determine the levels of extractable organic substances present in Norwegian erica-rich honeys and compare these data with those reported for New Zealand erica honeys, and the Portuguese *Erica arborea* honeys.

6.2. Experimental

6.2.1. Honey Extraction

Seven erica-rich honey samples (samples 1, 2, 3, 4, 5, 6 and 7) and five of eleven calluna-rich honey samples (samples 1, 2, 3, 4 and 6) were continuously liquid-liquid extracted (by Professor Wilkins in Norway during 2001) using the methodology reported in Section 2.2.1, other than that a surrogated standard (heptadecanoic acid) was not spiked into the samples just before the extraction. The remaining six calluna-rich honey samples (samples 5, 7, 8, 9, 10 and 11) [~ 5 g of honey (weighted accurately) dissolved in 150 mL of distilled water] were extracted with dichloromethane (4 x 75 mL) using a separating funnel (500 mL) due to the breakage of the liquid-liquid extractor. Extracts were concentrated *in vacuo* (~ 40 °C) and derivatized with diazomethane (Section 2.3) and forwarded to New Zealand for GC/MS chemical analysis.

6.2.3. GC/MS Analyses of Methylated Extracts

The methylated honey extracts which were qualitatively analysed in Oslo and subsequently quantitatively analysed in New Zealand following the addition of 50 µL [of a 1 mg/mL dichloromethane solution of heptadecanoic ethyl ester (17:0 Et)] as internal standard (post extraction) into each of sample. GC/MS analyses were conducted as described in Section 2.4.

6.3. Limitations

It was apparent from a comparison of the New Zealand and Norway GC/MS profiles that some losses of early eluting, volatile substances, mostly notably the methyl esters of benzoic acid and phenylacetic acid had occurred during the storage and transportation of the samples from Norway to New Zealand (Figure 6.1).

An estimate of the loss of benzoic acid (or phenylacetic acid) during the storage and transportation of samples can be made by comparing the ratio of the benzoic acid methyl ester (or phenylacetic acid methyl ester) and phenyllactic acid methyl ester peaks in the qualitative Norwegian and quantitative New Zealand GC/MS profiles, assuming no loss of phenyllactic acid methyl ester has occurred during storage and transportation.

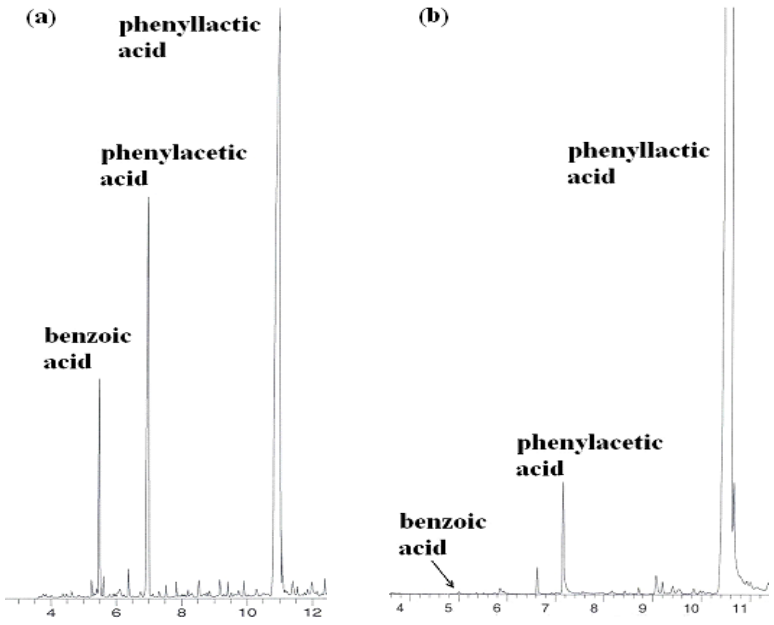


Figure 6.1. Partial GC/MS profiles recorded in (a) Norway and (b) New Zealand, of a representative Norwegian erica-rich honey extract showing the loss of volatile compounds (*e.g.* benzoic acid and phenylacetic acid) as a result of transportation between Norway and New Zealand.

A comparison of the two ratios gives an estimate of the extent to which benzoic acid methyl ester (or phenylacetic acid methyl ester) has been lost during storage and transportation, and the correction factor that can be applied to the New Zealand determined level of benzoic acid methyl ester (or phenylacetic acid methyl ester), when estimating the level of this compound that was initially present in the Norwegian extracts.

$$\text{Estimated correction factor} = \frac{[\text{benzoic acid}_{(Nor)}/\text{Phenyllactic acid}_{(Nor)}]}{[\text{benzoic acid}_{(NZ)}/\text{Phenyllactic acid}_{(NZ)}]}$$

where:

$\text{benzoic acid}_{(Nor)}$ = peak area of benzoic acid methyl ester in Norwegian GC/MS

$\text{benzoic acid}_{(NZ)}$ = peak area of benzoic acid methyl ester in New Zealand GC/MS

$\text{phenyllactic acid}_{(Nor)}$ = peak area of phenyllactic acid methyl ester in Norwegian GC/MS

$\text{phenyllactic acid}_{(NZ)}$ = peak area of phenyllactic acid methyl ester in New Zealand GC/MS

6.4. Results and Discussions

The levels of compounds identified in the methylated extracts of the Norwegian 1999-2000 season, 7 erica-rich and 5 calluna-rich liquid-liquid extracted and 6 separating funnel extracted calluna-rich honey samples analysed in this investigation are given in Tables 6.1, 6.2 and 6.3 respectively. The average concentrations, standard deviations and coefficients of variance of the methylated extractable organic substances detected in the three sets of samples are given in Tables 6.4 and 6.5 respectively. The detection of a methyl ester in the methylated extracts was considered to indicate the presence of the parent acid in original honey extracts.

The GC/MS profiles of typical methylated dichloromethane extracts of Norwegian erica-rich and calluna-rich honey extracts are shown in Figures 6.2 and 6.3 respectively.

Table 6.1. Concentrations (mg/kg) of components detected in dichloromethane extracts of seven (1999-2000 season) Norwegian erica-rich honey samples. Acids were quantified as the corresponding methyl esters.

	Name of compound	1	2	3	4	5	6	7
1	benzoic acid[a]	3.8	13	0.2	5.7	4.7	20	0.8
2	2,6,6-trimethyl-2-cyclohexene-1,4-dione	0.4	0.7	0.1	0.2	0.4	0.4	0.4
3	phenylacetic acid[a]	7.4	24	1.0	10	8.9	34	1.3
4	α-hydroxyphenylacetic acid	0.4	0.8	0.1	0.4	0.8	0.6	0.2
5	4-hydroxy-3,5,5-trimethylcyclohex-2-enone	0.2	0.3	0.3	0.2	0.2	0.2	0.2
6	phenyllactic acid	233	265	133	199	170	195	96
7	unknown (*m/z* 45, 91, 103, 131, 162, 191)[b]	1.2	0.9	0.3	0.8	1.8	1.0	0.4
8	unknown (*m/z* 43, 57, 137, 165, 180, 205, 221, 236)[b]	5.4	6.2	0.9	1.4	1.8	2.2	0.8
9	decanedioic acid	1.9	2.7	1.9	1.7	-	2.0	2.4
10	3-oxo-α-ionol	4.4	8.1	5.0	4.8	4.4	5.5	3.6
11	unknown (*m/z* 43, 57, 109, 151, 165, 183, 208)[b]	1.8	1.5	1.3	1.4	2.9	1.0	1.4
12	unknown (*m/z* 43, 55, 72, 111, 137, 82, 94, 162)[b]	1.9	2.1	1.6	1.6	2.1	1.4	1.2
13	tetradecanoic acid	1.5	2.7	1.5	1.7	2.7	11	0.7
14	methyl syringate	0.7	1.3	0.2	0.7	3.8	0.7	2.8
15	unknown (*m/z* 43, 55, 83, 98, 111, 126, 168)[b]	16	20	15	14	13	11	11
16	unknown (*m/z* 45, 93, 107, 152, 180, 224)[b]	1.1	1.4	0.5	1.1	1.0	1.8	0.3
17	4-hydroxy-3,3,5-trimethyl-2-cyclohexen-1-one	5.2	11	5.0	6.4	5.4	12	6.0
18	pentadecanoic acid	1.0	1.7	1.0	1.3	1.7	7.6	0.8
19	7-hexadecenoic acid	2.0	3.3	1.6	2.3	3.8	14	1.7
20	ericinic acid	1.2	1.6	0.8	0.9	1.6	1.1	0.7
21	hexadecanoic acid	7.3	11	6.8	9.0	11	43	5.5
22	isoericinic acid	0.2	0.4	0.3	0.3	0.3	0.5	0.2
23	unknown (*m/z* 123, 164,192, 280)[b]	0.5	0.9	0.7	0.7	0.8	0.4	0.5
24	7-octadecenenoic acid	12	11	7.8	9.9	11	20	5.2
25	*trans,cis*-abscisic acid	11	18	12	11	8.5	9.4	7.4
26	stearic acid	-	-	1.5	1.5	2.1	7.7	1.1
27	*trans,trans*-abscisic acid	2.0	3.6	1.6	2.0	1.9	1.3	1.1
28	docosanoic acid (0:22)	0.2	0.7	0.1	0.3	0.2	1.2	0.1

[a]corrected level (see text); [b] prominent ions observed in the mass spectra of unknown compounds are given in brackets.

Table 6.2. Concentrations (mg/kg) of components detected in dichloromethane extracts of five (1999-2000 season) Norwegian calluna-rich honey samples (liquid-liquid extracted). Acids were quantified as the corresponding methyl esters.

	Name of compound	1	2	3	4	6
1	benzoic acid[a]	25	12	17	21	15
2	2,6,6-trimethyl-2-cyclohexene-1,4-dione	2.2	1.5	1.2	2.7	2.2
3	phenylacetic acid[a]	40	28	33	43	34
4	phenyllactic acid	71	18	21	134	30
5	β-hydroxyphenylpropanoic acid	1.6	0.3	0.6	0.9	1.4
6	nonanedioic acid	0.4	0.1	0.1	0.4	0.6
7	3,4-dimethoxybenzoic acid	0.8	0.3	0.5	0.6	0.4
8	1,2,3-trimethoxybenzene	0.7	0.2	0.5	0.6	0.3
9	decanedioic acid	1.2	0.2	0.7	2.4	1.6
10	3-oxo-α-ionol	0.6	0.4	0.7	1.8	0.8
11	unknown (*m/z* 43, 77, 147, 162)[b]	6.0	4.9	4.8	7.0	7.0
12	unknown (*m/z* 82, 94, 150, 162)[b]	0.8	0.7	1.0	1.4	1.3
13	tetradecanoic acid	1.4	1.1	3.3	1.5	2.0
14	methyl syringate	1.0	0.6	1.0	1.3	1.1
15	unknown (*m/z* 45, 93, 107, 152,180, 224)[b]	6.3	4.9	6.7	7.1	9.6
16	4-hydroxy-3,3,5-trimethyl-2-cyclohexen-1-one	7.3	19	13	14	25
17	unknown (*m/z* 45, 73, 124, 219, 292)[b]	1.7	1.2	1.1	2.0	-
18	pentadecanoic acid	0.9	0.7	2.1	1.1	1.0
19	7-hexadecenoic acid	1.4	1.5	4.2	2.1	1.7
20	ericinic acid	1.1	0.4	0.5	0.8	0.5
21	hexadecanoic acid	8.9	6.0	13	8.1	9.7
22	isoericinic acid	0.1	0.1	0.2	0.1	0.1
23	heptadecanoic acid	0.3	0.2	0.6	0.4	0.3
24	7-octadecenenoic acid	8.9	5.2	7.9	4.4	6.6
25	*trans,cis*-abscisic acid	1.6	0.9	1.4	3.3	1.4
26	stearic acid	1.6	1.1	2.6	1.7	1.6
27	*trans,trans*-abscisic acid	0.7	0.3	0.4	1.2	0.5
28	docosanoic acid (0:22)	0.5	0.1	0.3	0.4	0.7

[a]corrected level (see text); [b]prominent ions observed in the mass spectra of unknown compounds are given in brackets.

Table 6.3. Concentrations (mg/kg) of components detected in dichloromethane extracts of six (1999-2000 season) Norwegian calluna-rich honey samples (separating funnel extracted). Acids were quantified as the corresponding methyl esters.

	Name of compound	5	7	8	9	10	11
1	benzoic acid[a]	5.2	3.6	1.2	6.2	1.9	2.2
2	2,6,6-trimethyl-2-cyclohexene-1,4-dione	2.3	0.5	1.2	2.7	1.6	1.2
3	phenylacetic acid[a]	12	8.7	3.3	11	3.8	5.2
4	phenyllactic acid	104	6.0	17	103	64	55
5	β-hydroxyphenylpropanoic acid	0.3	-	0.4	0.2	0.3	0.5
6	nonanedioic acid	0.3	0.1	0.1	0.3	0.2	0.2
7	3,4-dimethoxybenzoic acid	0.3	0.03	0.2	0.5	0.4	0.3
8	1,2,3-trimethoxybenzene	-	0.05	0.01	-	-	-
9	decanedioic acid	1.3	0.4	0.4	1.2	1.1	0.8
10	3-oxo-α-ionol	1.4	0.7	0.9	1.9	1.0	0.7
11	unknown (*m/z* 43, 77, 147, 162)[b]	12	4.1	5.5	7.7	6.7	5.7
12	unknown (*m/z* 82, 94, 150, 162)[b]	1.9	0.9	1.0	2.0	1.2	1.0
13	tetradecanoic acid	4.3	1.2	1.6	2.0	3.7	1.9
14	methyl syringate	1.5	0.6	1.1	0.9	0.7	1.4
15	unknown (*m/z* 45, 93,107,152,180, 224)[b]	18	6.6	11	10	14	9.9
16	4-hydroxy-3,3,5-trimethyl-2-cyclohexen-1-one	52	27	43	40	37	37
18	pentadecanoic acid	2.8	0.8	1.2	1.8	2.8	1.4
19	7-hexadecenoic acid	6.2	1.4	2.5	2.8	5.7	2.7
20	ericinic acid	0.6	0.3	0.5	0.9	0.5	0.5
21	hexadecanoic acid	18	6.6	9.7	14	18	12
22	isoericinic acid	0.3	0.1	0.2	0.2	0.2	0.2
23	heptadecanoic acid	0.7	0.2	0.3	0.4	0.8	0.6
24	7-octadecenenoic acid	9.3	5.3	11	9.9	8.8	9.2
25	*trans,cis*-abscisic acid	1.8	0.8	1.2	3.4	1.4	1.1
26	stearic acid	3.1	1.2	2.2	2.4	3.3	2.3
27	*trans,trans*-abscisic acid	0.6	0.2	0.2	1.0	0.8	0.6
28	docosanoic acid (0:22)	0.8	0.2	0.4	1.4	0.4	0.4

[a]corrected level (see text); [b]prominent ions observed in the mass spectra of unknown compounds are given in brackets.

Table 6.4. Mean concentration (mg/kg), standard deviation (stdev), and coefficient of variance (CV%) determined for compounds identified in the extracts of seven (1999-2000 season) Norwegian erica-rich honey samples. Acids were quantified as the corresponding methyl esters.

peak	name of compound	mean	stdev	n	CV (%)
1	benzoic acid[a]	6.9	7.1	7	104
2	2,6,6-trimethyl-2-cyclohexene-1,4-dione	0.4	0.2	7	51
3	phenylacetic acid[a]	12	12	7	99
4	α-hydroxyphenylacetic acid	0.5	0.3	7	61
5	4-hydroxy-3,5,5-trimethylcyclohex-2-enon	0.2	0.04	7	15
6	phenyllactic acid	184	58	7	31
7	unknown (*m/z* 45, 91, 103, 131, 162, 191)[b]	0.9	0.5	7	53
8	unknown (*m/z* 43, 57,137,165,180, 205, 221,236)[b]	2.7	2.2	7	82
9	decanedioic acid	2.1	0.4	6	18
10	3-oxo-α-ionol	5.1	1.4	7	28
11	unknown (*m/z* 43, 57, 109,151, 165, 183, 208)[b]	1.6	0.6	7	38
12	unknown (*m/z* 43, 55, 72, 111,137, 82, 94,162)[b]	1.7	0.3	7	20
13	tetradecanoic acid	3.1	3.5	7	114
14	methyl syringate	1.5	1.3	7	92
15	unknown (*m/z* 43, 55, 83, 98, 111,126,168)[b]	14	3.1	7	22
16	unknown (*m/z* 45, 93,107,152,180, 224)[b]	1.0	0.5	7	49
17	4-hydroxy-3,3,5-trimethyl-2-cyclohexen-1-one	7.3	2.9	7	40
18	pentadecanoic acid	2.2	2.4	7	112
19	7-hexadecenoic acid	4.1	4.4	7	108
20	ericinic acid	1.1	0.4	7	32
21	hexadecanoic acid	13	13	7	99
22	isoericinic acid	0.3	0.1	7	35
23	unknown (*m/z* 123,164,192,280)[b]	0.7	0.2	7	28
24	7-octadecenenoic acid	11	4.6	7	42
25	*trans,cis*-abscisic acid	11	3.5	7	31
26	stearic acid	2.8	2.8	5	100
27	*trans,trans*-abscisic acid	1.9	0.8	7	42
28	docosanoic acid (0:22)	0.4	0.4	7	98
29	tetracosanoic acid (0:24)	1.6	1.6	7	103

[a]corrected level (see text); [b]prominent ions observed in the mass spectra of unknown compounds are given in brackets.

Table 6.5. Mean concentration (mg/kg), standard deviation (stdev), and coefficient of variance (CV%) determined for compounds identified in the dichloromethane extracts of eleven (1999-2000 season) Norwegian calluna-rich honey samples. Acids were quantified as the corresponding methyl esters.

peak		liquid-liquid extraction				separating funnel extraction			
no	name of compound	mean	stdev	n	CV (%)	mean	stdev	n	CV (%)
1	benzoic acid[a]	18	5.1	5	28	3.4	2.0	6	59
2	2,6,6-trimethyl-2-cyclohexene-1,4-dione	2.0	0.6	5	31	1.6	0.8	6	51
3	phenylacetic acid[a]	36	5.9	5	17	7.3	3.7	6	51
4	phenyllactic acid	55	49	5	90	58	41	6	71
5	β-hydroxyphenylpropanoic acid	1.0	0.5	5	54	0.3	0.1	5	31
6	nonanedioic acid	0.3	0.2	5	59	0.2	0.1	6	46
7	3,4-dimethoxybenzoic acid	0.5	0.2	5	36	0.3	0.2	6	59
8	1,2,3-trimethoxybenzene	0.5	0.2	5	39	0.03	0.03	2	94
9	decanedioic acid	1.2	0.8	5	69	0.9	0.4	6	45
10	3-oxo-α-ionol	0.9	0.5	5	62	1.1	0.5	6	43
11	unknown (*m/z* 43, 77, 147, 162)[b]	5.9	1.1	5	18	7.0	2.8	6	40
12	unknown (*m/z* 82, 94, 150, 162)[b]	1.0	0.3	5	30	1.3	0.5	6	37
13	tetradecanoic acid	1.9	0.9	5	47	2.5	1.2	6	51
14	methyl syringate	1.0	0.3	5	26	1.0	0.4	6	36
15	unknown (*m/z* 45, 93, 107, 152, 180, 224)[b]	6.9	1.7	5	25	12	3.9	6	34
16	4-hydroxy-3,3,5-trimethyl-2-cyclohexen-1-one	16	6.7	5	43	39	8.2	6	21
17	unknown (*m/z* 45, 73, 124, 219, 292)[b]	1.5	0.4	4	28	-	-	-	-
18	pentadecanoic acid	1.2	0.5	5	47	1.8	0.8	6	46
19	7-hexadecenoic acid	2.2	1.2	5	53	3.6	1.9	6	54
20	ericinic acid	0.7	0.3	5	44	0.6	0.2	6	40
21	hexadecanoic acid	9.1	2.6	5	28	13	4.6	6	35
22	isoericinic acid	0.1	0.03	5	24	0.2	0.1	6	38
23	heptadecanoic acid	0.3	0.1	5	42	0.5	0.2	6	44
24	7-octadecenenoic acid	6.6	1.9	5	28	8.9	1.9	6	22
25	*trans,cis*-abscisic acid	1.7	0.9	5	53	1.6	0.9	6	58
26	stearic acid	1.7	0.5	5	32	2.4	0.7	6	31
27	*tran,trans*-abscisic acid	0.6	0.3	5	54	0.6	0.3	6	55
28	docosanoic acid (0:22)	0.4	0.2	5	51	0.6	0.4	6	70
29	tetracosanoic acid (0:24)	2.5	2.0	5	83	2.6	1.4	6	55

[a]corrected level (see text); [b]prominent ions observed in the mass spectra of unknown compounds are given in brackets.

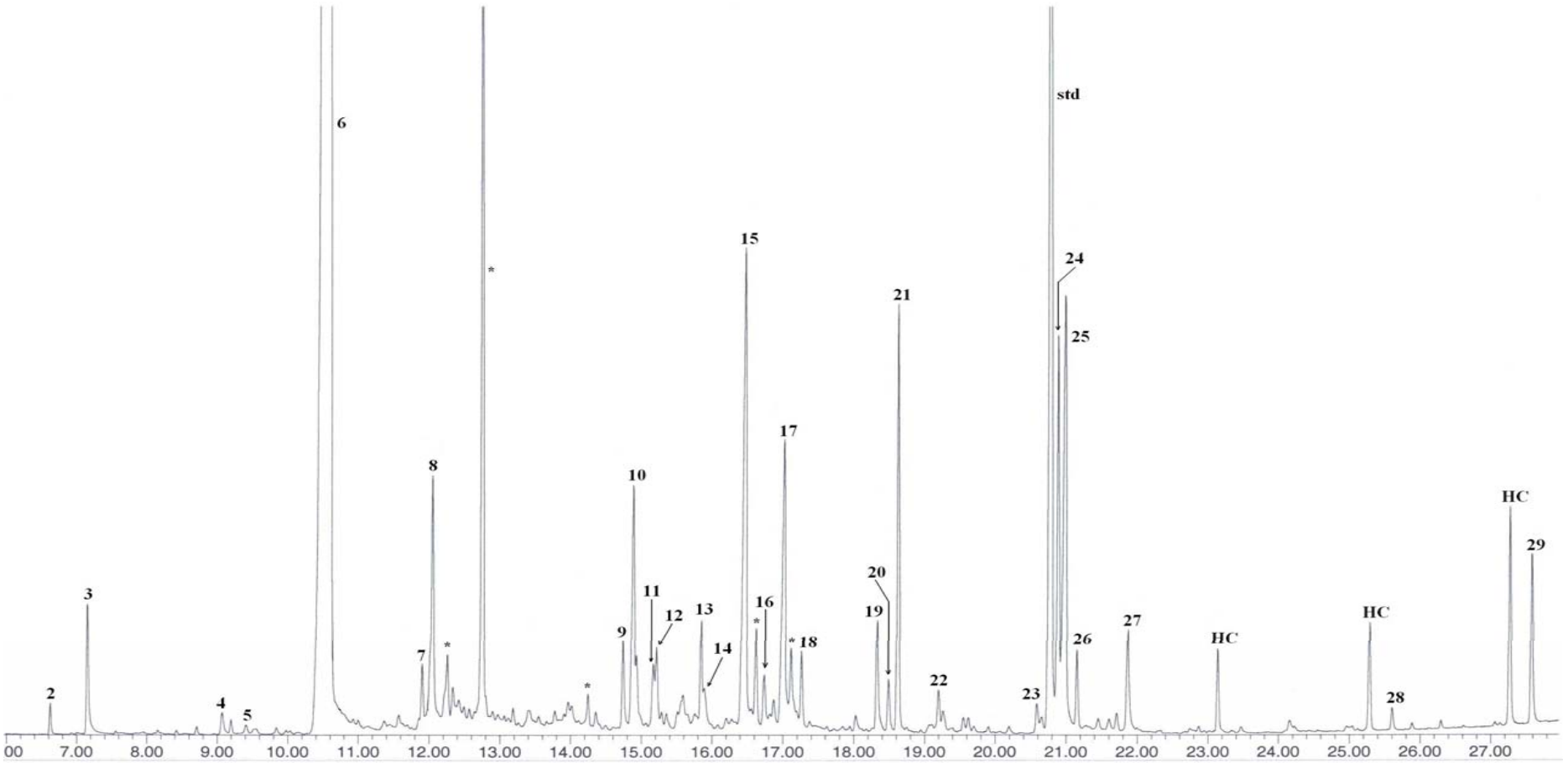


Figure 6.2. GC/MS profile of a representative methylated Norwegian erica-rich honey diethyl ether extract. Peak identifications are given Table 6.1 (Peaks marked with "HC" were hydrocarbon and the peaks marked with "*" were originated from phthalates and/or solvent stabilizers).

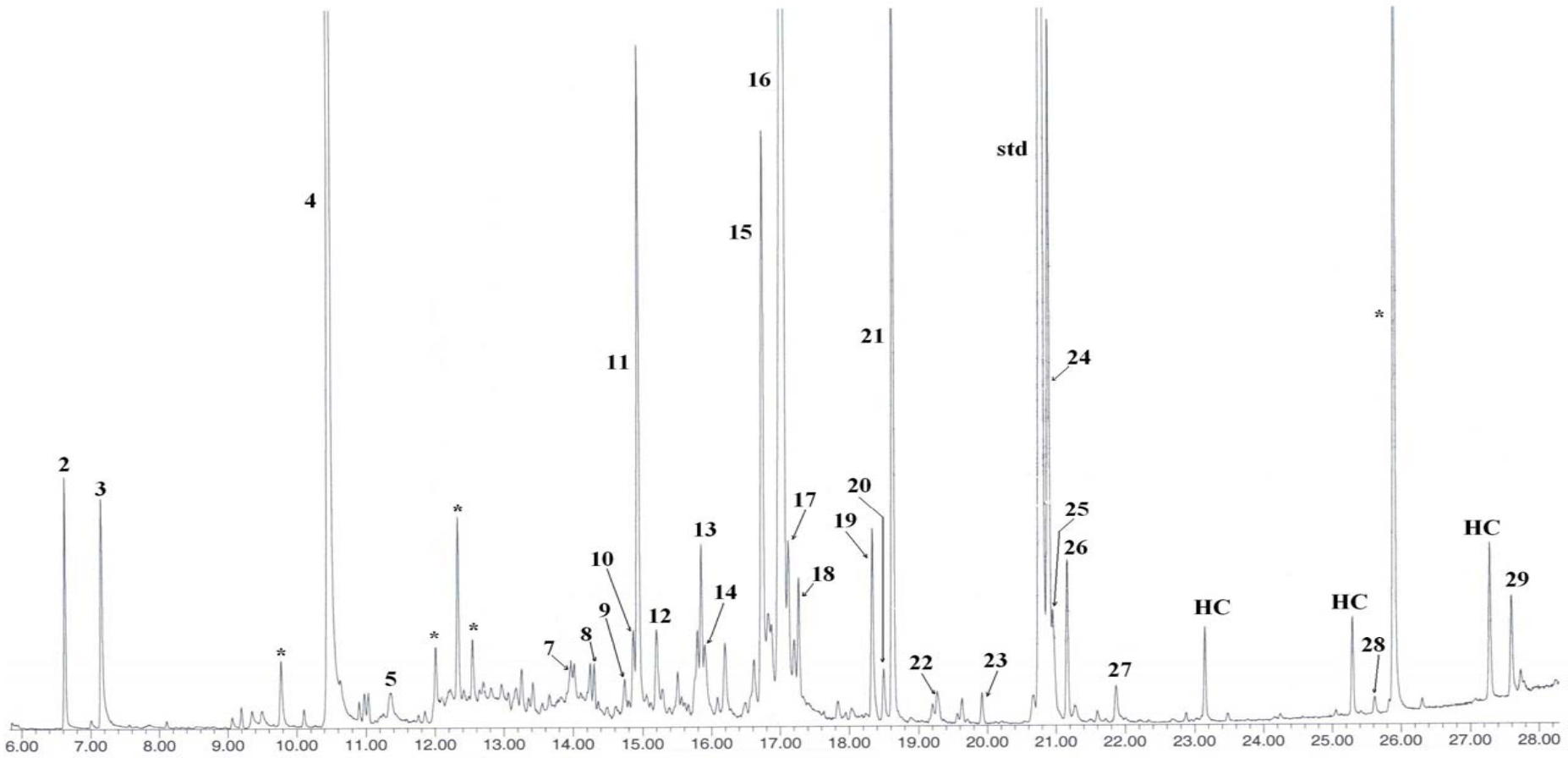


Figure 6.3. GC/MS profile of a representative methylated Norwegian calluna-rich honey diethyl ether extract. Peak identifications are given Table 6.2 (Peaks marked with "HC" were hydrocarbon and the peaks marked with "*" were originated from phthalates and/or solvent stabilizers).

The most prominent peak identified in the methylated dichloromethane Norwegian erica-rich honey extract was phenyllactic acid (peak 6) (average level 184 mg/kg). In addition, moderate levels of benzoic acid (peak 1) (average corrected level 6.9 mg/kg), phenylacetic acid (peak 3) (average corrected level 12 mg/kg), an unknown compound (peak 15) (average level 14 mg/kg), 4-hydroxy-3,3,5-trimethyl-2-cyclohexen-1-one (peak 17) (average level 7.3 mg/kg), 7-octadecenenoic acid (peak 24) (average level 11 mg/kg), *trans,cis*-abscisic acid (peak 25) (average level 11 mg/kg) and low levels of 3-oxo-α-ionol (peak 10) (average level 5.1 mg/kg), methyl syringate (peak 14) (average level 1.5 mg/kg), ericinic acid (peak 20) (average level 1.1 mg/kg), *trans,trans*-abscisic acid (peak 27) (average level 1.9 mg/kg) were observed. Trace levels of isoericinic acid (peak 22) (average level 0.32 mg/kg) were seen in the 7 of the erica-rich honey samples.

The principal peaks identified in the liquid-liquid extracted methylated Norwegian calluna-rich honey extracts were phenyllactic acid (peak 4) (average level 55 mg/kg) and phenylacetic acid (peak 3) (average corrected level 36 mg/kg), along with moderate level of benzoic acid (peak 1) (average corrected level 18 mg/kg), an unknown compound (peak 11) (average level 5.9 mg/kg), 4-hydroxy-3,3,5-trimethyl-2-cyclohexen-1-one (peak 16) (average level 16 mg/kg), 7-octadecenenoic acid (peak 24) (average level 6.6 mg/kg) and low to trace levels of 3-oxo-α-ionol (peak 10) (average level 0.87 mg/kg), ericinic acid (peak 20) (average level 0.67 mg/kg), isoericinic acid (peak 22) (average level 0.12 mg/kg), *trans,cis*-abscisic acid (peak 25) (average level 1.7 mg/kg) and *trans,trans*-abscisic acid (peak 27) (average level 0.64 mg/kg) .

6.4.1. Continuous Liquid-Liquid Extraction vs Separating Funnel Extraction

The levels of volatile aromatic acids extracted from the 6 Norwegian calluna-rich honey samples using dichloromethane and separating funnel recovery were lower than those observed in the continuous dichloromethane liquid-liquid extraction. For example, the average corrected levels of benzoic acid and phenylacetic acid extracted using the liquid-liquid extractor were 18 and 36 mg/kg respectively whereas the average levels of these compounds detected in the separating funnel extracts were 3.4 and 7.3 mg/kg respectively (Figure 6.4). An unknown compound (peak 17) was only

found in the liquid-liquid extracts of calluna-rich honeys (1.5 mg/kg), but this compound was not present in the separating funnel extracts.

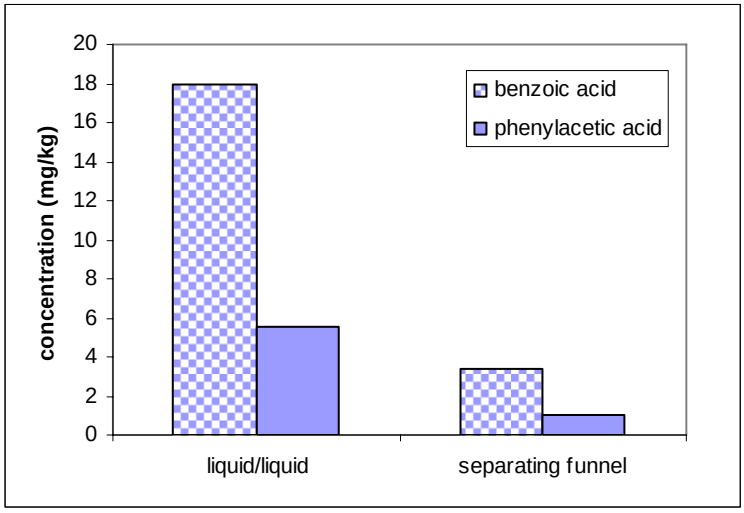


Figure 6.4. The average corrected levels of benzoic acid (peak 1) and phenylacetic acid (peak 3) detected in the continuous liquid-liquid extracts and separating funnel extracts of Norwegian calluna-rich honeys.

On the other hand, the levels of some of the compounds (*e.g.* 4-hydroxy-3,3,5-trimethyl-2-cyclohexen-1-one (peak 16) and an unknown (peak 15) detected in the separating funnel extracts (average levels 39 and 12 mg/kg respectively) of the calluna-rich honeys were higher than that observed in the continuous liquid-liquid extracts (average levels 16 and 6.9 mg/kg respectively).

There were no significant differences of the levels of ericinic acid, isoericinic, *trans,cis*-abscisic acid and *trans,trans*-abscisic acid found in the continuous liquid-liquid extracts (average levels 0.67, 0.12, 1.7 and 0.64 mg/kg respectively) and the separating funnel extracts (average levels 0.56, 0.20, 1.6 and 0.59 mg/kg respectively) of calluna-rich honeys. Similar levels of phenyllactic acid, methyl syringate, aliphatic acids (*e.g.* pentadecanoic acid and stearic acid) and diacids (nonanedioic acid and decanedioic acid) were also observed in both of the continuous liquid-liquid and separating funnel extracts of the calluna-rich honeys. However, it could be expected a low recovery of most of extracted organic substances in the separating funnel extraction method, as emulsions form during the extraction procedure (Emulsions may adsorb compounds with polar groups such as acids).

6.4.2. Ericinic Acid and Isoericinic Acid

Low levels of ericinic and isoericinic acids (average levels of 1.1 and 0.32 mg/kg respectively) were detected in methylated extracts of the 7 erica-rich honey samples. Approximately half of these levels were found in the calluna-rich honey samples (average levels of 0.61 and 0.16 mg/kg respectively), Figure 6.5.

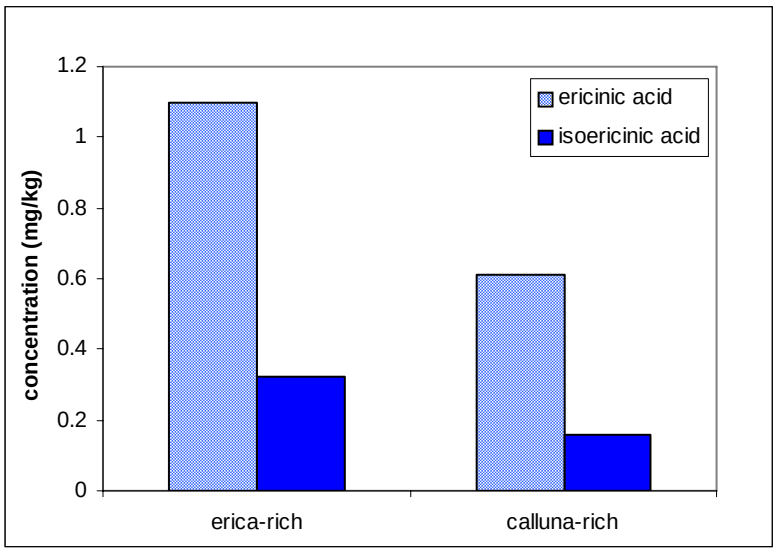


Figure 6.5. The average levels of ericinic acid and isoericinic acid present in the methylated dichloromethane extracts of 7 erica-rich and 11 calluna-rich honey samples.

Though the average levels of ericinic acid and isoericinic acid in methylated extracts of 7 Norwegian erica-rich honey samples were significantly lower that found in the methylated extracts of 4 New Zealand unifloral grade erica honey samples (average levels 363 and 34 mg/kg), these isomers were present in each of the seven extracts of Norwegian erica-rich honey. It is apparent much higher levels of erinic acid isomers are present in the New Zealand samples than the Norwegian samples. Possibly this is also the case for Portuguese erica honey, thus accounting for the apparent non-detection of ericinic acid isomers in the honey samples investigated by Ferreres *et al.* (1996a).

The hypothesis that ericinic acid isomers could be artefacts, or by-products, of the liquid-liquid extraction procedure is discounted by the observation that ericinic acid was detected in the unheated dichloromethane extract.

6.4.3. Abscisic Acid Isomers

The seven Norwegian erica-rich honey samples contained moderate concentrations of *trans,cis* and *trans,trans*-abscisic acid isomers (average levels 11 and 1.9 mg/kg respectively. These levels were higher than those found in the 11 calluna-rich honey samples (average levels 1.7 and 0.61 mg/kg respectively), Figure 6.6. These levels can be compared with the much greater levels of abscisic acid isomers found in the methylated diethyl ether extracts of New Zealand erica honeys (average levels 302 and 224 mg/kg respectively).

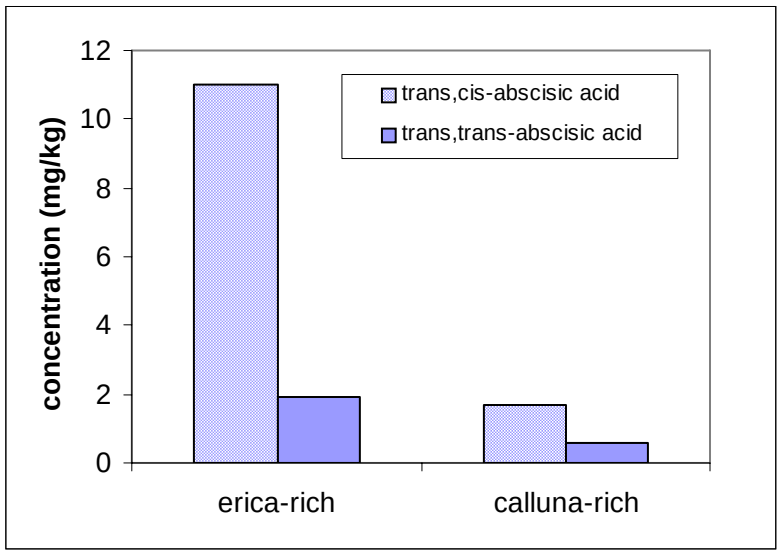


Figure 6.6. The average levels of *trans,cis*-abscisic acid and *trans,trans*-abscisic acid detected in the methylated extracts of 7 erica-rich and 11 calluna-rich honey samples.

6.4.4. Benzoic Acid

The corrected concentration ranges of benzoic acid recovered from Norwegian erica-rich and calluna-rich honeys (liquid-liquid extracted) were 0.23-20 and 12-25 mg/kg respectively while the average levels of this compound detected in 7 erica-rich honey samples (6.9 mg/kg) were lower than those detected in 5 liquid-liquid extracted calluna-rich honey samples (18 mg/kg). These levels are much lower (200-300-fold lower) than those found in New Zealand erica honeys (average level 6950 mg/kg) and higher than those found in other New Zealand manuka, clover and noddling thistle honeys (see Section 5.3.3).

6.4.5. Phenylacetic Acid

Relatively high levels of phenylacetic acid (average corrected level 36 mg/kg) were detected in the 5 liquid-liquid extracted calluna-rich honey samples while moderate levels of this compound (average corrected level 12 mg/kg) were found in the 7 erica-rich honey samples. The average corrected level of phenylacetic acid found in Norwegian erica-rich honey samples was most similar to that found in 4 New Zealand unifloral grade erica honey samples (average level 16 mg/kg) (Section 5.3.5) and also to the single collection of erica honey analysed by Hyink (1998) (12 mg/kg).

6.4.6. Degraded Carotenoid-Like Compounds

Trace levels (average level 0.37 mg/kg) of 2,6,6-trimethyl-2-cyclohexene-1,4-dione were detected in the 7 erica-rich honey samples while low levels of this compound were detected the 11 calluna-rich honey samples (average levels 2.0 and 1.6 mg/kg for liquid-liquid and separating funnel extracted samples respectively). The levels of this compound that were detected in the Norwegian erica-rich and the calluna-rich honey samples were much lower than the levels found New Zealand erica honey samples (average level 22 mg/kg) (see Section 5.3.6).

Significant levels of 4-hydroxy-3,3,5-trimethyl-2-cyclohexen-1-one were detected in the 7 erica-rich (average levels 7.3 mg/kg) and the 11 calluna-rich honey extracts (average levels 16 and 39 mg/kg for liquid-liquid extracts and separating funnel extracts respectively).

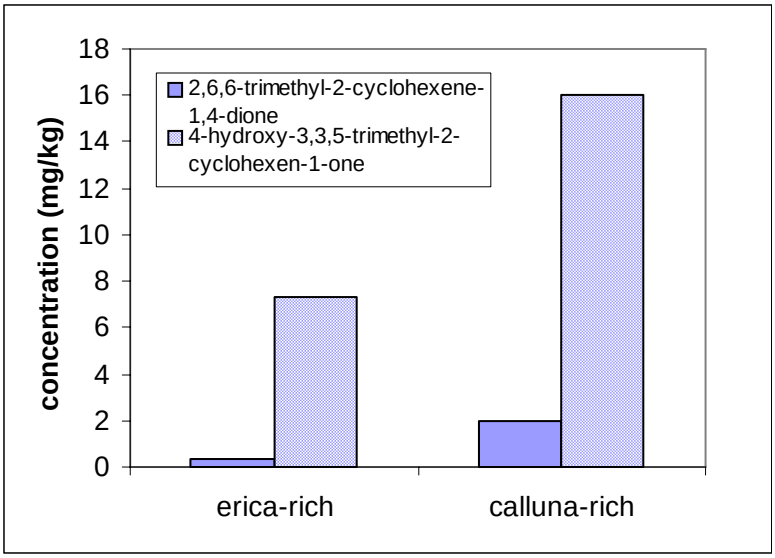


Figure 6.7. The average levels of 2,6,6-trimethyl-2-cyclohexene-1,4-dione and 4-hydroxy-3,3,5-trimethyl-2-cyclohexen-1-one detected in the methylated dichloromethane extracts of 7 erica-rich and 5 calluna-rich honey samples (liquid-liquid extracted).

6.4.7. Phenyllactic Acid

The major constituent of methylated extracts of 7 Norwegian erica-rich (average level 184 mg/kg) and 11 Norwegian calluna-rich (average level 56 mg/kg) honey samples was phenyllactic acid (peak 6 and peak 4 respectively). The levels of this compound found in Norwegian erica-rich honey extracts were lower than that found in the New Zealand erica honey extracts (average level 295 mg/kg) (Section 5.3.8) while always higher than that observed in New Zealand ling-heather honey extracts (*Calluna*) (average level 4.3 mg/kg) (Tan *et al*., 1989b) (Figure 6.8).

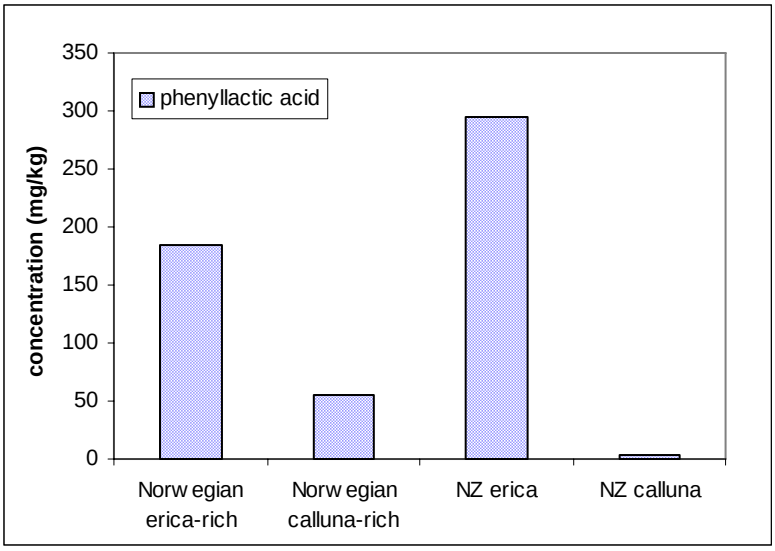


Figure 6.8. The average levels of phenyllactic acid detected in the Norwegian 7 erica-rich, 11 calluna-rich, New Zealand 4 erica (Section 5.3.8) (Table 5.4) and 4 calluna (ling-heather) (Tan *et al*., 1989b) honey samples.

6.4.8. Fatty Acids

An array of aliphatic acids and diacids were detected as their methyl esters in both Norwegian erica-rich and calluna-rich honey extracts. Low levels of aliphatic acids and diacids including decanedioic acid, tetradecanoic acid, pentadecanoic acid, 7-hexadecenoic acid, hexadecanoic acid, 7-octadecenenoic acid, stearic acid and docosanoic acid (average levels 2.1, 3.1, 2.2, 4.1, 13, 11, 2.8 and 0.41 mg/kg respectively) were identified in the seven 1999-2000 season erica-rich honey samples.

Calluna-rich honey extractives (liquid-liquid extracted) included low levels of aliphatic acids and diacids including nonanedioic acid, decanedioic acid, tetradecanoic acid, pentadecanoic acid, 7-hexadecenoic acid, hexadecanoic acid, heptadecanoic acid, 7-octadecenoic acid, stearic acid and docosanoic acid (average levels 0.32, 1.2, 1.9, 1.2, 2.2, 9.1, 0.35, 6.6, 1.7 and 0.42 mg/kg respectively for the 5 liquid-liquid extracted calluna-rich honey samples).

6.4.9. Other Compounds

Unknown (peak 7) (average level 0.93 mg/kg), unknown (peak 8) (average level 2.7 mg/kg), 3-oxo-α-iono (peak 10) (average level 5.1 mg/kg), unknown (peak 11) (average level 1.6 mg/kg), unknown (peak 12) (average level 1.7 mg/kg), methyl syringate (peak14) (average level 1.5 mg/kg), unknown (peak 15) (average level 14 mg/kg), unknown (peak 16) (average level 1.0 mg/kg) and unknown (peak 23) (average level 0.66 mg/kg) were also detected in the all of the erica-rich honey extracts.

β-Hydroxyphenylpropanoic acid (peak 5) (average level 1.0 mg/kg), 3,4-dimethoxybenzoic acid (peak7) (average level 0.54 mg/kg), 1,2,3-trimethoxybenzene (peak 8) (average level 0.48 mg/kg), 3-oxo-α-ionol (peak 10) (average level 0.87 mg/kg), unknown (peak 11) (average level 5.9 mg/kg), unknown (peak 12) (average level 1.0 mg/kg), methyl syringate (peak 14) (average level 1.0 mg/kg), unknown (peak 15) (average level 6.9 mg/kg) and unknown (peak 17) (average level 1.5 mg/kg) were found in most of the calluna honey samples (average levels of these compounds were only reported for liquid-liquid extracted calluna-rich honey samples).

6.5. Comparison between Norwegian Erica-Rich and New Zealand Erica Honeys

The percentage contributions of ericinic acid, isoericinic acid, *trans,cis*-abscisic acid and *trans,trans*-abscisic acid relative to the total level of extractable compounds in Norwegian erica-rich and New Zealand erica honey samples are compared in Table 6.6 and presented graphically in Figure 6.9.

It is apparent that ericinic acid contributions to the Norwegian erica-rich honey samples is lower, relative to New Zealand erica honey samples, both in terms of the levels present (Tables 6.1) and the % contribution of these compounds to total extractives (Table 6.6).

Table 6.6. Percentage contributions of ericinic acid, isoericinic acid, *trans,cis*-abscisic acid and *trans,trans*-abscisic acid in Norwegian erica-rich and New Zealand erica honeys.

compound	Norwegian calluna-rich	Norwegian erica-rich	New Zealand erica
ericinic acid	0.5	0.4	3.6
isoericinic acid	0.1	0.1	0.3
trans,cis-abscisic acid	1.2	3.9	3.0
trans,trans-abscisic acid	0.4	0.7	2.2
% of total extractives[a]	2.2	5.1	8.1

[a]sum of ericinic and abscisic acid isomers contributions.

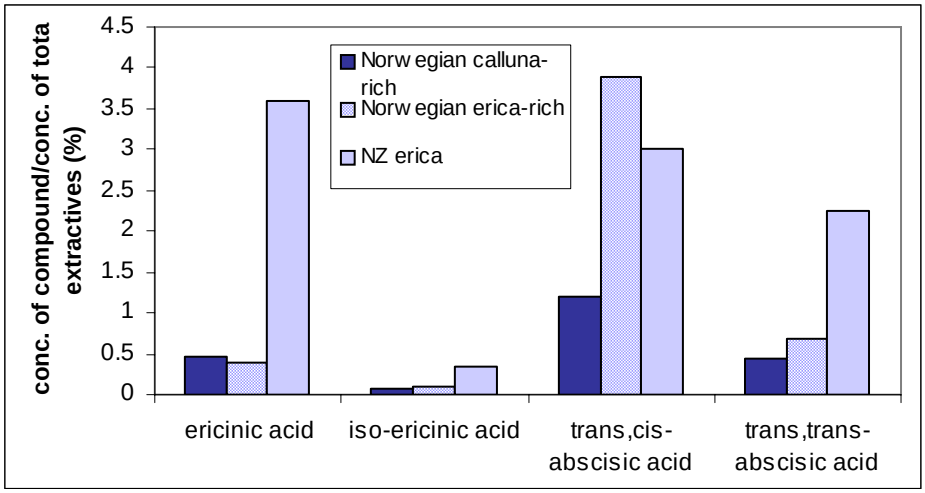


Figure 6.9. Percentage contributions of ericinic acid, isoericinic acid, *trans,cis*-abscisic acid and *trans,trans*-abscisic acid to total extractives in Norwegian erica-rich and New Zealand erica honeys.

On the other hand the % contribution to *trans,cis*-abscisic acid to total extractives recovered from in the Norwegian erica-rich and New Zealand erica samples are comparable (3.9 and 3.0% respectively).

A *ca* 1:10 ratio of ericinic acid: *trans,cis*-abscisic acid were present in the Norwegian erica-rich honey samples (average levels 1.1 and 11 mg/kg respectively) while the ratio of these compounds in New Zealand erica honey samples were *ca* 1.2:1.0 (average levels 363 and 302 mg/kg respectively).

Appreciable levels of phenyllactic acid were found in both Norwegian erica-rich and New Zealand erica honey samples (average levels 184 and 295 mg/kg).

However this compound cannot be suggested as a floral marker compound for the erica honeys, since it has been found in several New Zealand honey types including manuka (average level 775 mg/kg) (Wilkins *et al*., 1993b), ling-heather (average level 4.3 mg/kg) (Tan *et al.,* 1989b) and honeydew honeys (average level 165 mg/kg) (Section 4.3.2) honeys.

While New Zealand erica honeys contained elevated levels of benzoic acid, only moderate levels of this compound were found in Norwegian erica-rich honey samples (average levels 6950 and 6.9 (corrected) mg/kg respectively). Thus the presence of elevated levels of benzoic acid cannot be considered to be a 'universal' characteristic of erica honeys.

trans,cis-Abscisic acid, *trans,trans*-abscisic acid, ericinic acid, and isoericinic acid can however be suggested as floral marker compounds for erica honeys since these compounds were found in moderate levels both Norwegain erica-rich and New Zealand erica honey extracts.

6.6. Conclusions

Norwegian erica-rich honey possesses generally lower levels of extractable organic substances compared erica honeys from New Zealand and the Portuguese *Erica arborea* honeys investigated by Ferreres *et al.* (1996a).

The results presented here indicate that ericinic and isoericinic acids are likely to be universally present in erica honeys at levels which may range from as low as 1 mg/kg or less as found in some Norwegian samples, and up to >100 mg/kg as found in some New Zealand samples (Chapter 5). Possibly the level of ericinic acids is species dependent (with especially high levels in New Zealand *Erica lusitanica* honeys) and also regionally influenced. A further condition is that the distribution of varietal types of *Erica* species may be strongly linked to geographical factors.

It would be interest to re-analyse the *Erica arborea* honeys examined by Ferreres *et al.* (1996a and 1996b) and determine if moderate to low (trace) levels of ericinic acids were present in the extracts of the Portuguese erica honeys.

Chapter Seven

Extractable Organic Substances from Active and Inactive New Zealand Manuka (*Leptospermum scoparium*) Honeys

Chapter Seven

Extractable Organic Substances from Active and Inactive New Zealand Manuka (*Leptospermum scoparium*) Honeys

7.1. Introduction

It is now well established that some New Zealand manuka (*Leptospermum scoparium*) honeys display antibacterial activity significantly greater than that which can be ascribed merely to the high osmolarity and the presence of hydrogen peroxide (Russell, 1983; Molan and Russell, 1988) (Section 1.4). This additional antibacterial agent is known as the "unique manuka factor" (UMF). Very little information presently exists as to the nature or mode of action of the additional antibacterial factor (s).

Recent reports have demonstrated that the UMF activity of honey appears to be correlated with the presence of highly water-soluble components rather than ether-extracted organic substances (Tan, 1989; Russell *et al*., 1990; Weston *et al*., 1999). The UMF activity may also depend on the presence of a range of chemical substances rather than one or two specific components.

Although previous workers have reported that the organic extractives of active manuka honey with UMF activity could not be distinguished from the inactive manuka honey extractives (Tan, 1989; Weston, 2000), a visual inspection of the GC/MS profiles of a series of active and inactive manuka honeys suggested they might differ to a degree that could be correlated with antibacterial activity. It was recognised at the outset of these investigations that it was not likely that the compounds which are responsible for the UMF activity of manuka honeys would appear in GC/MS profiles, but some compounds identified in the GC/MS profiles still need to be evaluated for such activity. Rather, it can be hypothesised that substances contributing to the GC/MS might serve as marker substances for the presence in the honey of unidentified, and presumably polar, substances (see above) responsible for the UMF activity. The possibility that such a 'marker'

relationship might exist between UMF values and the ether-extracted organic compounds has not been investigated in previous studies. The account presented in this Chapter appears to be first time such an analysis has been attempted for New Zealand manuka honeys.

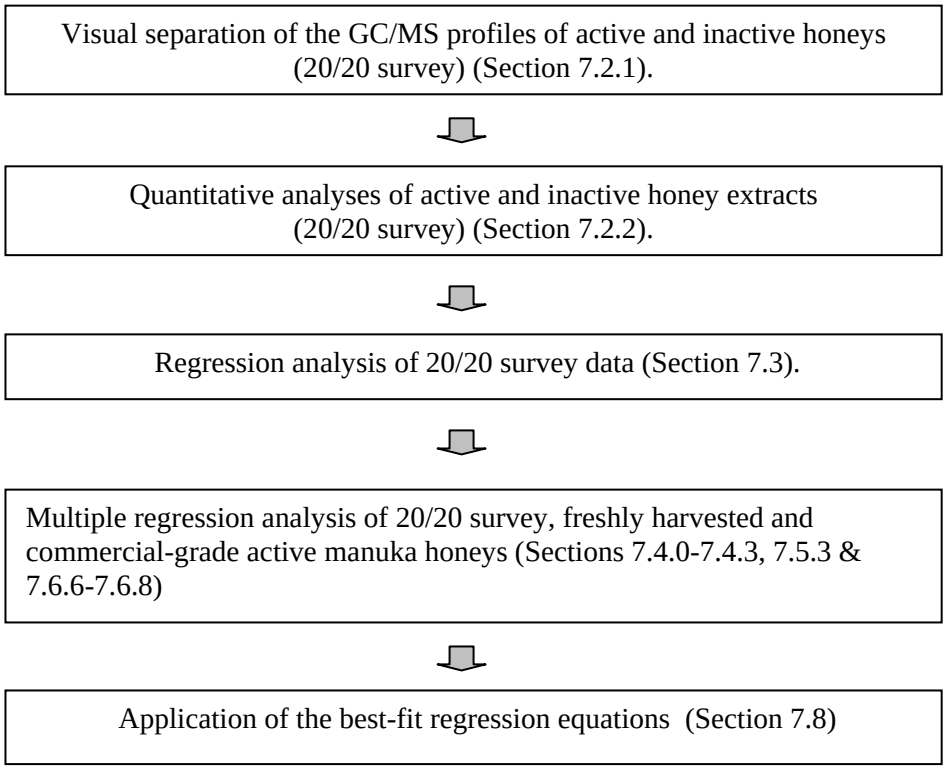
The principal objectives of the work reported in this chapter were:

1. -to determine if active and inactive manuka honeys could be reliably distinguished by their GC/MS profiles

2. -to identify possible GC-detectable activity-related marker substances

3. -to determine if statistical correlations existed between the ether-extracted marker compounds of active manuka honeys and the UMF values.

Processing of the results of the investigations reported in this Chapter is summarized in the flow diagram below.



7.2. 20/20 Survey

The hypothesis that active and inactive manuka honeys might afford visually different marker compounds in GC/MS profiles was explored by comparing the GC/MS profiles of 20 active manuka honey samples (UMF activities >10) and 20 inactive (either no UMF activity, or UMF values < 5). The 20 active manuka honey samples possessed medium (UMF = 10-15) or high (UMF = 15-20) UMF activities based on the diameter of the area of inhibition of growth in the standard agar well diffusion assay.

Honey samples were supplied by the Professor P. C. Molan and Ms K. Allen (Honey Research Unit, The University of Waikato, Hamilton, New Zealand). The 40 samples had been stored for various times (up to 10 years) at refrigerator temperature (~ 5 ºC). The samples originated from sites in both the North and South Islands of New Zealand, however the details of the honey samples (when and where the honey samples have been collected) are unknown.

The diethyl ether extracts of the organic substances of the 40 manuka honey samples were methylated and quantitatively analysed. The levels of 17 compounds (benzoic acid, phenylacetic acid, 2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, 4-methoxybenzoic acid, octanedioic acid, *cis*-cinnamic acid, *trans*-cinnamic acid, nonanedioic acid, an unknown compound (*m/z* 196, 165), 4-methoxyphenyllactic acid, decanedioic acid, 2-decenedioic acid, methyl syringate, palmitic acid and stearic acid) identified in the methylated diethyl ether extracts of the 20 active and 20 inactive manuka honey samples are given in Tables 7.1 and 7.2 respectively (see Section 7.2.2). Acids and some phenols were detected as their methyl esters, or ether derivatives (Tan *et al*., 1988, 1989b), unless otherwise indicated. Comparison of ethylated and methylated diethyl ether extracts showed that methyl syringate (but not syringic acid) was present in these manuka honey extracts.

7.2.1. Visual Comparison of the GC/MS Profiles of the Extracts of 20 Active and 20 Inactive Manuka Honeys

Visual examination of the GC/MS profiles of the 20 active and 20 inactive honeys revealed two predominant GC/MS profiles which broadly arose from active and inactive honeys (Figure 7.1). Specifically 17 of the 20 active honey samples exhibited GC/MS profiles which can be readily distinguished from 18 of the 20 inactive honeys.

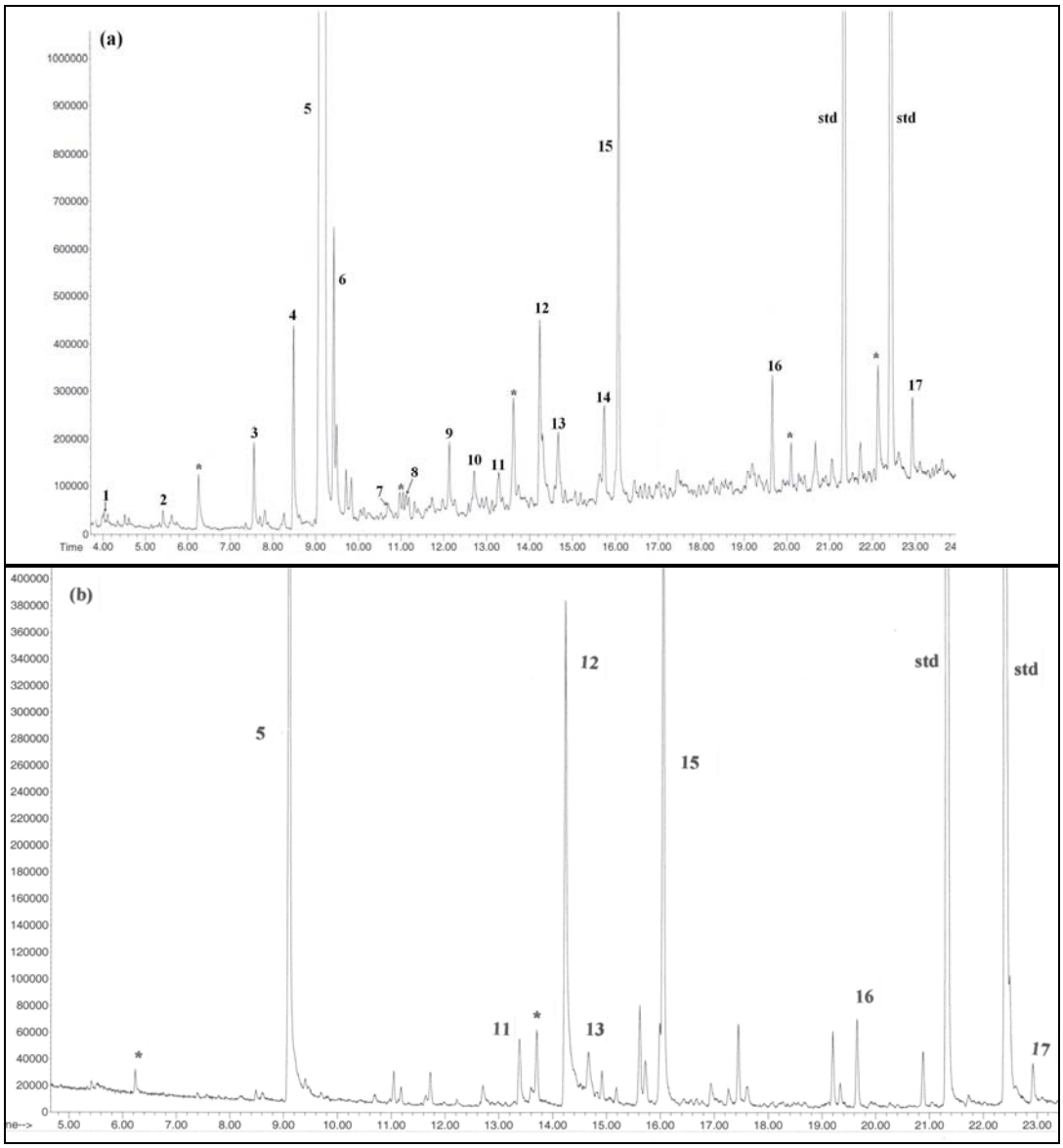


Figure 7.1. GC/MS profiles of representative methylated (a) active and (b) inactive New Zealand manuka honey diethyl ether extracts. Peak identifications are given Tables 7.1 and 7.2.

The levels of some compounds [*e.g.* phenyllactic acid (peak 5), 2-methoxybenzoic acid (peak 4), 2-methoxyacetophenone (peak 3), 4-methoxybenzoic acid (peak 6) and cinnamic acid isomers (peaks 8 and 9)] appeared to be higher in active honeys than was the case for inactive honeys. Quantitative data (Section 7.2.2) substantiated this conclusion.

The extent to which the GC/MS profiles of the active and inactive honeys could visually divided into two sub-sets (> 80% separation) supports the view that it might be possible to use GC fingerprint data (based on the distribution of floral markers and other extractable organic compounds) to distinguish active and inactive manuka honeys, and possibly predict the UMF value of active manuka honeys.

7.2.2. Quantitative Analyses of the 20 Active and 20 Inactive Manuka Honeys

Quantitative data for the 40 samples are presented in Tables 7.1 and 7.2. The mean concentrations, standard deviations and coefficients of variances of the methylated extractable organic substances detected in extracts of these two sets of manuka honey samples are given in Table 7.3. The results of student's *t*-test that give the probability which indicating the significant difference between the levels of compounds in active and inactive honey samples are given in Table 7.4.

The results of student's *t*-test (Table 7.4) showed that the levels of phenylacetic acid (peak 2) (p = 0.003), 2-methoxyacetophenone (peak 3) (p = 0.000), 2-methoxybenzoic acid (peak 4) (p = 0.001), phenyllactic acid (peak 5) (p = 0.000), and 4-methoxybenzoic acid (peak 6) (p = 0.000), *cis* and *trans*-cinnamic acid (peaks 8 and 9) (p = 0.021 & 0.000 respectively) in the active honey samples (average levels 9.9, 58, 1113, 22, 6.1, 9.3 mg/kg respectively) were significantly higher than those found in the inactive honey samples (average levels 3.0, 8.0, 520, 6.6, 3.0, 0 mg/kg respectively), while the levels of 4-methoxyphenyllactic acid (peak 12) (p = 0.003) and methyl syringate (peak 15) (p = 0.040) in the active honey samples (average levels 20, 61 mg/kg respectively) were significantly lower than that found in the inactive honey samples (average levels 282, 83 mg/kg respectively). Concentrations of most fatty acids were remarkably similar in both active and inactive honey samples.

Table 7.1. UMF values (% phenol equivalent) and concentrations (mg/kg) of compounds detected in methylated diethyl ether extracts of 20 New Zealand active manuka honey samples. Acids were quantified as the corresponding methyl esters.

peak	name of compound	sample number										
		1	2	3	4	5	6	7	8	9	10	11
1	benzoic acid	-	0.3	0.8	0.8	1.0	1.3	0.7	1.3	-	1.2	1.1
2	phenylacetic acid	0.5	2.8	3.0	3.9	2.5	3.8	2.4	1.6	1.3	2.7	3.9
3	2-methoxyacetophenone	5.2	8.0	8.3	9.4	11	6.4	21	20	10	9.4	11
4	2-methoxybenzoic acid	129	137	144	145	18	42	16	15	21	19	178
5	phenyllactic acid	1584	1235	1447	1828	1007	700	1334	1271	4.6	1495	1769
6	4-methoxybenzoic acid	25	6.4	10	20	23	10	23	10	9.0	34	36
7	octanedioic acid	1.8	1.1	-	1.0	2.5	-	2.8	1.0	-	1.5	2.4
8	*cis*-cinnamic acid	42	9.9	7.8	7.9	6.5	9.6	4.1	1.8	-	6.2	8.5
9	*trans*-cinnamic acid	49	25	12	12	9.8	5.9	4.8	1.8	-	8.2	17
10	nonanedioic acid	3.2	3.5	4.0	1.9	5.2	6.5	8.2	7.8	-	4.9	9.3
11	unknown (*m/z* 196, 165)	2.2	0.9	3.3	1.4	2.9	6.0	5.2	4.5	8.5	8.8	3.9
12	4-methoxyphenyllactic acid	9.4	5.9	10	12	95	7.7	15	19	10	20	10
13	decanedioic acid	2.7	3.3	5.4	3.7	7.1	-	-	-	-	4.0	13
14	2-decenedioic acid	3.0	1.8	7.8	4.0	2.7	-	-	24	-	-	29
15	methyl syringate	2.7	1.7	12	11	109	94	75	71	97	54	17
16	palmitic acid	8.9	5.8	9.9	9.0	5.7	31	21	20	22	11	11
17	stearic acid (18:0)	4.9	3.8	5.9	5.1	3.6	26	27	23	24	8.4	13
i	pimaric acid	tr	tr	tr	tr	tr	1.1	1.3	1	2.5	0.3	1.2
ii	sandaracopimaric acid	tr	tr	tr	tr	tr	0.4	0.4	0.2	0.6	0.1	0.4
iii	isopimaric acid	0	0	tr	tr	tr	3.5	2.6	2.3	6.9	0.5	2.5
iv	dehydroabietic acid	1.3	0.5	0.5	0.5	0.2	5	4.2	2.1	10	1.1	6.0
v	abietic acid	-	-	-	-	-	-	1.3	0.6	6.7	-	-
	UMF value[a]	21.7	22.0	22.8	23.3	14.3	11.2	15.0	15.0	11.3	13.9	22.2

[a]Unique Manuka Factor (% phenol equivalent) measured by K. Allen, Honey Research Unit, The University of Waikato; tr = trace.

Table 7.1. continued (samples 12-20).

peak	name of compound	sample number								
		12	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1	benzoic acid	1.7	1.6	1.8	1.5	1.1	4.2	0.5	0.4	2.3
2	phenylacetic acid	3.1	1.7	2.7	7.1	4.2	3.8	1.0	0.3	1.6
3	2-methoxyacetophenone	17	10	7.9	9.6	5.8	16	4.8	6.0	7.2
4	2-methoxybenzoic acid	37	39	17	180	8.2	26	26	4.4	13
5	phenyllactic acid	1516	848	893	2014	804	1545	470	559	665
6	4-methoxybenzoic acid	53	15	15	46	12	62	18	13	20
7	octanedioic acid	4.4	3.0	3.1	4.2	2.1	3.0	1.7	0.8	2.3
8	*cis*-cinnamic acid	5.3	2.9	2.1	16	2.8	5.9	3.0	0.8	3.5
9	*trans*-cinnamic acid	17	2.0	2.0	20	4.7	8.8	2.4	0.9	4.3
10	nonanedioic acid	13	6.3	9.4	9.0	3.9	5.9	2.7	2.6	4.8
11	unknown (*m/z* 196, 165)	6.8	6.6	5.5	1.6	1.1	3.2	1.8	1.7	1.4
12	4-methoxyphenyllactic acid	-	36	122	16	36	15	8.2	28	96
13	decanedioic acid	-	12	-	7.5	2.3	9.3	4.2	2.0	6.7
14	2-decenedioic acid	-	-	-	15	6.3	18	6.6	12	-
15	methyl syringate	84	90	91	23	36	95	58	37	57
16	palmitic acid	16	5.6	6.2	6.4	4.1	4.6	3.4	4.5	4.9
17	stearic acid (18:0)	13	2.4	2.4	4.2	2.2	3.1	2.4	4.8	2.3
i	pimaric acid	tr	tr	tr	tr	tr	tr	0.2	0.7	0.6
ii	sandaracopimaric acid	tr	tr	tr	tr	tr	tr	0.1	0.2	0.2
iii	isopimaric acid	0.1	0.2	0.2	0.3	0.1	tr	0.4	1.3	1.3
iv	dehydroabietic acid	1.6	0.7	0.9	1.6	0.5	0.5	0.9	2.7	2.0
	UMF value[a]	14.1	16.0	15.9	26.5	13.0	18.5	17.0	10.3	12.7

[a]Unique Manuka Factor (% phenol equivalent) measured by K. Allen, Honey Research Unit, The University of Waikato; tr = trace.

Table 7.2. Concentrations (mg/kg) of compounds detected in methylated diethyl ether extracts of 20 New Zealand inactive manuka honey samples. Acids were quantified as the corresponding methyl esters.

peak	name of compound	sample number									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	benzoic acid	0.8	1.4	0.4	0.8	0.8	1.1	6.4	-	-	-
2	phenylacetic acid	1.2	2.8	1.5	1.3	2.2	2.8	0.9	2.1	3.6	1.0
3	2-methoxyacetophenone	1.3	1.1	-	-	0.5	3.9	1.5	2.1	12	1.4
4	2-methoxybenzoic acid	2.0	6.0	10	1.3	8.7	8.3	1.9	4.6	21	1.9
5	phenyllactic acid	380	1496	676	2.3	217	1049	1.7	242	1343	162
6	4-methoxybenzoic acid	4.0	15	5.9	-	1.6	3.8	10	4.7	6.9	1.1
7	octanedioic acid	2.3	2.1	-	3.5	2.7	3.0	2.7	4.0	3.6	4.2
8	*cis*-cinnamic acid	-	4.3	3.9	-	-	3.2	-	2.9	3.6	2.6
9	*trans*-cinnamic acid	-	-	-	-	-	-	-	-	-	-
10	nonanedioic acid	3.7	5.5	3.2	7.5	3.8	3.8	3.5	7.9	8.3	7.7
11	unknown (*m/z* 196, 165)	2.7	3.2	-	3.4	2.0	0.8	2.6	3.1	12	3.6
12	4-methoxyphenyllactic acid	333	652	189	35	65	614	34	116	12	68
13	decanedioic acid	-	-	-	-	-	-	-	-	-	-
14	2-decenedioic acid	-	-	-	31	-	-	-	-	-	-
15	methyl syringate	71	193	70	62	58	121	15	61	72	43
16	palmitic acid	22	14	14	16	12	15	16	15	40	16
17	stearic acid (18:0)	21	10	9.2	10	9.5	9.2	11	11	50	16
i	pimaric acid	1.3	1.0	tr	0.9	0.5	0.3	0.6	1	1.6	0.8
ii	sandaracopimaric acid	0.4	0.2	tr	0.3	0.1	0.1	0.2	0.3	0.5	0.3
iii	isopimaric acid	2.5	1.5	0.4	1.7	1.0	0.6	1.2	1.7	2.3	1.1
iv	dehydroabietic acid	3.4	2.4	1.5	2.9	1.3	1.1	1.6	2.9	4.5	2.1

tr = trace

Table 7.2 continued (samples 11-20).

peak	name of compound	sample number									
		11	12	13	14	15	16	17	18	19	20
1	benzoic acid	-	3.3	3.1	2.8	0.3	0.6	0.8	0.8	0.8	0.9
2	phenylacetic acid	-	1.2	1.4	1.3	0.5	0.7	0.6	0.9	1.0	1.0
3	2-methoxyacetophenone	1.0	9.0	3.0	3.3	-	-	-	1.4	-	0.9
4	2-methoxybenzoic acid	2.2	5.5	50	-	1.0	-	1.5	2.9	-	-
5	phenyllactic acid	510	497	437	366	125	92	155	773	870	998
6	4-methoxybenzoic acid	16	5.7	1.8	2.4	1.5	3.2	2.7	6.9	1.6	30
7	octanedioic acid	1.7	0.9	2.3	1.6	0.3	1.1	0.5	1.2	-	1.1
8	*cis*-cinnamic acid	3.7	2.6	3.0	2.8	2.6	2.5	2.2	3.3	2.4	3.0
9	*trans*-cinnamic acid	-	-	-	-	-	-	-	-	-	-
10	nonanedioic acid	4.2	2.4	4.1	3.2	1.9	1.7	1.2	2.3	3.0	2.0
11	unknown (*m/z* 196, 165)	2.6	1.9	3.7	4.2	-	-	0.2	-	-	-
12	4-methoxyphenyllactic acid	377	160	10	12	68	62	61	475	1141	1160
13	decanedioic acid	-	-	-	-	-	-	-	-	-	-
14	2-decenedioic acid	-	-	-	-	-	-	-	-	-	-
15	methyl syringate	96	64	85	78	59	53	57	71	163	169
16	palmitic acid	23	7.5	4.9	7.6	6.8	2.3	0.9	7.8	2.9	7.7
17	stearic acid (18:0)	23	4.1	3.6	4.3	3.2	0.7	0.4	2.0	0.8	3.1
i	pimaric acid	0.8	0.3	0.2	0.2	0.3	tr	tr	tr	tr	0.1
ii	sandaracopimaric acid	0.2	0.1	0.1	0.1	0.1	tr	tr	tr	tr	0.04
iii	isopimaric acid	1.2	0.2	0.2	0.2	0.3	tr	0.1	tr	tr	0.05
iv	dehydroabietic acid	2.5	1.3	1.1	1.1	1.1	0.5	0.2	0.7	0.3	0.5

tr = trace

Table 7.3. Mean concentration (mg/kg), standard deviation (stdev), and coefficient of variance (CV%) determined for compounds identified in the diethyl ether extracts of 20 active and 20 inactive manuka honey samples. Acids were detected and quantified as the corresponding methyl esters.

		active samples				inactive samples			
Peak	**compound**	**mean**	**stdev**	**n**	**CV (%)**	**mean**	**stdev**	**n**	**CV (%)**
1	benzoic acid	1.2	0.4	19	30	1.6	1.6	-	102
2	phenylacetic acid	2.6	1.3	20	48	1.5	0.7	19	50
3	2-methoxyacetophenone	9.9	2.0	20	20	3.0	1.5	14	50
4	2-methoxybenzoic acid	58	57	20	97	8.0	3.7	16	46
5	phenyllactic acid	1113	408	20	37	520	558	20	107
6	4-methoxybenzoic acid	22	7.7	20	35	6.6	5.2	19	79
7	octanedioic acid	2.3	0.7	17	31	2.1	0.5	19	26
8	*cis*-cinnamic acid	6.1	3.7	20	61	3.0	0.6	16	19
9	*trans*-cinnamic acid	9.3	8.6	20	92	-	-	0	-
10	nonanedioic acid	5.9	1.6	19	27	4.1	1.6	20	40
11	unknown (*m/z* 196, 165)	3.9	1.8	20	47	3.3	1.0	14	32
12	4-methoxyphenyllactic acid	20	9.2	20	45	282	268	20	95
13	decanedioic acid	6.0	1.8	14	30	-	-	0	-
14	2-decenedioic acid	11	2.3	12	22	31	-	1	-
15	methyl syringate	61	52	19	86	83	53	20	64
16	palmitic acid	10	9.5	20	91	12	3.5	20	28
17	stearic acid (18:0)	8.9	8.9	20	100	10	4.5	20	45

Table 7.4. The results of student's *t*-test performed for 20 active and 20 inactive manuka honey samples.

	name of compound	probability [a]	significance
2	phenylacetic acid	0.003	highly significant
3	2-methoxyacetophenone	0.000	highly significant
4	2-methoxybenzoic acid	0.001	highly significant
5	phenyllactic acid	0.000	highly significant
6	4-methoxybenzoic acid	0.000	highly significant
8	*cis*-cinnamic acid	0.021	significant
9	*trans*-cinnamic acid	0.000	highly significant
12	4-methoxyphenyllactic acid	0.003	highly significant
15	methyl syringate	0.040	significant

[a]data predicted by using Eton statistical and math tables.

Phenyllactic Acid and 4-Methoxyphenyllactic Acid

In previous studies, phenyllactic acid (peak 5) has been found by Wilkins *et al*. 1993b to be the most abundant compound amongst the extractable organic compounds in manuka honey and they proposed this compound as floral marker substance for manuka honey. Tan (1989) has shown that phenyllactic acid contributed only 1.6-3.2% of the UMF activity of active manuka honey. Recently, Lavermicocca *et al*. (2003) have reported that phenyllactic acid which occurs in cultures of *Lactobacillu plantarum*, showed antifungal activity in sourdough breads while Dieuleveux (1997) has found that phenyllactic acid inhibited the growth of *Geotrichum candidum*. Weston *et al*. (1999) have concluded that aromatic substances are only partly responsible for the UMF antibacterial activity.

Phenyllactic acid is a significant constituent of a number of other honeys. For example, Steeg and Montag (1987) have shown that phenyllactic acid is one of the major acids in some European honeys. These honeys do not however exhibit UMF activity, thus (as noted previously by Tan 1989) it appears unlikely that phenyllactic acid is a significant contributor to the UMF activity characteristic of manuka honey.

Some of the inactive honeys contained relatively high levels of 4-methoxyphenyllactic acid. The levels of this compound in the 20 active and 20 inactive honeys are compared in Figure 7.2.

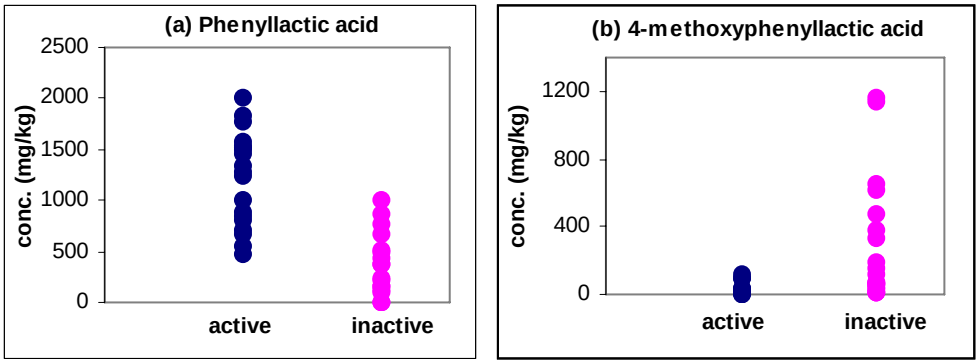


Figure 7.2. Levels of (a) phenyllactic acid and (b) 4-methoxyphenyllactic acid in active and inactive manuka honey samples.

cis- and trans-Cinnamic Acids

cis- and *trans*-Cinnamic acids can be viewed as dehydrated analogues of phenyllactic acid. As described in Section 7.2.2, the levels of *cis* and *trans*-cinnamic acids (peaks 8 and 9) in some, but not all, of the active honey samples (average levels 6.1 mg/kg and 9.3 mg/kg respectively), were significantly higher than those found in the inactive honey samples (average levels 3.0 and 0 mg/kg respectively) (Figure 7.3). Weston *et al*. (1999) however reported the presence of cinnamic acid isomers in extracts of the active manuka honey that did not exhibit UMF activity.

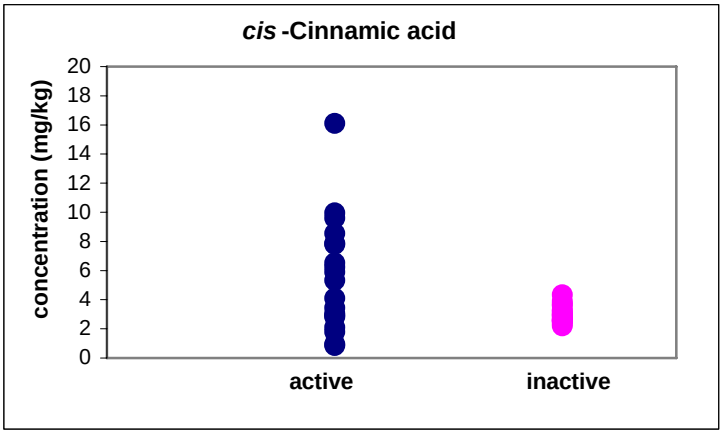


Figure 7.3. Levels of *cis*-cinnamic acid in active and inactive manuka honey samples.

Cinnamic acid is known to be antibacterial and has been used as an antibacterial wound treatment agent (Aljadi *et al*., 2003). Prior to the 9th century,

pungent smelling cinnamon bark was used to dress and scent foul-smelling wounds while ancient Chinese people used Cinnamon bark to treat external fungal infections (Tillotson *et al*., 2001). Tonari *et al*. (2002) have reported the strong antibacterial activity of some compounds related to cinnamic acid (*e.g.* a compound having the substituted phenyl group in propiolic aldehyde or its ester form) against *Bacillus subtilis* and *Escherichia coli*.

It has been found that honey antibacterial activity can increase with storage period ((Molan, personal communication, 2005). It is possible that during storage, dehydration of phenyllactic acid proceeds to afford *cis* and *trans*-cinnamic acids, and that this may influence the UMF value of a honey.

2-Methoxybenzoic Acid

The levels of 2-methoxybenzoic acid (peak 4) in 17 of the 20 active honey samples (average level 58 mg/kg) were significantly higher than those found in 18 of the 20 inactive honey samples (average level 8.0 mg/kg) (Figure 7.4).

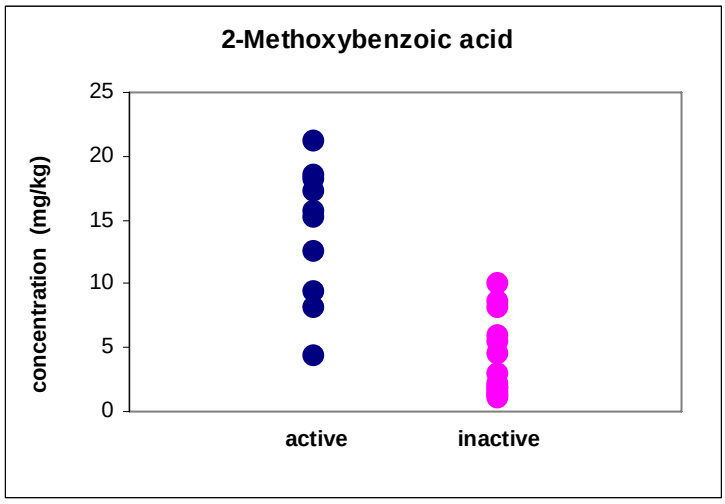


Figure 7.4. Levels of 2-methoxybenzoic acid in active and inactive manuka honey samples.

The GC/MS results of methylated and ethylated sub-samples of active manuka honey extracts indicated that both 2-methoxybenzoic acid and 2-

hydroxybenzoic acid were present in the active honey samples and that the levels of 2-methoxybenzoic acid was always higher than 2-hydroxybenzoic acid.

2-Hydroxybenzoic acid (salicylic acid) and salicylic acid derivatives (*e.g.* 6-[8'(*Z*),11'(*Z*),14'-pentadecatrienyl]salicylic acid) are also known to be antibacterial agents (Kubo *et al*., 2003). Tan (1989) has reported that 2-hydroxybenzoic acid contributed only 0.2-0.3% of the UMF activity of active manuka honeys.

Methyl Syringate

The levels of methyl syringate (peak 15) (average level 61 mg/kg) in the active honey samples was somewhat lower than that found in the inactive honey samples (average level 83 mg/kg).

Previously Russell *et al*. (1990) and Wilkins *et al*. (1993b) have found that methyl syringate was one of the more abundant extractable organic components in manuka honey extracts and proposed this compound (along with phenyllactic acid) as a floral marker for manuka honeys, although it was not unique to manuka. It is, for example, abundant in grape vine (Spencer *et al*., 1990) and rape honey (Joerg and Sontag, 1993).

Weston *et al*. (1999, 2000) reported that methyl syringate was the dominant component in the active phenolic extracts of the manuka honey. They suggested that methyl syringate, while not a significant contributor to the UMF activity of the manuka honey, could be partly responsible for UMF activity.

Phenylacetic Acid

The level of phenylacetic acid (peak 2) in the most of the active honey samples (average level 2.6 mg/kg) was higher than that found in the inactive honey samples (average level 1.5 mg/kg).

Fatty Acids

Some differences were apparent in the levels of some of the short chain C_8-C_{10} fatty acids, diacids (*e.g.* octanedioic acid, nonanedioic and decanedioic acid) and/or hydroxy-fatty acids determined for active and inactive honey samples. On the other hand the levels of long chain fatty acids such as palmitic acid and stearic acid did not show recognizable differences between active and inactive honey samples.

Possibly short chain fatty acids levels may indirectly contribute to UMF values *via* synergistic effects since they can act as carriers for other molecules which normally cannot penetrate cell walls (Maharishi, 2005). Most of short chain C_8-C_{10} hydroxy-fatty acids and diacids were found in royal jelly and they act as good detergents and antimicrobial agents since these compounds have active functionalities at the end of the molecule (Schmidt, 1996). Usually they are universally present in honeys and the level of these compounds may depend on seasonal, geographical or storage factors.

7.3. Correlations between UMF Values and Levels of Ether-Extracted Organic Components

Since there appeared to be some visual differences between the GC/MS profiles of active and inactive manuka honeys, simple regression analysis was carried out in order to find the relationship between the levels of selected ether-extracted compounds and UMF activity. Longer chain fatty acids were excluded from this analysis since they are universally present in honey extracts and it was considered unlikely that they would be correlated with UMF values. The levels of such long chain fatty acids are usually dependent on the extraction technique, particular on time and temperature. On the other hand, medium-chain diacids (C_8-C_{10}) were included since variable levels of diacids were found in honey samples and they have been known as the part of royal jelly compounds.

Prior to the detailed statistical analyses, Pearson's correlation coefficients for the levels of selected ether-extracted organic compounds and UMF values were determined (using Data Desk 6.1) to find association among these variables either positive or negative. The correlation between UMF values and selected organic compound concentrations and the list of the significant correlations are listed in Table 7.5.

2-Methoxybenzoic acid (Figure 7.5), *cis* and *trans*-cinammic acids showed strongly positive correlations with UMF value (coefficients of 0.858, 0.678, 0.692 respectively) while phenyllactic acid showed moderate positive correlation with UMF value (coefficients of 0.505). One compound (methyl syringate) was moderately negatively correlated with UMF value (-0.650) and the remaining compounds showed very low level association with UMF value.

Table 7.5. Correlations between UMF values and selected extractable organic manuka honey compounds (n = 20).

		Correlation		
Peak	Correlation (UMF *vs* substance)	coefficient	probability[a]	significance
1	benzoic acid	0.198	> 0.1	no
2	phenylacetic acid	0.376	> 0.1	no
3	2-methoxyacetophenone	0.046	> 0.1	no
4	2-methoxybenzoic acid	0.858	< 0.001	strong
5	phenyllactic acid	0.505	< 0.02	moderate
6	4-methoxybenzoic acid	0.268	> 0.1	no
7	octanedioic acid	0.232	> 0.1	no
8	*cis*-cinammic acid	0.678	< 0.001	strong
9	*trans*-cinammic acid	0.692	< 0.001	strong
10	nonanedioic acid	0.183	> 0.1	no
11	unknown (*m/z* 196, 165)	-0.349	> 0.1	no
12	4-methoxyphenyllactic acid	-0.298	> 0.1	no
13	decanedioic acid	0.500	< 0.05	weak
14	2-decenedioic acid	0.444	< 0.05	weak
15	methyl syringate	-0.650	< 0.01	moderate
16	palmitic acid	-0.189	> 0.1	no
17	stearic acid (18:0)	-0.241	> 0.1	no

[a]data predicted by using Eton statistical and math tables.

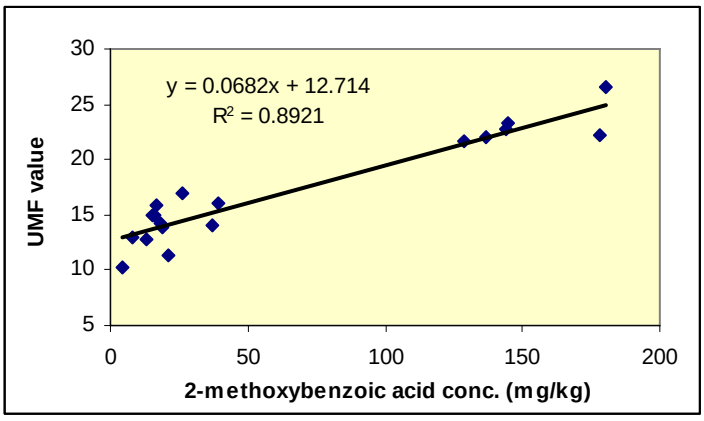


Figure 7.5. Relationship between the levels of 2-methoxybenzoic acid (mg/kg) and UMF values of active manuka honey samples.

7.4. Multiple Regression Analysis

The purpose of multiple regression analyses is to elucidate relationships between the response variable and number of predictor variables. In the case of manuka honey samples it can be used to explore possible relationships between UMF values and levels of selected extractable organic compounds. Some of the extractable organic components may be the better predictors of the UMF values and some of them may not.

7.4.1. Multiple Regression Analysis of Active Honey Samples

The UMF values of the 20 active manuka honey samples obtained from the Honey Research Unit, Waikato University were utilised in the regression analyses (Section 2.12). Seventeen compounds had retention times less than that of palmitic and or stearic acid and were present at mean levels of *ca* 1 mg/kg, or greater, in the extracts of active manuka honeys (Table 7.3). An additional requirement was that target compounds had to be present in all of the samples examined, including groups of manuka honey sample used in other regression analyses (Sections 7.5.3 and 7.6.6) at levels > 1 mg/kg.

It was anticipated that compounds which were present at levels of < 1 mg/kg were unlikely to be strongly correlated with activity and that, at least in initial statistical analyses, the integrity of the regression analyses would not be compromised by excluding minor components. Moreover, it is difficult to reliably quantify compounds which are only present in the sub-ppm range.

UMF values for the 20 active honey samples which ranged from 10 to 22 (expressed as % phenol equivalent) were regressed against the levels of 11 ether-extracted organic compounds namely, phenylacetic acid, 2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, octanedioic acid, *cis*-cinnamic acid, *trans*-cinnamic acid, nonanedioic acid, 4-methoxyphenyllactic acid, decanedioic acid and methyl syringate which satisfied these conditions (see above).

Since UMF values of inactive honey samples are not measurable, multiple regression analysis of inactive honey samples cannot not performed.

7.4.2. Results and Discussion: Active Honeys

The residual and probability outputs of the regression analysis for the 20 active manuka honey data are given in Tables 7.6. The normal probability plot and the scatter plot of residuals against predicted UMF values are shown in Figures 7.6 and 7.7 respectively.

The regression analysis yielded nearly normally distributed residuals and the normal probability plot (Figure 7.6) was under perfect normality as the plot represented a 45 –degree line. The scatter plot of residuals against predicted UMF values (Figure 7.7) showed that 95% of residuals fall between –2 and +2 and thus it revealed that there were no obvious defects in the model.

Table 7.6. The residual and probability outputs of the regression analysis for the 20 active manuka honey samples.

Residual output			**Probability output**	
Observation	Predicted UMF	Residuals	Percentile	UMF
1	21.7	0.0	2.5	10.3
2	20.4	1.6	7.5	11.2
3	21.2	1.6	12.5	11.3
4	24.8	-1.5	17.5	12.7
5	15.1	-0.8	22.5	13.0
6	11.8	-0.6	27.5	13.9
7	15.5	-0.5	32.5	14.1
8	13.5	1.5	37.5	14.3
9	12.6	-1.3	42.5	15.0
10	14.8	-0.9	47.5	15.0
11	23.6	-1.4	52.5	15.9
12	15.3	-1.2	57.5	16.0
13	16.1	-0.1	62.5	17.0
14	14.3	1.6	67.5	18.5
15	26.6	-0.1	72.5	21.7
16	12.8	0.2	77.5	22.0
17	16.5	2.0	82.5	22.2
18	14.2	2.8	87.5	22.8
19	12.4	-2.1	92.5	23.3
20	13.7	-1.0	97.5	26.5

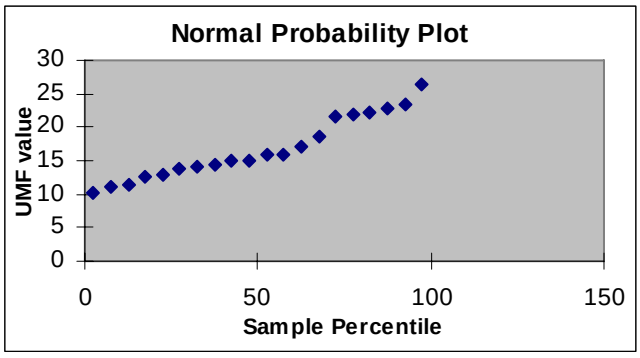


Figure 7.6. The normal probability plot (percentile *vs* UMF values) for the 20 active manuka honey samples.

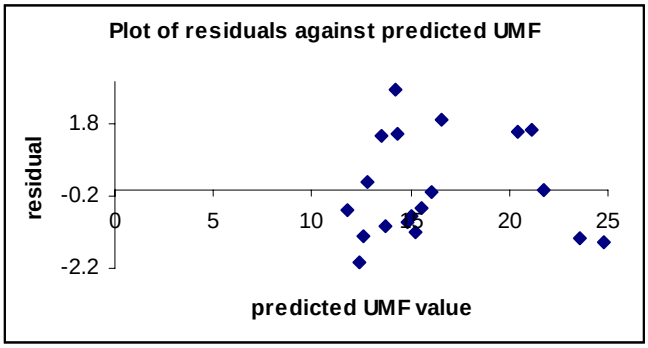


Figure 7.7. Plots of residuals against predicted UMF values for the 20 active manuka honey samples.

The regression equation for the 11 compounds was found to be:

UMF value = 10.06 – 0.34 (phenylacetic acid conc. mg/kg) + 0.04 (2-methoxyacetophenone conc. mg/kg) + 0.07 (2-methoxybenzoic acid conc. mg/kg) + 0.003 (phenyllactic acid conc. mg/kg) + 1.31 (octanedioic acid conc. mg/kg) - 0.05 (*cis*-cinnamic acid conc. mg/kg) - 0.12 (t*rans*-cinnamic acid conc. mg/kg) - 0.52 (nonanedioic acid conc. mg/kg) + 0.01 (4-methoxyphenyllactic acid conc. mg/kg) - 0.10 (decanedioic acid conc. mg/kg) + 0.01 (methyl syringate conc. mg/kg).

R values, standard error and regression *p*-value determined in the multiple regression equation for the 20 active honey samples are given in Table 7.7.

Table 7.7. R values, standard errors and regression *p*-values determined for the multiple regression equation for the 20 active manuka honey samples.

Regression Statistics	
multiple R	0.96
multiple R^2	0.91
adjusted R	0.89
adjusted R^2	0.80
standard error	2.11
regression *p*-value[a]	0.003

[a]*p*-value for the total regression output.

R, R^2 values, adjusted R and R^2 values (0.96, 0.91, 0.89 and 0.80 respectively) and the regression *p*-value (0.003) showed a significant positive and linear relationship between the concentrations of the 11 selected organic

compounds and UMF value. Overall, a moderate standard error (2.11) was observed (Table 7.7).

Coefficients, standard errors and *p*-values for the individual organic compounds for the regression equations performed for the 20 active samples are given in Table 7.8.

Table 7.8. Coefficients, standard errors and *p*-values of multiple regression analysis between the levels of 11 organic compounds and the UMF values of 20 active manuka honey samples.

	coefficients	standard error	*p*-value
intercept	10.06	2.22	0.002
phenylacetic acid	-0.34	0.57	0.57
2-methoxyacetophenone	0.04	0.20	0.84
2-methoxybenzoic acid	0.07	0.02	0.001
phenyllactic acid	0.003	0.003	0.24
octanedioic acid	1.31	0.74	0.11
cis-cinnamic acid	0.05	0.17	0.76
trans-cinnamic acid	-0.12	0.16	0.47
nonanedioic acid	-0.52	0.28	0.10
4-methoxyphenyllactic acid	0.01	0.02	0.68
decanedioic acid	-0.10	0.18	0.59
methyl syringate	0.01	0.03	0.77

p-Values (Probability)

The lowest *p*-value (0.001) was determined for 2-methoxybenzoic acid. This value implies that there is a significant relation between UMF values and the level of this compound. The relatively high *p*-values determined for 2-methoxyacetophenone (0.84), methyl syringate (0.77), *cis*-cinnamic acid (0.76) and 4-methoxyphenyllactic acid (0.68) implied that there were no significant relationship between the levels of these compounds and the UMF values.

Estimated Regression Coefficients

The regression coefficients determined for individual compounds (Table 7.8) represent the extent to which UMF changes when the level of a particular organic compound changes (Section 2.13.1).

The regression coefficient value for 2-methoxybenzoic acid was 0.07. This value represents the relative influence on the 2-methoxybenzoic acid concentration to the UMF value. The positive sign indicates that the concentration of this compound increases as the levels of the UMF value increases.

Compounds which have positive UMF correlation coefficients were octanedioic acid (1.31), *cis*-cinnamic acid (0.05), 2-methoxyacetophenone (0.04), 4-methoxyphenyllactic acid (0.01), methyl syringate (0.01) and phenyllactic acid (0.003).

Some of the estimated regression coefficients were negative, implying that the UMF value increases as the concentrations of the species decreases. Nonanedioic acid (-0.52), phenylacetic acid (-0.34), *trans*-cinnamic acid (-0.12), and decanedioic acid (-0.01) showed negative regression coefficients.

Standard Errors of the Estimators

Standard errors are measures of the precision of the regression coefficients. The closer standard error values are to zero the more precise are the measured values. The relatively high standard errors determined for octanedioic acid (0.74), phenylacetic acid (0.57) and nonanedioic acid (0.28) showed that the regression coefficients for these compounds (1.31, -0.34 and -0.52 respectively) have limited precision, whereas the moderate standard errors determined for 2-methoxyacetophenone (0.20), decanedioic acid (0.18), *cis*-cinnamic acid (0.17), *trans*-cinnamic acid (0.16) showed that the regression coefficients for these compounds (0.04, -0.10, 0.05 and -0.12 respectively) are moderately precise. On the other hand the low standard errors determined for 4-methoxyphenyllactic acid (0.02), methyl syringate (0.03), 2-methoxybenzoic acid (0.02), and phenyllactic acid (0.003) showed the regression coefficients for these compounds (0.01, 0.01, 0.07 and 0.003 respectively) were comparatively precise.

2-Methoxybenzoic acid showed the strongest positive correlations to the UMF values. This compound had lower *p*-values (0.001), lower standard errors (0.02), relatively moderate regression coefficients (0.07) and therefore high partial contributions to predicted UMF scores (*e.g.* 0.07 x 58 mg/kg = 4.06) [partial

contribution of a compound to predicted UMF value = regression coefficient x concentration (mg/kg)].

The *p*-values, regression coefficients and standard errors determined for some of the other 11 compounds had little significance. For example, octanedioic acid showed the highest regression coefficient (1.31), along with a high standard error (0.74). 2-Methoxyacetophenone, *cis*-cinnamic acid, 4-methoxyphenyllactic acid and methyl syringate also showed high *p*-values (p = 0.84, 0.76, 0.68 and 0.77 respectively).

In general, compounds with high *p*-values can be neglected since they do not contribute significantly to UMF values estimation. In such circumstances the number of variables (compounds contributing to UMF value estimation) can be reduced step-wise in subsequent cycles of the regression analyses.

7.4.3. Backwards Stepwise Multiple Regression Analysis for Active Honey Samples

Having shown that there was a considerable correlation (R = 0.96; *p*-value = 0.003; and the standard error = 2.11) between observed UMF values and the levels of 11 selected organic compounds (Section 7.4.1), the possibility that a more satisfactory correlation between UMF value and a reduced set of variables (reduced number organic compounds) might be obtained. This was achieved by programming backwards selection into the S-Plus 6 for Windows software and Microsoft Excel 2002.

The variables (compound levels), which showed the highest *p*-values, were omitted from the data set stepwise until the best equation with the highest R^2 value, the lowest standard error and the highest significant regression *p*-value were identified.

Statistics of the stepwise (steps 1-5) backwards selection multiple regression analysis performed for the 20 active samples are given in Tables 7.9 and 7.10.

Table 7.9. *p*-Values of steps 1-5 of the backwards selection multiple regression.

	original[a]	step 1	step 2	step 3	step 4	step 5
	p-value	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value
intercept	0.002	0.001	0.0005	0.0001	3E-07	1E-07
phenylacetic acid	0.57	0.42	0.41	0.39	0.45	0.50
2-methoxyacetophenone	0.84					
2-methoxybenzoic acid	0.007	0.004	0.002	0.002	0.001	0.000
phenyllactic acid	0.24	0.06	0.05	0.04	0.04	0.04
octanedioic acid	0.11	0.08	0.07	0.05	0.05	0.04
cis-cinnamic acid	0.76	0.79				
trans-cinnamic acid	0.47	0.45	0.27	0.24	0.25	0.29
nonanedioic acid	0.10	0.08	0.06	0.05	0.05	0.05
4-methoxyphenyllactic acid	0.68	0.70	0.65			
decanedioic acid	0.59	0.50	0.48	0.49	0.58	
methyl syringate	0.77	0.60	0.54	0.53		

[a]the results of multiple regression analysis in Section 7.4.1.

Table 7.10. R values, standard errors and regression *p*-values for the 1-5 steps of backwards selection stepwise multiple regression (n = 20).

Regression Statistics	original[a]	step 1	step 2	step 3	step 4	step 5
multiple R	0.96	0.96	0.96	0.96	0.95	0.95
multiple R^2	0.91	0.91	0.91	0.91	0.91	0.91
adjusted R	0.89	0.91	0.92	0.92	0.93	0.93
adjusted R^2	0.80	0.82	0.84	0.85	0.86	0.86
standard error	2.11	2.00	1.90	1.83	1.79	1.74
regression *p*-value[b]	0.003	0.001	0.0003	8E-05	2E-05	5E-06

[a]statistics for the original 11 variable analyses the results of multiple regression analysis in Section 7.4.1;
[b]*p*-value for the total regression output.

Variables (compounds) omitted were 2-methoxyacetophenone (original step; $p = 0.84$), *cis*-cinnamic acid (step 1; $p = 0.79$), 4-methoxyphenyllactic acid (step 2; $p = 0.65$), methyl syringate (step 3; $p = 0.53$), decanedioic acid (step 4; $p = 0.59$) and phenylacetic acid (step 5; $p = 0.50$) (Table 7.9).

It is of note that the overall correlation coefficient (R and R^2 values) remained essentially constant (*ca* R = 0.96-0.95) as the number of variables (compounds) was reduced (Table 7.10). This shows that the eliminated compounds contributed little to the overall correlation.

Optimum Regression Line

The best-fit regression equation for the 20 active honeys data set (in step 6) showed a normal distribution (Figure 7.8) while the plot of residuals against predicted UMF values (Figure 7.9) indicated that the spread of the residuals around the predicted UMF values was even along the length of the line. These observations showed that there was no obvious model defects and that a reliable correlation could be obtained from the reduced data sets.

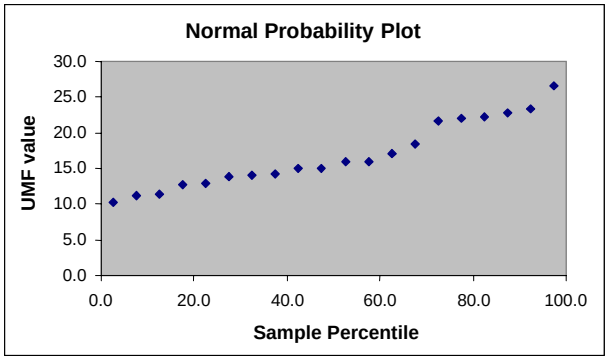


Figure 7.8. The normal probability plot: percentile *vs* UMF values

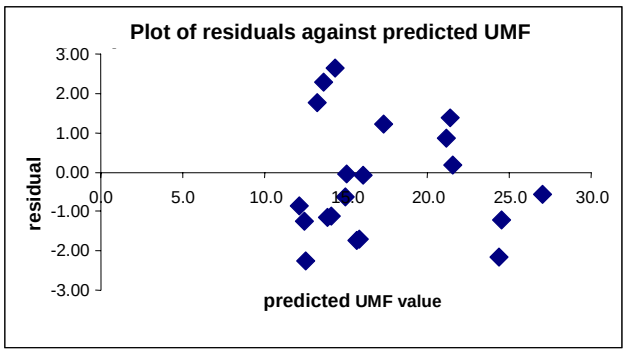


Figure 7.9. Plots of residuals against predicted UMF values

The generated best-fit regression equation in step 6 (with highest the R^2 value, lowest standard error and the highest significant regression *p*-value) was:

UMF value = 10.93 + 0.058 (2-methoxybenzoic acid) + 0.003 (phenyllactic acid) + 1.040 (octanedioic acid) -0.044 (*trans*-cinnamic acid) - 0.427 (nonanedioic acid).

Five marker compounds (2-methoxybenzoic acid, phenyllactic acid, octanedioic acid, *trans*-cinnamic acid and nonanedioic acid) were retained in the best-fit regression equation derived using data determined for the 20 active honeys. R values, standard error and regression *p*-value determined for the best-fit regression equation for the 20 active honeys are given in Table 7.11.

Table 7.11. R values, standard error and regression *p*-value determined for the best-fit regression equation in step 6.

Regression Statistics	
multiple R	0.95
multiple R^2	0.91
adjusted R	0.93
adjusted R^2	0.87
standard error	1.71
regression *p*-value[a]	1.0E-06

[b]*p*-value for the total regression output.

The values of the multiple correlation coefficient (R = 0.95; R^2 = 0.91), adjusted multiple correlation coefficient (R = 0.93; R^2 = 0.87) and regression *p*-value (0.000) for the 5 compounds analyses shows that there is a strong positive linear relationship between UMF values and levels of the five retained compounds (2-methoxybenzoic acid, phenyllactic acid, octanedioic acid, *trans*-cinnamic acid and nonanedioic acid) (Table 7.11).

Individual Effects of Five Compounds

Coefficients, standard errors and *p*-values for the individual marker compounds of the best-fit regression equation performed for the 20 active honeys data set is given in Tables 7.11.

The *p*-values for the compounds showed that 2-methoxybenzoic acid (*p* = 0.000), phenyllactic acid (*p* = 0.04), octanedioic acid (*p* = 0.04) and nonanedioic acid (*p* = 0.04) levels were highly correlated, whereas the *trans*-cinnamic acid (*p* = 0.38) was only very weakly correlated, however inclusion reduces the standard error and contributes to the quality of the regression line fit (Table 7.12).

Table 7.12. Coefficients, standard errors and *p*-values of the best-fit regression equation in step 6.

	coefficients	standard error	*p*-value
intercept	10.93	1.02	3.8E-08
2-methoxybenzoic acid	0.058	0.01	0.00001
phenyllactic acid	0.003	0.001	0.04
octanedioic acid	1.040	0.46	0.04
trans-cinnamic acid	-0.044	0.05	0.38
nonanedioic acid	-0.427	0.19	0.04

The regression coefficient values of 2-methoxybenzoic acid (0.058), phenyllactic acid (0.003) and octanedioic acid (1.040) implied there were positive partial effects on UMF values while nonanedioic acid (-0.427) showed negative partial effect on the UMF values (Table 7.12). The regression coefficient value of *trans*-cinnamic acid (-0.040) has little significance due to the low concentration of this compound and its minor negative contribution to the UMF value.

The lower values of standard errors (closer to zero except octanedioic acid) also implied that the relationship between these selected ether-extracted organic compounds and the UMF values were true. Though the standard error of octanedioic acid (0.46) to the regression coefficient of 1.04 implied quite the opposite, coupled with the fact the estimated sign contradicts commonsense or reality, the level of *p*-value was significant ($p < 0.05$).

Observed and Predicted UMF values

The observed and the predicted UMF values, predicted as a linear combinations of five retained compounds, namely 2-methoxybenzoic acid, phenyllactic acid, octanedioic acid, *trans*-cinnamic acid and nonanedioic acid concentrations are given in Table 7.13.

The results of the Table 7.13 showed that, the predicted UMF values were comparable to that of observed UMF values.

Table 7.13. Observed and predicted UMF values for 20 active honey samples

sample no.	observed UMF	predicted UMF[a]	difference[b]
1	21.7	21.5	-0.2
2	22	21.1	-0.9
3	22.8	21.4	-1.4
4	23.3	24.5	+1.2
5	14.3	14.9	+0.6
6	11.2	12.5	+1.3
7	15	15.1	+0.1
8	15	13.2	-1.8
9	11.3	12.2	+0.9
10	13.9	15.6	+1.7
11	22.2	24.3	+2.1
12	14.1	15.8	+1.7
13	16	16.1	+0.1
14	15.9	13.6	-2.3
15	26.5	27.1	+0.6
16	13	14.1	+1.1
17	18.5	17.3	-1.2
18	17	14.4	-2.6
19	10.3	12.6	+2.3
20	12.7	13.8	+1.1

[a]predicted levels based on intercept value (10.9) plus individual compound contributions (Section 2.13.1).
[b]difference = (predicted UMF value - observed UMF value).

7.5. Analysis of Freshly Harvested Manuka Honey Samples

While the findings presented in Sections 7.3 and 7.4 point to the existence of a correlation between UMF values and levels of selected extractable organic substances, at least for the set of 20 historic manuka honey samples investigated in Section 7.2, the generality of this finding for freshly harvested manuka honeys also needed to be explored prior to the acceptance by other workers that such a relationship exists.

Accordingly 17 active and 3 inactive freshly harvested manuka honey samples were obtained from beekeepers with hives in the Northland, Waikato, Coromandel, Central North Island, and the East Coast regions of New Zealand. While the samples investigated in Section 7.2 (20/20 survey) originated from sites

in both the North and South Islands, the samples utilized in this Section originated only from 5 geographic regions from the North Island of New Zealand.

7.5.1. Results and Discussions: Freshly Harvested Samples

The organic substances present in the diethyl ether extracts of 17 active and 3 inactive manuka honey samples (provided by Mr. Jon Stephens, Honey Research Unit) were determined using the methodology described in Section 2.4. The UMF values of the 17 active samples were in the low (5-10) to medium (10-15) ranges (Table 7.14).

The levels of phenylacetic acid, 2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, octanedioic acid, *cis*-cinnamic acid, *trans*-cinnamic acid, nonanedioic acid, an unknown compound (*m/z* 196, 165), 4-methoxyphenyllactic acid, decanedioic acid, methyl syringate, palmitic acid and stearic acid identified in the methylated diethyl ether extracts of the 17 active and 3 inactive manuka honey samples are given in Table 7.14. The mean concentrations, standard deviations and coefficients of variances of the methylated extractable organic substances detected in the extracts of the 20 honey samples are given in Tables 7.14. Acids and some phenols were detected as their methyl esters, or ether derivatives (Tan *et al*., 1988, 1989b), unless otherwise indicated.

In the diethyl ether extracts, 14 substances were dominant (present in > 60% of samples) and the statistical analyses were conducted using the levels of these compounds. A degree of caution should however be exercised in interpreting the results presented here since most of the samples analysed in this investigation contained relatively low levels of extractives.

Table 7.14. UMF values (% phenol equivalent) and concentrations (mg/kg) of dominant compounds detected in methylated diethyl ether extracts of 17 active and 3 inactive manuka honey samples collected from North Island of New Zealand. Acids were quantified as the corresponding methyl esters.

peak	name of compound	sample number									
no		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	phenylacetic acid	0.05	0.1	0.01	0.1	0.2	tr	0.01	-	-	-
2	2-methoxyacetophenone	0.8	1.1	0.2	0.7	1.4	0.6	0.6	0.1	0.1	0.3
3	2-methoxybenzoic acid	5.3	4.8	3.1	4.9	5.6	6.1	1.9	0.4	0.6	0.6
4	phenyllactic acid	337	279	220	286	358	119	159	44	59	58
5	octanedioic acid	0.4	0.4	0.1	0.1	0.4	0.1	0.04	0.05	0.1	0.2
6	*cis*-cinnamic acid	1.2	1.3	1.6	0.8	0.8	1.0	1.2	-	0.3	-
7	*trans*-cinnamic acid	2.9	3.0	1.5	2.8	2.5	1.2	1.3	-	0.6	-
8	nonanedioic acid	0.4	0.3	0.5	0.3	0.9	0.5	0.4	0.4	0.6	0.5
9	unknown (*m/z* 196, 165)[b]	1.9	1.5	0.9	1.7	2.2	1.3	0.5	0.4	0.4	0.4
10	4-methoxyphenyllactic acid	8.3	6.5	4.5	6.5	11	0.8	14	1.3	1.9	0.2
11	decanedioic acid	2.2	1.6	1.8	1.4	1.9	1.1	1.0	0.6	0.9	0.9
12	methyl syringate	28	27	17	19	30	39	17	5.0	6.8	6.1
13	palmitic acid	4.9	4.0	2.4	5.1	3.9	2.2	3.3	2.9	2.8	3.3
14	stearic acid (18:0)	2.9	2.4	2.0	3.0	2.6	1.8	2.5	1.8	1.8	2.6
	UMF value[a]	14.2	14.1	12.6	14.1	14.6	15.2	7.7	10.8	16.1	5.3

[a]Unique Manuka Factor measured by Mr Jon Stephens, Honey Research Unit, The University of Waikato; tr = trace;

[b]prominent ions observed in the mass spectra of unknown compound are given in brackets.

Table 7.14 continued (samples 11-20).

peak no	name of compound	sample number									
		11	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1	phenylacetic acid	-	0.01	-	-	-	0.1	-	0.02	0.04	0.03
2	2-methoxyacetophenone	-	0.1	0.1	-	-	5.8	1.0	0.6	1.0	1.3
3	2-methoxybenzoic acid	0.1	-	0.5	-	-	6.1	2.7	2.0	1.8	2.7
4	phenyllactic acid	15	95	102	0.8	3.3	334	252	191	179	306
5	octanedioic acid	0.4	0.1	1.0	-	-	0.4	-	-	-	0.04
6	*cis*-cinnamic acid	-	1.0	0.5	-	-	0.8	0.9	0.5	0.4	0.5
7	*trans*-cinnamic acid	-	1.0	0.5	-	-	4.2	3.1	2.2	2.3	3.6
8	nonanedioic acid	0.5	0.6	0.1	-	-	0.4	0.5	0.4	0.4	0.1
9	unknown (*m/z* 196, 165)[b]	0.1	0.6	0.2	0.3	-	1.4	1.0	0.9	0.9	1.2
10	4-methoxyphenyllactic acid	0.2	8.0	1.7	-	-	26	23	4.7	3.2	2.4
11	decanedioic acid	0.7	0.9	0.8	0.02	-	2.1	0.9	2.0	0.5	1.0
12	methyl syringate	2.4	11	8.3	0.5	1.8	27	33	17	11	31
13	palmitic acid	2.5	4.1	7.7	7.1	3.8	7.5	4.4	7.6	5.9	5.6
14	stearic acid (18:0)	1.6	2.6	3.9	2.9	1.5	5.2	3.4	4.3	4.5	3.2
	UMF value[a]	0.0	4.6	4.8	0.0	0.0	13.7	8.5	7.4	9.5	11.1

[a]Unique Manuka Factor measured by Jon Stephens, Honey Research Unit, The University of Waikato; tr = trace; [b] prominent ions observed in the mass spectra of unknown compound are given in brackets.

Table 7.15. Mean concentration (mg/kg), standard deviation (stdev), and coefficient of variance (CV%) determined for dominant compounds identified in the 17 active and 3 inactive freshly harvested manuka honey samples. Acids were detected and quantified as the corresponding methyl esters.

		active				inactive			
peak no	**compound**	**mean**	**stdev**	**n**	**CV (%)**	**mean**	**stdev**	**n**	**CV (%)**
1	phenylacetic acid	0.1	0.1	11	99	-	-	-	-
2	2-methoxyacetophenone	0.9	1.3	17	143	-	-	-	-
3	2-methoxybenzoic acid	3.1	2.1	16	69	0.1	-	1	-
4	phenyllactic acid	199	107	17	54	6.4	7.5	3	118
5	octanedioic acid	0.3	0.3	14	108	0.4	-	1	-
6	*cis*-cinnamic acid	0.9	0.4	15	42	-	-	-	-
7	*trans*-cinnamic acid	2.2	1.1	15	51	-	-	-	-
8	nonanedioic acid	0.4	0.2	17	45	0.5	-	1	-
9	unknown (*m/z* 196, 165)[a]	1.0	0.6	17	57	0.2	0.1	2	47
10	4-methoxyphenyllactic acid	7.3	7.6	17	104	0.2	-	1	-
11	decanedioic acid	1.3	0.6	17	44	0.4	0.5	2	135
12	methyl syringate	20	11	17	54	1.6	1.0	3	63
13	palmitic acid	4.6	1.8	17	39	4.5	2.3	3	52
14	stearic acid (18:0)	3.0	1.0	17	34	2.0	0.8	3	38

[a]prominent ions observed in the mass spectra of unknown compound are given in brackets.

7.5.2. GC/MS Profiles of Freshly Harvested Samples

As observed for in 20/20 survey (Section 7.2.1), it proved possible to visually distinguish the GC/MS profiles of the 17 active and 3 inactive honeys.

The principle results obtained were:

1. The 17 active manuka honey samples contained relatively high levels of extractive substances compared to the 3 inactive honey samples (samples 11, 14 and 15).

2. The levels of phenyllactic acid (peak 4), 4-methoxyphenyllactic acid (peak 10), phenylacetic acid (peak 1) 2-methoxybenzoic acid (peak 3), and *cis* and *trans*-cinnamic acid isomers (peaks 6 and 7) in the extracts of active honey samples (average levels 199, 0.9, 3.1, 0.9 and 2.2 mg/kg respectively) were significantly higher than in extracts of the inactive honeys (average levels 6.4, 0, 0.1, 0 and 0 mg/kg respectively).

3. The Pearson's correlation coefficients matrix for UMF values and organic compound concentrations showed that the relationship between the levels of 2-methoxybenzoic acid and UMF values was in the "very" ($p < 0.01$) significant category (coefficient = 0.608) (Figure 7.10) while the relationship between the concentrations of phenyllactic acid and UMF values was in the 'weak' ($p < 0.05$) category. No other organic compounds showed a significant correlation with the UMF value.

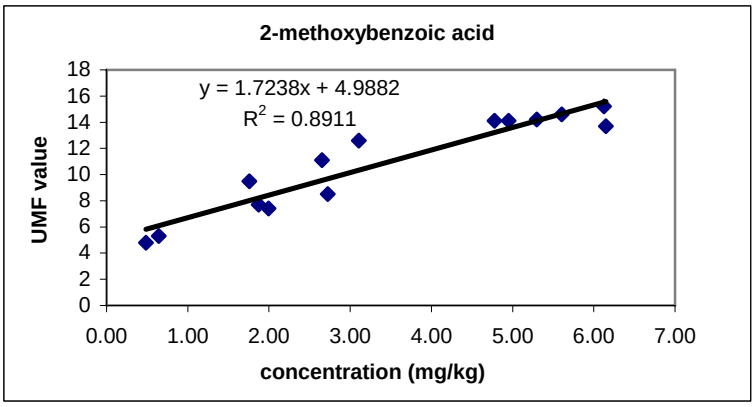


Figure 7.10. The relationship between the levels (mg/kg) of 2-methoxybenzoic acid and UMF values of freshly harvested active samples.

7.5.3. Multiple Regression Analysis for the Freshly Harvested Samples

As was described in the Section 7.4.1 for the stored samples of active manuka honey, the UMF values for the 17 samples of active freshly harvested manuka honey were regressed against the levels of the 11 statistically significant compounds identified in the 20/20 survey (phenylacetic acid, 2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, octanedioic acid, *cis*-cinnamic acid, *trans*-cinnamic acid, nonanedioic acid, 4-methoxyphenyllactic acid, decanedioic acid and methyl syringate). Subsequently, backwards selection multiple regression analysis was performed to eliminate weakly correlated substances from the data set (Section 7.4.3).

Statistics of the stepwise (steps 1-5) backwards selection multiple regression analysis performed for the 17 active samples are given in Tables 7.16 and 7.17.

Table 7.16. *p*-Values of steps 1-4 of backwards selection stepwise multiple regression analysis performed for the freshly harvested 17 active samples.

	original	step 1	step 2	step 3	step 4
	p-value	*p*-value	*p*-value	*p*-value	*p*-value
intercept	0.16	0.06	0.04	0.03	0.004
phenylacetic acid	0.55	0.43	0.31	0.27	0.40
2-methoxyacetophenone	0.23	0.18	0.15	0.11	0.08
2-methoxybenzoic acid	0.07	0.04	0.02	0.02	0.01
phenyllactic acid	0.98	0.97	-	-	-
octanedioic acid	0.99	-	-	-	-
cis-cinnamic acid	0.48	0.43	0.34	0.22	0.21
trans-cinnamic acid	0.70	0.61	0.39	0.41	-
nonanedioic acid	0.32	0.14	0.11	0.10	0.12
4-methoxyphenyllactic acid	0.48	0.42	0.34	0.33	0.50
decanedioic acid	0.72	0.68	0.65	-	-
methyl syringate	0.30	0.24	0.19	0.19	0.27

Octanedioic acid (original step; $p = 0.99$), phenyllactic acid (step 1; $p = 0.97$), decanedioic acid (step 2; $p = 0.65$), *trans*-cinnamic acid (step 3; $p = 0.41$) and 4-methoxyphenyllactic acid (step 4; $p = 0.50$) were progressively eliminated since they showed the higher *p*-values (Table 7.15). Finally, the best-fit regression equation with the highest R^2 value, the lowest standard error and the highest significant regression *p*-value was identified in step 5 (Table 7.17).

Table 7.17. R values, standard errors and regression *p*-values for the 1-5 steps of backwards selection stepwise multiple regression analysis performed for the 17 active freshly harvested honey samples.

Regression Statistics	original	step 1	step 2	step 3	step 4	step 5
multiple R	0.88	0.88	0.88	0.88	0.87	0.86
multiple R^2	0.78	0.78	0.78	0.77	0.75	0.74
adjusted R	0.55	0.65	0.71	0.74	0.75	0.76
adjusted R^2	0.30	0.42	0.50	0.55	0.56	0.58
standard error	3.2	3.0	2.7	2.6	2.6	2.5
regression *p*-value[a]	0.30	0.18	0.09	0.05	0.03	0.01

[a]*p*-value for the total regression output.

Optimum Regression Line

The data set in step 5 which gave the optimum results, showed a normal distribution (Figure 7.11) and the plot of residuals against predicted UMF values (Figure 7.12) also indicated that the spread of the residuals around the predicted UMF values was even along the length of the line and showed there were no obvious defects in the model.

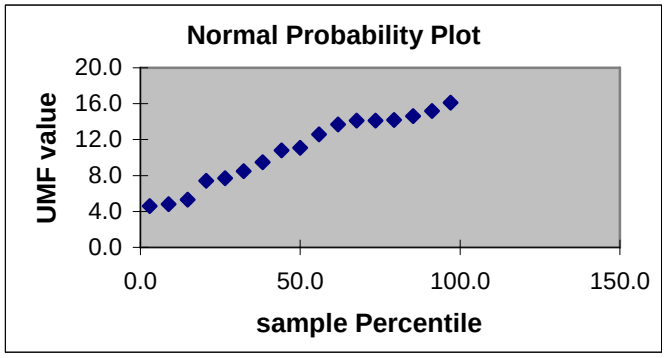


Figure 7.11. The normal probability plot (percentile *vs* UMF value) performed for the 17 active freshly harvested honey samples.

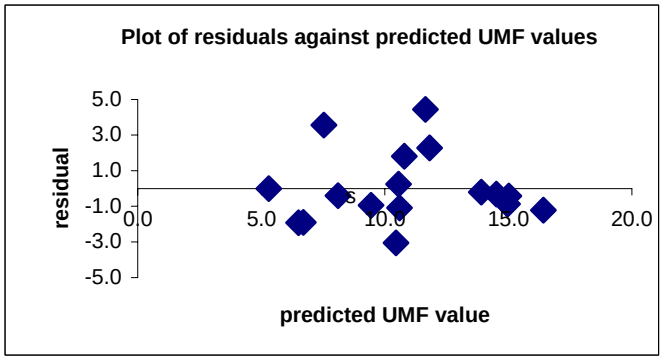


Figure 7.12. Plots of residuals against predicted UMF values performed for the 17 active freshly harvested honey samples.

Phenylacetic acid, 2-methoxyacetophenone, 2-methoxybenzoic acid, *cis*-cinnamic acid and nonanedioic acid were retained in the best-fit regression equation derived from data determined for the 17 freshly harvested active honeys.

The best-fit regression equation for the 17 active honey samples (with highest R^2 value, lowest standard error and the highest significant regression *p*-value) was:

UMF value = 8.3 -12.7 (phenylacetic acid) - 0.1 (2-methoxyacetophenone) + 1.3 (2-methoxybenzoic acid) - 1.7 (*cis*-cinnamic acid) + 6.3 (nonanedioic acid) – 0.1(methyl syringate).

R values, standard error and regression *p*-value are given in Table 7.18.

Table 7.18. R values, standard error and regression *p*-value determined for the best-fit regression equation in step 5 (n = 17).

Regression Statistics	step 5
multiple R	0.86
multiple R^2	0.74
adjusted R	0.76
adjusted R^2	0.58
standard error	2.5
regression *p*-value[a]	0.01

[a]*p*-value for the total regression output.

The R values (R = 0.86; R^2 = 0.74; adjusted R = 0.76; and adjusted R^2 = 0.58) and the regression *p*-value (0.01) for the six-compound analysis implied that the degree of relationship between the predicted UMF values and the levels of the 6 retained compounds (phenylacetic acid, 2-methoxyacetophenone, 2-methoxybenzoic acid, *cis*-cinnamic acid, nonanedioic acid and methyl syringate) was quite strong and positive (Table 7.18).

Retained Marker Compounds

Coefficients, standard errors and *p*-values for the six retained marker compounds retained in the best-fit regression equation are given Table 7.19.

Table 7.19. Coefficients, standard errors and *p*-values of the best-fit regression equation in step 5.

	coefficient	standard error	*p*-value
intercept	8.3	2.07	0.003
phenylacetic acid	-12.7	9.25	0.20
2-methoxyacetophenone	-0.1	0.05	0.06
2-methoxybenzoic acid	1.3	0.36	0.005
cis-cinnamic acid	-1.7	0.98	0.12
nonanedioic acid	6.3	3.54	0.11
methyl syringate	-0.1	0.08	0.14

Among the six retained organic compounds, the *p*-value of 2-methoxybenzoic acid ($p = 0.005$) implied that there was a significant relationship between the level of this compound and UMF values. The *p*-values of 2-methoxyacetophenone ($p = 0.06$), *cis*-cinnamic acid ($p = 0.12$), nonanedioic acid ($p = 0.11$) and methyl syringate ($p = 0.14$) implied that their levels were moderately related to the UMF values, while phenylacetic acid showed ($p = 0.20$) a weak correlation to the UMF value (Table 7.19). Inclusion of the weakly related compounds (*e.g.* phenylacetic acid) decreases the standard error and the increases the quality of the regression line, since they well fit into the regression line.

As seen in the 20/20 survey (Section 7.4.3), the lowest *p*-value (0.005) and highest regression coefficient (1.3) were generated for 2-methoxybenzoic acid (Table 7.19).

Observed and Predicted UMF values

The observed and the predicted UMF values, predicted as a linear combinations of six retained compounds, namely phenylacetic acid, 2-methoxyacetophenone, 2-methoxybenzoic acid, *cis*-cinnamic acid, nonanedioic acid and methyl syringate concentrations are given in Table 7.20.

The results presented in Table 7.20 showed that, the predicted UMF values of most of the samples (13/17 samples) were comparable to that of observed UMF values. Considerable variances between the observed and the predicted UMF values were observed for 3 low activity samples (samples 11, 12 and 15).

Table 7.20. Observed and predicted UMF values for 17 active honey samples.

sample no.	observed UMF	predicted UMF[a]	difference[b]
1	14.2	14.5	+0.3
2	14.1	11.8	-2.3
3	12.6	10.8	-1.8
4	14.1	15	+0.9
5	14.6	15	+0.4
6	15.2	16.4	+1.2
7	7.7	8.1	+0.4
8	10.8	10.6	-0.2
9	16.1	11.7	-4.4
10	5.3	5.3	0.0
11	4.6	6.5	+1.9
12	4.8	6.7	+1.9
13	13.7	13.9	+0.2
14	8.5	9.5	+1.0
15	7.4	10.5	+3.1
16	9.5	10.6	+1.1
17	11.1	7.6	-3.5

[a]predicted levels based on intercept value (8.26) plus individual compound contributions (Section 2.13.1);
[b]difference = (predicted UMF value - observed UMF value).

7.6. Analysis of Commercial-Grade Manuka Honey Samples

The statistical data of the previous two data sets (Sections 7.4-7.4.3 and 7.5.3) supports the hypothesis that the GC/MS data might distinguish active and inactive honeys and that UMF values might be correlated to the levels of a small set of statistically significant marker compounds.

A limitation of the results presented in Sections 7.2 -7.5 is that they are based on data determined for a moderate number of historic manuka samples from diverse (unknown) locations, and 17 freshly harvested North Island samples of variable quality (*e.g.* mixtures of honey and combs).

It was of interest to also investigate 30 commercial-grade and freshly harvested (2003 season) manuka honey samples (24 active and 6 inactive) for which commercial UMF values (determined by Gribbles Ltd. for Comvita

NewZealand Ltd.) were available. The 30 samples, which were supplied by Comvita New Zealand Ltd., originated from 7 North Island regions (Northland, East Coast, Coromandel, Keri Keri, Whangarei, Central North Island and Wellington).

7.6.1. Results and Discussions: Commercial-Grade Samples

The methylated diethyl ether extracts of the 24 active and 6 inactive manuka honey samples were analysed using the methodology described in Section 2.4. The UMF values of these manuka honey samples determined commercially were in the ranges negligible (< 5), very low (~ 5), low (~ 10), medium (~ 15) and high (~ 20) based on the diameter of the area of inhibition of growth in the standard agar well diffusion assay (Table 7.21).

The levels of 16 dominant compounds (2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, 3,5-dimethoxybenzoic acid, 3,4-dimethoxybenzoic acid, *cis*-cinnamic acid, 4-methoxyphenyllactic acid, decanedioic acid *trans*-cinnamic acid, tetradecanoic acid, methyl syringate, palmitic acid, 8-octadecenoic acid, stearic acid, ricinoleic acid (12-hydroxy-(*cis*)-9-octadecenoic acid) and unknown (*m/z* 91, 162)) which were present in > 60% of the samples are given in Tables 7.21 and 7.22. The mean concentrations, standard deviations and coefficients of variances of these methylated extractable organic substances are given in Table 7.23. Acids and some phenols were detected as their methyl esters, or ether derivatives (Tan *et al*., 1988, 1989b), unless otherwise indicated.

Table 7.21. UMF values (% phenol equivalent) and concentrations (mg/kg) of 16 compounds detected in the methylated diethyl ether extracts of 24 commercial-grade active manuka honey samples. Acids were quantified as the corresponding methyl esters.

	name of compound	sample number											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	2-methoxyacetophenone	2.1	2.5	5.4	2.6	2.5	3.5	3.9	3.5	0.9	1.4	1.5	1.6
2	2-methoxybenzoic acid	36	48	9.9	5.6	4.7	8.5	1.3	1.3	11	1.6	3.0	7.3
3	phenyllactic acid	451	443	625	388	303	323	135	151	341	132	194	327
4	3,5-dimethoxybenzoic acid	1.3	1.0	4.5	1.2	1.4	2.2	1.1	1.5	1.5	1.3	1.2	0.7
5	3,4-dimethoxybenzoic acid	0.3	0.6	6.0	1.5	1.7	2.1	0.9	0.6	0.8	0.8	1.0	0.8
6	*cis*-cinnamic acid	1.0	0.5	0.6	0.3	-	0.8	0.3	0.3	0.4	0.2	-	-
7	*trans*-cinnamic acid	0.4	0.5	-	0.4	0.4	0.7	0.4	0.5	0.4	-	0.1	0.3
8	4-methoxyphenyllactic acid	3.2	3.3	11	59	73	6.5	48	44	12	14	52	12
9	decanedioic acid	1.1	1.6	1.0	3.1	3.6	1.7	1.5	1.1	0.8	0.9	0.9	1.1
10	tetradecanoic acid	1.6	1.9	3.4	1.8	2.3	1.7	2.0	1.5	1.7	1.2	1.8	1.0
11	methyl syringate	9.9	9.2	47	70	76	37	32	28	14	19	32	17
12	palmitic acid	5.4	6.4	13	8.7	7.1	6.9	5.9	6.0	12	6.0	7.6	6.7
13	8-octadecenoic acid	21	22	33	20	20	21	14	17	27	21	21	22
14	stearic acid	2.3	2.3	5.5	3.9	3.1	2.4	2.3	2.3	3.0	2.8	3.6	3.3
15	ricinoleic acid (C:18 unst + OH)	7.0	6.7	8.7	7.3	6.5	9.1	6.3	5.2	12	4.6	6.4	4.8
16	unknown (*m/z* 91, 162)[b]	3.3	2.8	tr	0.5	0.6	2.5	0.3	0.4	0.3	0.2	0.5	0.8
	UMF value[a]	19.9	19.1	18.9	20.1	18.6	30.1	14.5	14.8	16.4	13.5	14.5	14.8

tr = trace; [a]Unique Manuka Factor measured by Gribbles Ltd. for Comvita New Zealand Ltd; [b]prominent ions observed in the mass spectra of unknown compound are given in brackets.

Table 7.21. continued (samples 13-24).

		sample number											
	name of compound	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
1	2-methoxyacetophenone	2.2	2.5	1.5	1.7	4.0	3.2	1.4	1.6	2.9	0.3	1.2	1.7
2	2-methoxybenzoic acid	1.1	0.9	9.9	10	2.1	1.8	3.4	4.0	2.3	0.5	1.6	0.9
3	phenyllactic acid	111	129	336	346	311	312	228	346	108	34	85	113
4	3,5-dimethoxybenzoic acid	0.4	0.4	0.5	0.4	0.5	0.5	0.8	0.5	1.1	0.3	0.8	0.2
5	3,4-dimethoxybenzoic acid	1.1	0.7	0.7	0.7	0.8	1.3	1.2	1.2	-	0.1	0.5	0.5
6	*cis*-cinnamic acid	-	-	0.2	0.1	-	-	-	-	-	0.1	0.3	0.1
7	*trans*-cinnamic acid	0.2	0.2	0.2	0.1	-	-	0.1	0.4	-	0.1	0.4	0.2
8	4-methoxyphenyllactic acid	56	44	54	53	211	154	21	36	20	4.8	26	5.5
9	decanedioic acid	1.3	0.7	1.3	1.6	-	-	1.5	1.8	2.1	3.7	0.7	1.9
10	tetradecanoic acid	1.9	2.3	2.0	1.7	1.8	1.5	1.7	1.5	3.1	1.1	1.5	1.3
11	methyl syringate	28	27	18	17	46	52	18	40	20	5.8	15	10
12	palmitic acid	7.1	6.9	10	9.3	9.2	7.4	9.4	7.7	21	5.4	6.3	5.3
13	8-cctadecenoic acid	20	23	30	28	24	18	26	22	33	19	28	14
14	stearic acid	2.9	2.7	3.2	2.8	2.8	2.0	3.2	2.0	7.1	2.4	2.0	1.6
15	ricinoleic acid	6.0	7.4	7.3	7.8	10	8.1	7.3	7.9	15	3.9	7.5	4.3
16	unknown (*m/z* 91, 162)[b]	0.4	0.3	0.2	0.2	0.7	0.8	0.7	0.8	-	-	0.2	0.1
	UMF value[a]	11.4	11.5	9.9	9.5	7.1	5.8	7.2	6.4	6.7	5.1	5.3	7.1

tr = trace; [a]Unique Manuka Factor measured by Gribbles Ltd. for Comvita New Zealand Ltd; [b]prominent ions observed in the mass spectra of unknown compound are given in brackets.

Table 7.22. Concentrations (mg/kg) of 16 compounds detected in the methylated diethyl ether extracts of 6 commercial-grade inactive manuka honey samples. Acids were quantified as the corresponding methyl esters.

		sample number					
	name of compound	**1**	**2**	**3**	**4**	**5**	**6**
1	2-methoxyacetophenone	2.6	0.7	0.9	0.7	0.2	1.2
2	2-methoxybenzoic acid	1.6	2.1	3.6	1.2	0.3	0.9
3	phenyllactic acid	122	124	260	54	29	67
4	3,5-dimethoxybenzoic acid	1.5	0.6	0.6	0.2	0.4	0.5
5	3,4-dimethoxybenzoic acid	0.6	1.3	0.7	0.5	0.2	0.2
6	*cis*-cinnamic acid	-	0.2	0.2	0.2	-	-
7	*trans*-cinnamic acid	-	0.3	0.2	0.1	0.1	0.1
8	4-methoxyphenyllactic acid	94	12	48	2.2	9.3	1.3
9	decanedioic acid	2.0	1.3	1.7	1.2	0.8	0.4
10	tetradecanoic acid	1.5	1.7	1.0	1.2	1.2	1.6
11	methyl syringate	20	32	25	9.9	8.7	7.9
12	palmitic acid	5.6	7.7	4.9	5.2	5.3	7.1
13	8-octadecenoic acid	20	24	14	17	18	23
14	stearic acid	1.6	2.3	1.5	2.1	2.1	2.2
15	ricinoleic acid	6.5	7.3	4.8	4.9	4.3	6.5
16	unknown (*m/z* 91, 162)[a]	0.1	0.05	0.4	-	-	-

[a]prominent ions observed in the mass spectra of unknown compound are given in brackets.

Table 7.23. Mean concentrations (mg/kg), standard deviations (stdev), and coefficient of variance (CV%) determined for 16 compounds identified in the diethyl ether extracts of 24 active and 6 inactive commercial-grade manuka honey samples. Acids were detected and quantified as the corresponding methyl ester.

		active				inactive			
	name of compound	**mean**	**stdev**	**n**	**CV (%)**	**mean**	**stdev**	**n**	**CV (%)**
1	2-methoxyacetophenone	2.3	1.2	24	50	1.0	0.8	6	80
2	2-methoxybenzoic acid	7.4	11	24	154	1.6	1.1	6	71
3	phenyllactic acid	261	144	24	55	109	83	6	76
4	3,5-dimethoxybenzoic acid	1.1	0.9	24	83	0.6	0.4	6	67
5	3,4-dimethoxybenzoic acid	1.1	1.2	23	103	0.6	0.4	6	71
6	*cis*-cinnamic acid	0.4	0.3	14	72	0.2	0.01	3	4.1
7	*trans*-cinnamic acid	0.3	0.2	19	51	0.1	0.1	5	67
8	4-methoxyphenyllactic acid	43	49	24	114	28	37	6	132
9	decanedioic acid	1.6	0.8	22	54	1.2	0.6	6	49
10	tetradecanoic acid	1.8	0.5	24	31	1.4	0.3	6	21
11	methyl syringate	29	19	24	65	17	9.9	6	58
12	palmitic acid	8.2	3.4	24	41	5.9	1.2	6	20
13	8-octadecenoic acid	23	5.1	24	22	20	3.8	6	19
14	stearic acid	3.0	1.2	24	40	2.0	0.3	6	15
15	ricinoleic acid	7.4	2.5	24	34	5.7	1.2	6	21
16	unknown (*m/z* 91, 162)[a]	0.8	0.9	21	115	0.2	0.2	3	93

[a]prominent ions observed in the mass spectra of unknown compound are given in brackets.

7.6.2. GC/MS Profiles of 24 Active and 6 Inactive Honey Samples

The GC/MS profiles of the active and inactive honeys could not be reliably distinguished visually. However, Pearson's correlation coefficients of 2-methoxybenzoic acid, phenyllactic acid, 3,5-dimethoxybenzoic acid, *cis*-cinnamic acid, *trans*-cinnamic acid and an unknown compound (*m/z* 91, 162) exhibiting ions reminiscent of phenyllactic acid showed positive significant correlation with UMF activity (0.531, 0.559, 0.606, 0.716, 0.674 and 0.536 respectively).

The coefficients determined for the remaining 10 compounds (2-methoxyacetophenone, 3,4-dimethoxybenzoic acid, 4-methoxyphenyllactic acid, decanedioic acid, tetradecanoic acid, methyl syringate, palmitic acid, 8-octadecenoic acid, stearic acid and ricinoleic acid) showed that there was no significant relationship to the UMF activity (0.296, 0.370, -0.260, -0.043, 0.171, 0.272, -0.131, -0.112, 0.063 and 0.007 respectively).

7.6.3. UMF Classification into Three Groups

Since it was difficult to visually differentiate GC/MS profiles determined for the 30 active and inactive commercial-grade honey samples, the honey samples were sub-divided into 3 groups according their degree of UMF activity (low, medium and high) (UMF values of < 9, 10-17 and >17 respectively). The mean concentrations of the selected 16 organic substances detected in each of the low, medium and high UMF samples are given in Table 7.24 and the average levels of 2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, 3,5-dimethoxybenzoic acid, *cis*-cinnamic acid, 4-methoxyphenyllactic acid, *trans*-cinnamic acid and unknown (*m/z* 91, 162) in low, medium and high activity honey samples are graphically shown in Figure 7.13.

Table 7.24. Mean concentrations (mg/kg) and standard deviations of the selected 16 compounds present in three groups of samples classified according the degree of UMF activity.

		low activity[b]			medium activity[c]			high activity[d]		
peak	name of compound	mean	SD	n	mean	SD	n	mean	SD	n
1	2-methoxyacetophenone	1.3	0.8	17	2.3	1.0	13	2.8	1.4	7
2	2-methoxybenzoic acid	1.2	0.9	16	3.7	3.3	13	18	17	7
3	phenyllactic acid	158	108	17	235	96	13	410	111	7
4	3,5-dimethoxybenzoic acid	0.6	0.3	14	0.8	0.4	13	1.9	1.2	7
5	3,4-dimethoxybenzoic acid	1.1	2.1	15	0.9	0.2	13	1.9	1.9	7
6	*cis*-cinnamic acid	0.2	0.1	9	0.2	0.1	5	0.6	0.2	6
7	*trans*-cinnamic acid	0.2	0.1	10	0.2	0.1	10	0.4	0.1	6
8	4-methoxyphenyllactic acid	91	102	17	61	57	13	24	29	7
9	decanedioic acid	2.1	1.7	12	1.3	0.3	11	1.8	1.1	7
10	tetradecanoic acid	1.5	0.5	17	1.7	0.3	13	2.1	0.6	7
11	methyl syringate	41	37	17	29	12	13	38	28	7
12	palmitic acid	9.1	6.2	17	7.6	1.4	13	8.5	2.9	7
13	8-octadecenoic acid	26	12	17	22	4.4	13	23	4.9	7
14	stearic acid	4.0	4.2	17	2.8	0.5	13	3.2	1.2	7
15	ricinoleic acid	6.0	2.7	17	6.9	1.5	13	8.2	1.9	7
16	unknown (*m/z* 91, 162) [a]	0.3	0.1	9	0.5	0.3	13	1.4	1.4	7

[a]prominent ions observed in the mass spectra of unknown compound are given in brackets;
[b]low activity (UMF < 9); [c]medium activity (UMF 10-17) and; [d]high activity (UMF > 17).

Table 7.24 shows that the average levels of nine compounds (2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, 3,5-dimethoxybenzoic acid, *cis*-cinnamic acid, *trans*-cinnamic acid, tetradecanoic acid, ricinoleic acid and an unknown compound (*m/z* 91, 162) generally increases with increasing the UMF value, while the level of 4-methoxybenzoic acid decreases with increasing UMF value.

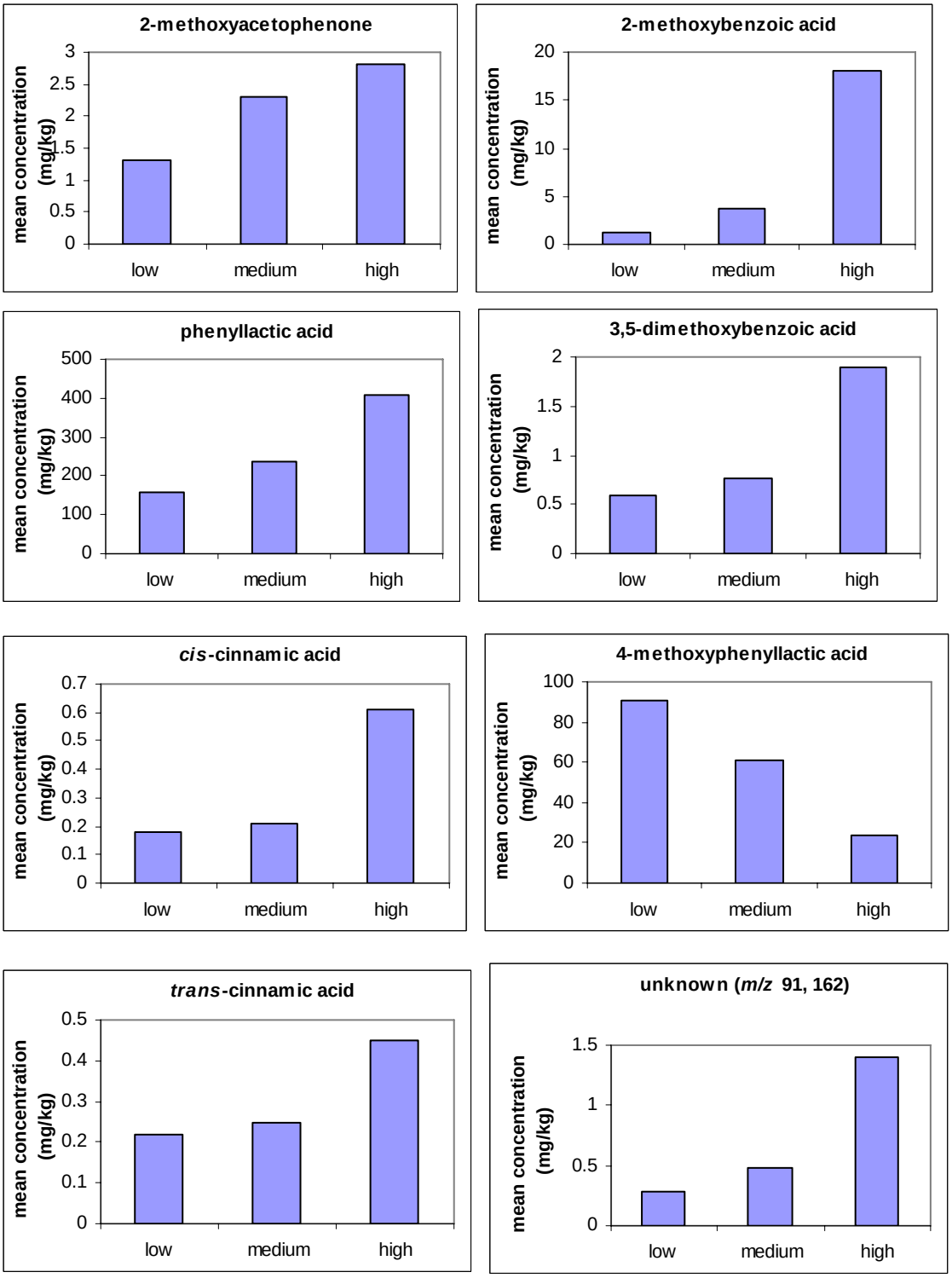


Figure 7.13. The average levels of 2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, 3,5-dimethoxybenzoic acid, *cis*-cinnamic acid, 4-methoxyphenyllactic acid, *trans*-cinnamic acid and an unknown compound (*m/z* 91, 162) in low, medium and high activity honeys.

7.6.4. Regional Classification

The 30 commercial-grade honey samples can also be grouped according to the regions they originated from. Thus, a characteristic GC/MS fingerprint for each region was observed (Figure 7.14). The average levels of 16 compounds detected in each group honey samples are given in Table 7.25.

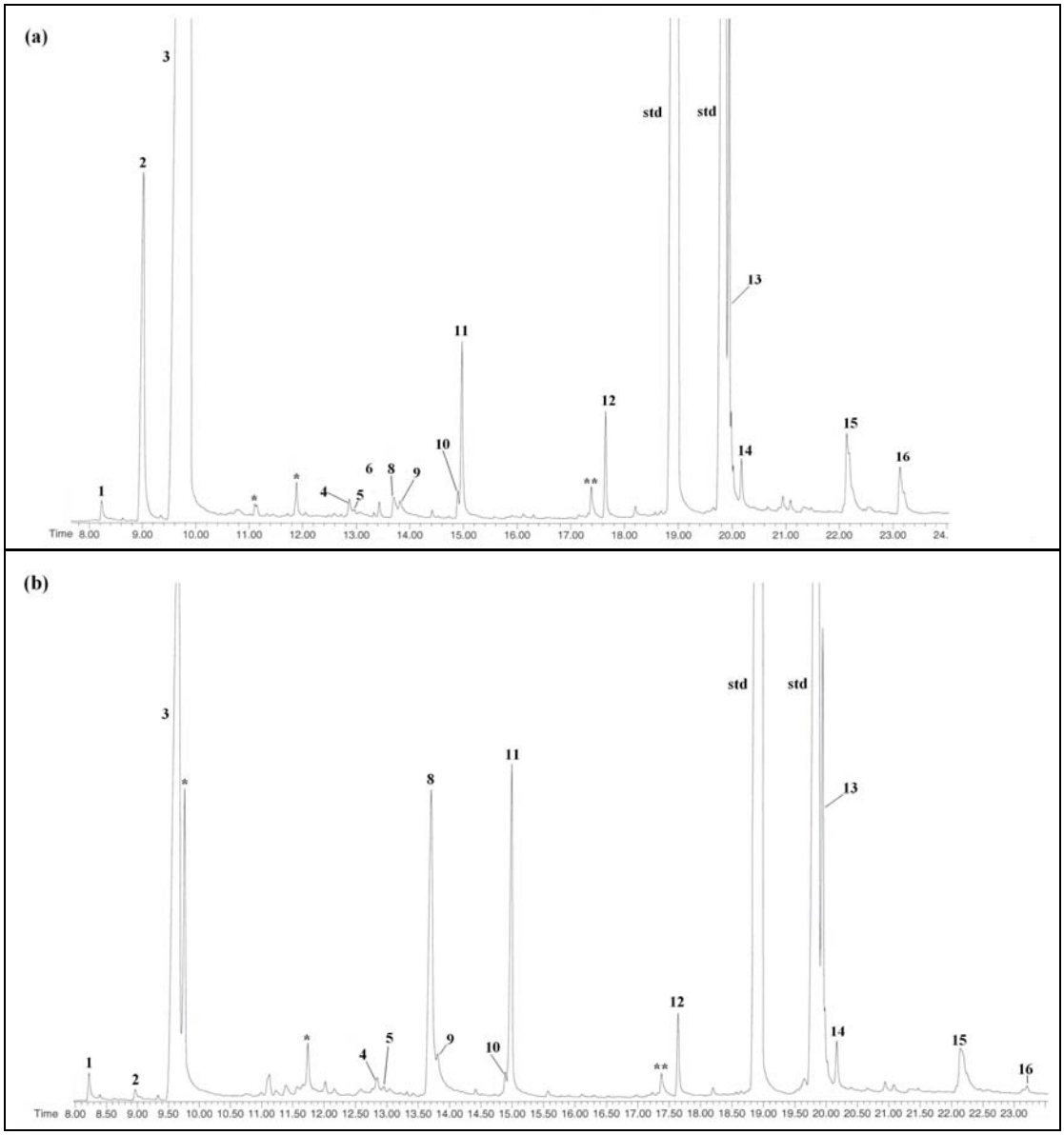


Figure 7.14. GC/MS profiles of representative methylated diethyl ether extracts of manuka honeys (a) Northland region (b) East Coast region (Peak identification is given Table 7.21) (*phthalates and/or solvent stabilizers) (** 9-hexadecenoic acid).

Table 7.25. The average levels of 16 compounds of each group of regional commercial-grade honey samples.

	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Northland																
mean	2.0	19	366	1.1	0.8	0.5	0.4	20	1.3	1.7	17	8.1	24	2.8	7.8	1.4
stdev	0.8	16	55	0.6	0.6	0.3	0.2	23	0.3	0.3	9.2	2.4	3.7	0.4	2.2	1.4
n	7	7	7	7	7	6	7	7	7	7	7	7	7	7	7	
East Coast																
mean	2.6	1.4	153	0.7	0.7	0.2	0.3	68	1.6	1.9	28	8.3	22	2.9	7.8	0.4
stdev	1.2	0.6	96	0.4	0.4	0.1	0.1	68	1.0	0.6	14	4.8	5.9	1.6	3.4	0.2
n	9	9	9	9	8	4	6	9	7	9	9	9	9	9	9	7
Coromandel																
mean	0.9	1.5	102	0.8	0.6	0.2	0.1	19	0.9	1.3	17	6.0	19	2.6	5.1	0.4
stdev	0.6	1.1	75	0.5	0.4	0.05	0.03	22	0.2	0.3	11	1.1	1.9	0.7	0.9	0.2
n	4	4	4	4	4	2	3	4	4	4	4	4	4	4	4	2
Keri Keri																
mean	2.0	4.6	317	1.4	2.1	0.4	0.2	26	1.5	1.8	32	8.5	24	2.9	7.2	0.4
stdev	1.9	3.0	190	1.7	2.2	0.2	0.1	16	0.3	0.9	12	2.9	6.8	1.6	1.5	0.4
n	5	5	5	5	5	3	4	5	5	5	5	5	5	5	5	5
Whangarei																
mean	2.6	5.1	345	1.3	1.6	0.3	0.4	66	3.3	2.0	73	7.9	20	3.5	6.9	0.6
stdev	0.1	0.6	60	0.1	0.2	-	0.01	9.4	0.3	0.3	4.1	1.2	0.2	0.6	0.6	0.01
n	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	2
Central North Island																
mean	1.5	0.9	90	0.4	0.3	0.1	0.1	3.4	1.1	1.4	8.9	6.2	19	1.9	5.4	0.1
stdev	0.3	0.03	32	0.2	0.2	-	0.1	2.9	1.1	0.2	1.4	1.3	6.3	0.4	1.6	-
n	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	1
Wellington[b]																
level	2.6	1.6	122	1.5	0.6	-	-	94	2.0	1.5	20	5.6	20	1.6	6.5	0.1

[a]compounds 1-16 are (1) 2-methoxyacetophenone, (2) 2-methoxybenzoic acid, (3) phenyllactic acid, (4) 3,5-dimethoxybenzoic acid, (5) 3,4-dimethoxybenzoic acid, (6) *cis*-cinnamic acid, (7) *trans*-cinnamic acid, (8) 4-methoxyphenyllactic acid, (9) decanedioic acid, (10) tetradecanoic acid, (11) methyl syringate (12) palmitic acid, (13) 8-octadecenoic acid, (14) stearic acid, (15) ricinoleic acid and (16) unknown (*m/z* 91, 162) respectively; [b]only one sample.

Northland honey samples possessed considerably higher levels of 2-methoxybenzoic acid (19 mg/kg) compared with the East Coast, Coromandel, Keri Keri, Whangarei, Central North Island and Wellington honey samples (1.4, 1.5, 4.6, 5.1, 0.9 and 1.6 mg/kg respectively).

The levels of phenyllactic acid present in the Northland (366 mg/kg), Keri Keri (317 mg/kg) and Whangarei (345 mg/kg) honey samples were 2 to 3 times higher than in the East Coast (153 mg/kg), Coromandel (102 mg/kg), Central North Island (90 mg/kg) and Wellington (122 mg/kg) honey samples.

The levels of 4-methoxyphenyllactic acid present in the East Coast (68 mg/kg), Whangarei (66 mg/kg) and Wellington (94 mg/kg) samples were higher than in other regional honey samples. Higher levels of methyl syringate were detected in the Whangarei honey samples (73 mg/kg) than in Northalnd, East Coast, Coromandel, Keri Keri, Central North Island and Wellington honey samples (17, 28, 17, 32, 8.9 and 20 mg/kg respectively). There were no significant differences between the levels of the other selected 12 substances (2-methoxyacetophenone, 3,5-dimethoxybenzoic acid, 3,4-dimethoxybenzoic acid, *cis*-cinnamic acid, *trans*-cinnamic acid, decanedioic acid, tetradecanoic acid, palmitic acid, 8-octadecenoic acid, stearic acid, ricinoleic acid and an unknown compound) and the region where the samples were collected.

7.6.5. Correlation Coefficients

The Pearson's correlation coefficients between the UMF values and the levels of the selected 16 compounds of the 7 Northland honey samples showed that 9 compounds were significantly correlated with UMF activity. These correlation coefficients indicated that UMF activity increased as the levels 2-methoxyacetophenone (0.802), 3,5-dimethoxybenzoic acid (0.930), 3,4-dimethoxybenzoic acid (0.685), *cis*-cinnamic acid (0.716) and *trans*-cinnamic acid (0.974), and an unknown (*m/z* 91, 162) (0.734) increased. On the other hand levels of 4-methoxyphenyllactic acid (-0.758), 8-octadecenoic acid (-0.769) and stearic acid (-0.653) were negatively correlated with UMF values. Figure 7.15 shows that, for the 7 Northland samples, UMF activity and the levels of *trans*-cinammic acid and 3,5-dimethoxybenzoic acid were highly correlated (0.974 and 0.930 respectively).

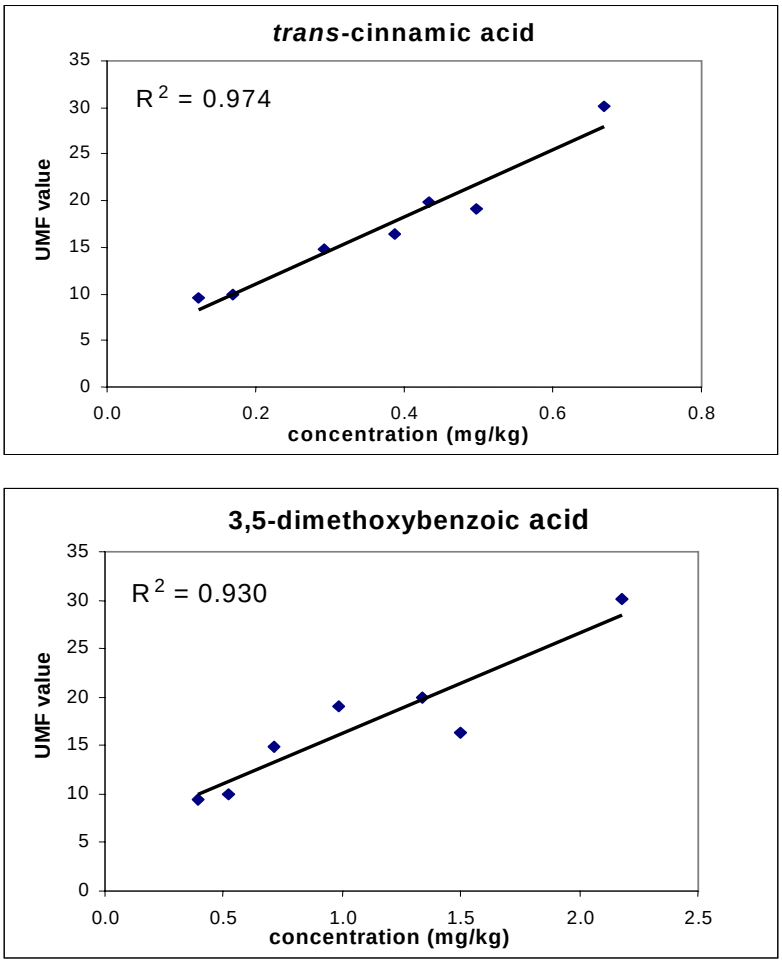


Figure 7.15. Relationship between UMF values and levels of (a) *trans*-cinnamic acid and (b) 3,5-dimethoxybenzoic acid for the 7 active Northland region manuka honey samples.

The Pearson's correlation coefficients determined for honey samples from other regions (*e.g.* East Coast, Keri Keri, etc) showed that there were no significant correlation between the levels of selected compounds and the UMF activity. Thus, only the GC/MS fingerprints for the extracts of active honey samples produced from the Northland region were seen to be distinguishable from those of inactive honeys produced in this region.

7.6.6. Multiple Regression Analysis for Commercial-Grade Honey Samples

The levels of 8 principal compounds (2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, *cis*-cinnamic acid, *trans*-cinnamic acid, 4-methoxyphenyllactic acid, decanedioic acid and methyl syringate) identified in the

diethyl ether extracts of the 23 commercial-grade active manuka honey samples were regressed against the UMF values. Three compounds (phenylacetic acid, octanedioic acid and nonanedioic acid) were excluded from this analysis since there were only present in the samples at low or trace levels (<1 mg/kg).

The data for sample 6 (a Northland region sample) was omitted since the UMF value for this sample (30.1) was substantially higher than that any of the other samples (max level of 20.1), possibly pointing to the inadvertent inclusion of some peroxide activity, or activity from an inadvertently introduced agent or substance during processing of the sample. The levels of extractable organic substances present in this sample were however similar to other Northland region samples.

Backwards Stepwise Multiple Regression Analysis

Backwards stepwise multiple regression analysis was performed by eliminating the weakly correlated depending variables (organic substances) from data sets as described in Section 7.4.3. Phenyllactic acid (p = 0.85 in the original analyses), 2-methoxyacetophenone (p = 0.79 in step 1) and *trans*-cinnamic acid (p = 0.42 in step 2) were progressively eliminated as they showed the higher p-values.

Optimum Regression Line

The optimum resulting data set (in step 3) showed a normal distribution (Figure 7.16) and the plot of residuals against predicted UMF values (Figure 7.17) also indicated that the spread of the residuals around the predicted UMF values was even along the length of the line, thus there was no obvious model defects. The total number of observations used for the analysis was 23.

The generated best-fit regression equation in step 3 (with highest the R^2 value and lowest standard error) was:

UMF value = 7.54 + 0.187 (2-methoxybenzoic acid) + 5.41 (*cis*-cinnamic acid) - 0.055 (4-methoxyphenyllactic acid) - 0.88 (decanedioic acid) + 0.204 (methyl syringate).

2-Methoxybenzoic acid, *cis*-cinnamic acid, 4-methoxyphenyllactic acid, decanedioic acid and methyl syringate were retained in the best-fit regression equation.

R values, standard error and regression *p*-value determined for the best-fit regression equation for the 23 commercial-grade active honey data set is given in Table 7.26.

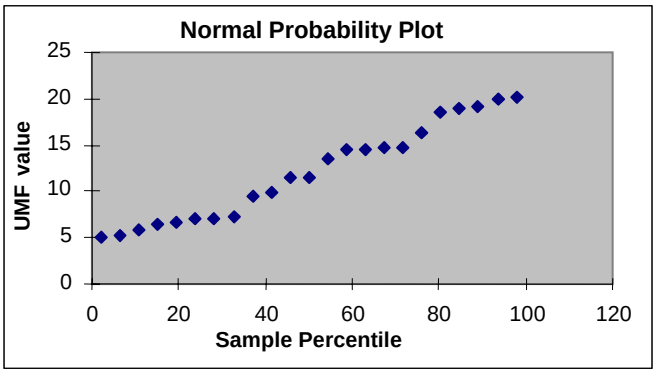


Figure 7.16. The normal probability plot of in step 3: percentile *vs* UMF value for the 23 commercial-grade active manuka honey samples.

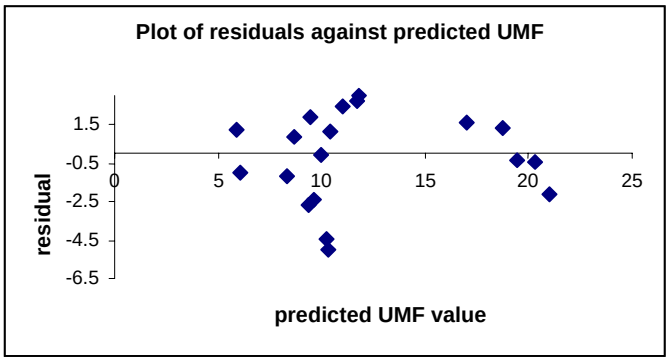


Figure 7.17. Plots of residuals against predicted UMF values in step 3 for the 23 commercial-grade active manuka honey samples.

Table 7.26. Summary output from step 3 of the best-fit regression equation for the 23 commercial-grade active honey samples.

regression statistics	step 3
multiple R	0.83
multiple R^2	0.70
adjusted R	0.77
adjusted R^2	0.60
standard error	3.26
regression *p*-value[a]	0.0005

[a]*p*-value for the total regression output.

The multiple correlation coefficients ((R, R^2, adjusted R and adjusted R^2 values = 0.83, 0.70, 0.77 and 0.60 respectively) and regression the *p*-value (0.000) determined for the five retained compounds (2-methoxybenzoic acid, *cis*-cinnamic acid, 4-methoxyphenyllactic acid, decanedioic acid and methyl syringate) indicated that there was a significant relationship between UMF values and the levels of the five compounds.

Individual effects of Five Components

Coefficients, standard errors and *p*-values for the individual marker compounds of the best-fit regression equation performed for the 20 active honeys data set is given in Table 7.27.

Table 7.27. Coefficients, standard errors and *p*-values of the best-fit regression equation in step 3 for the 23 commercial-grade active honey samples.

	coefficient	**standard error**	***p*-value**
intercept	7.54	2.05	0.002
2-methoxybenzoic acid	0.18	0.09	0.05
cis-cinnamic acid	5.41	4.61	0.26
4-methoxyphenyllactic acid	-0.055	0.02	0.02
decanedioic acid	-0.88	0.94	0.36
methyl syringate	0.20	0.05	0.001

The *p*-values and the standard errors for the compounds showed that methyl syringate (p = 0.001; standard error = 0.05), 4-methoxyphenyllactic acid (p = 0.02; standard error = 0.02) and 2-methoxybenzoic acid (p = 0.05; standard error = 0.09) were significantly correlated with the predicted UMF values, whereas, *cis*-cinnamic acid (p = 0.26; standard error = 4.61), and decanedioic acid (p = 0.36; standard error = 0.94) were only weakly correlated.

Observed and Predicted UMF values

The observed and the predicted UMF values, predicted as a linear combination of five retained compounds, namely 2-methoxybenzoic acid, *cis*-cinnamic acid, 4-methoxyphenyllactic acid, decanedioic acid and methyl syringate concentrations are also given in Table 7.28.

Table 7.28. Observed (measured by Gribbles Ltd. for Comvita New Zealand Ltd.) and predicted UMF values for 23 commercial-grade active manuka honey samples.

sample no.	observed UMF	predicted UMF[a]	difference[b]
1	19.9	20.4	+0.5
2	19.1	19.5	+0.4
3	18.9	21.1	+2.2
4	20.1	18.8	-1.3
5	18.6	17.1	-1.5
7	14.5	11.8	-2.7
8	14.8	11.8	-3.0
9	16.4	13.2	-3.2
10	13.5	11.1	-2.4
11	14.5	11.2	-3.3
12	14.8	11	-3.8
13	11.4	9.5	-1.9
14	11.5	10.5	-1.0
15	9.9	10	-0.1
16	9.5	9.2	-0.3
17	7.1	6	-1.1
18	5.8	10.3	-4.5
19	7.2	9.7	+2.5
20	6.4	13.1	+6.7
21	6.7	9.4	+2.7
22	5.1	6.1	+1.0
23	5.3	10.3	+5.0
24	7.1	8.3	+1.2

[a]predicted levels based on intercept value (7.54) plus individual compound contributions (Section 2.13.1);

[b]difference = (predicted UMF value - observed UMF value).

The results of the Table 7.28 showed that, the predicted UMF values of high and medium activity samples were comparable to that of observed UMF values. As seen in freshly harvest sample survey (Section 7.5.3), considerable variances between the observed and the predicted UMF values were observed for low activity samples (samples18, 20 and 23).

7.6.7. Comparison of Commercial and University-Determined UMF values

Given the variations often observed for UMF values determined in different laboratories, it was of interest to ascertain if there was a greater degree of correlation between selected marker compounds and University-determined UMF values, as opposed to commercially determined UMF values.

Though the same protocol was in principle used for the determination of UMF values of active manuka honeys in both commercial and the University procedures, the University procedure included multiple replicates (> 10) per sample and the mean value of the diameters of the clearing of replicates is used to derive the UMF value of the sample. Only a single assay is routinely used in commercial analyses (carried out by Gribbles Ltd.). One portion of each homogenized honey sample was tested in two labs. The University and Gribble's UMF values are compared numerically in Table 7.29 and graphically in Figure 7.18. The University values were obtained by Miss Carol Goss, and have been reported to Comvita New Zealand Ltd. by Dr Merilyn Manley-Harris.

Table 7.29. Commercial and University UMF values observed for 24 active manuka honey samples.

sample no	commercial UMF value[a]	University UMF value[b]	difference[c]
1	19.9	25.4	+5.5
2	19.1	28.0	+8.9
3	18.9	24.3	+5.4
4	20.1	22.9	+2.8
5	18.6	20.9	+2.3
6	30.1	29.7	-0.4
7	14.5	16.9	+2.4
8	14.8	17.8	+3.0
9	16.4	19.4	+3.0
10	13.5	16.8	+3.3
11	14.5	17.7	+3.2
12	14.8	17.7	+2.9
13	11.4	15.7	+4.3
14	11.5	16.0	+4.5
15	9.9	17.9	+8.0
16	9.5	18.2	+8.7
17	7.1	15.9	+8.8
18	5.8	15.6	+9.8
19	7.2	14.8	+7.6
20	6.4	16.1	+9.7
21	6.7	13.0	+6.3
22	5.1	10.0	+4.9
23	5.3	10.4	+5.1
24	7.1	10.6	+3.5

[a]Unique Manuka Factor measured by Gribbles Ltd. for Comvita New Zealand Ltd.; [b]UMF values measured by Miss Carol Goss, Chemistry Department, The University of Waikato; [c]difference = (University UMF value - commercial UMF value).

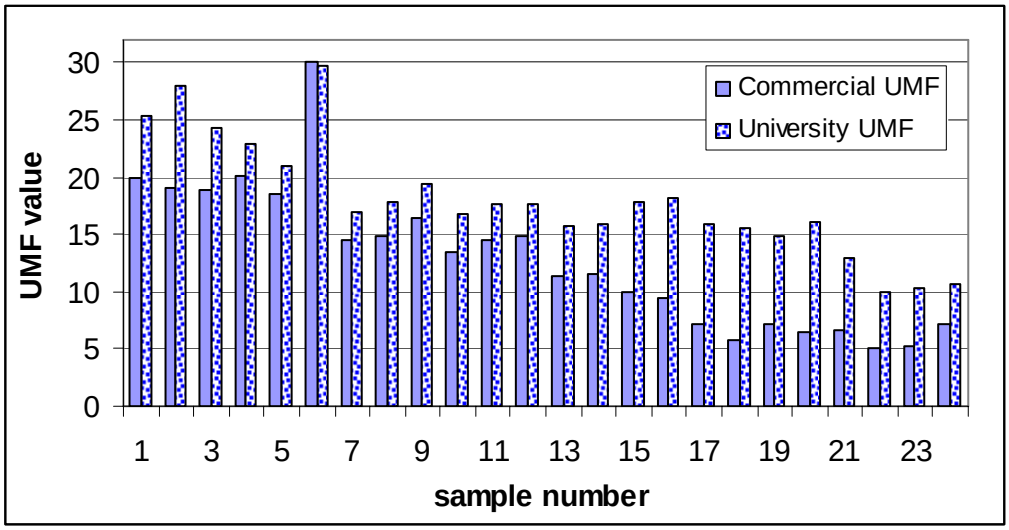


Figure 7.18. Comparison of commercial and University-determined UMF values for 24 commercial-grade manuka honey samples.

Using a 25% dilution of the honey, the University procedure was able to determine UMF values greater than 7.6. This value corresponded to a clearance diameter (zone of inhibition) of 8.5 mm (including the well diameter of 8.0 mm).

A notable feature of the results presented in Table 7.29 was the significantly higher University UMF values (10.0-18.2) determined for samples 15-23, compared to commercial UMF values (5.1-9.9). Deviations of this magnitude (variances of 8.0-9.8) (Table 7.29) were only observed for low activity samples. On the other hand similar UMF values were obtained for the fourteen high activity samples (samples 1-14) and a low activity sample (sample 24) (< 10 UMF value).

It is known that the UMF activity of the manuka honey increases slowly with the storage time. Possibly this may be the reason (at least in part) for the somewhat higher University values, since the samples had been stored for a further 9 months after UMF values were determined by Gribbles Ltd.

7.6.8. Multiple Regression Analyses Using University UMF values

The levels of the set of 8 principal compounds (2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, *cis*-cinnamic acid, *trans*-cinnamic acid, 4-methoxyphenyllactic acid, decanedioic acid and methyl syringate) identified as possible activity marker substances when using commercial UMF data, (Section 7.6)

were regressed against University UMF values using the procedure described in Section 2.13.

Backwards Stepwise Multiple Regression Analysis

When the backwards selection multiple regression analysis was performed using commercial UMF values, 2-methoxyacetophenone (p = 0.95 in the original analyses), *trans*-cinnamic acid (p = 0.90 in step 1) and phenyllactic acid (p = 0.43 in step 2) were progressively eliminated since they weakly correlated dependent variables. This also proved to be case using University-determined UMF values. The weakly correlated dependent variables from the data set with commercial UMF values with their p-values were phenyllactic acid (p = 0.84), 2-methoxyacetophenone (p = 0.78) and *trans*-cinnamic acid (p = 0.42).

Optimum Regression Line

The resulting data set showed a normal distribution (Figure 7.19) and the plot of residuals against predicted UMF values (Figure 7.20) showed that the spread of the residuals around the predicted UMF values was even along the length of the line.

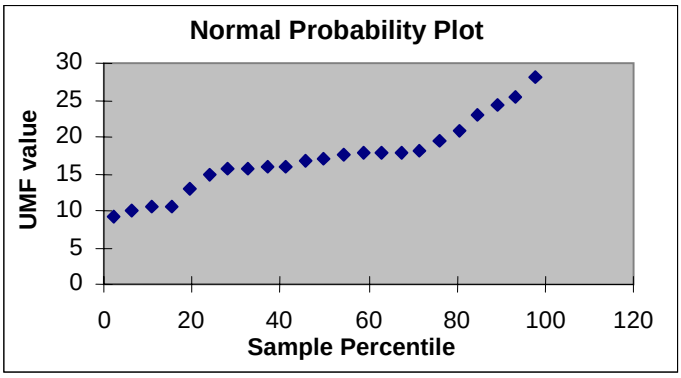


Figure 7.19. Normal probability plot for step 3 of the regression analyses for the 23 commercial-grade active honey samples with University UMF values.

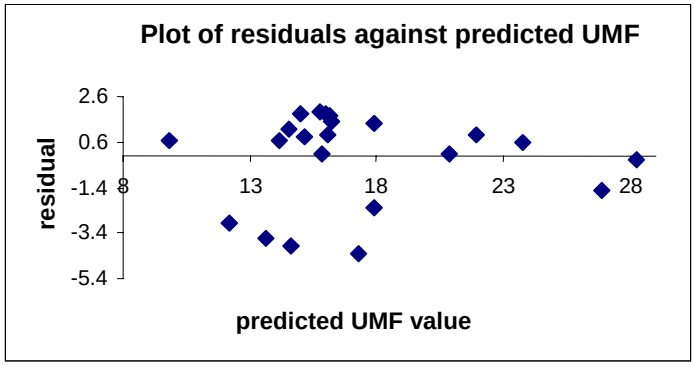


Figure 7.20. Plots of residuals against predicted UMF values for step 3 of the regression analysis for the 23 commercial-grade active samples with University UMF values.

The best-fit regression equation identified in step 3 (with highest R^2 value, lowest standard error and significant regression *p*-value) was:

UMF value = 11.96 + 0.31 (2-methoxybenzoic acid) + 3.49 (*cis*-cinnamic acid) - 0.021 (4-methoxyphenyllactic acid) - 0.99 (decanedioic acid) + 0.16 (methyl syringate).

The retained compounds in the best-fit regression equation identified in step 3 were 2-methoxybenzoic acid, *cis*-cinnamic acid, 4-methoxyphenyllactic acid, decanedioic acid and methyl syringate.

R values, standard error and regression *p*-value determined for the best-fit regression equation for the 23 commercial-grade active samples with University UMF value data set is given in Table 7.30.

Table 7.30. R values, standard error and regression *p*-value determined for the best-fit regression equations in step 3 (n = 23).

Regression Statistics	step 3
multiple R	0.91
multiple R^2	0.82
adjusted R	0.88
adjusted R^2	0.77
standard error	2.35
regression *p*-value[a]	0.0001

[a] *p*-value for the total regression output.

The final R, R^2, adjusted R, adjusted R^2 values (0.91, 0.82, 0.77 and 0.88 respectively) and regression *p*-value (0.000) showed that there is a significant positive relationship between the levels of 5 retained marker compounds and the UMF values.

Individual Effects of Five Compounds

Coefficients, standard errors and *p*-values for the individual marker compounds of the best-fit regression equation performed for the 23 active honeys with University UMF values is given in Tables 7.30.

Table 7.31. Coefficients, standard errors and *p*-values of the best-fit regression equation in step 3.

	coefficients	**standard error**	***p*-value**
intercept	11.96	1.51	0.00004
2-methoxybenzoic acid	0.31	0.06	0.0001
cis-cinnamic acid	3.49	3.42	0.32
4-methoxyphenyllactic acid	-0.021	0.01	0.22
decanedioic acid	-0.99	0.69	0.17
methyl syringate	0.16	0.04	0.0002

As seen in Section 7.6.6, 2-methoxybenzoic acid and methyl syringate showed stronger correlations to the predicted UMF values, as these compounds had lower *p*-values (0.000 and 0.000 respectively) and lower standard errors (0.06 and 0.04 respectively). *cis*-Cinnamic acid, 4-methoxyphenyllactic acid and decanedioic acid (*p*-values = 0.32, 0.22 and 0.17 respectively) were only weakly correlated (Table 7.31).

The correlations determined using University UMF values were comparable to those obtained using commercial UMF values (Section 7.6.6). The degree of correlation determined for the 23 active manuka honey samples using the five retained marker compounds and University UMF values was somewhat higher (R = 0.91; R^2 = 0.83) than that determined using commercial UMF values (R = 0.83; R^2 = 0.70) in Section 7.6.6.

Observed and Predicted UMF values

The observed and the predicted UMF values, predicted as a linear combination of five retained compounds, namely 2-methoxybenzoic acid, *cis*-cinnamic acid, 4-

methoxyphenyllactic acid, decanedioic acid and methyl syringate concentrations are given in Table 7.32.

Table 7.32. Observed (measured by the University of Waikato group) and predicted UMF values for 23 active manuka honey samples.

sample no.	observed UMF	predicted UMF[a]	difference[b]
1	25.4	26.9	+1.5
2	28	28.2	+0.2
3	24.3	23.7	-0.6
4	22.9	21.9	-1.0
5	20.9	20.8	-0.1
7	16.9	16	-0.9
8	17.8	16	-1.8
9	19.4	17.9	-1.5
10	16.8	15.0	-1.8
11	17.7	16.2	-1.5
12	17.7	15.8	-1.9
13	15.7	14.5	-1.2
14	16	15.2	-0.8
15	17.9	16.1	-1.8
16	18.2	15.5	-2.7
17	15.9	15.8	-0.1
18	15.6	18.0	+2.4
19	14.8	14.2	-0.6
20	13	17.3	+4.3
21	10	13.6	+3.6
22	10.4	9.8	-0.6
23	10.6	14.6	+4.0
24	9.2	12.2	+3.0

[a]predicted levels based on intercept value (11.96) plus individual marker compound contributions (Section 2.13.1); [b]difference = (predicted UMF value - observed UMF value).

The data presented in Table 7.32 showed that, most of the predicted UMF values (> 80%) were comparable to that of observed UMF values.

7.7. Multiple Regression Analysis of Pooled Manuka Honey Samples

Data from the 60 samples utilized in the previous studies (Sections 7.2-7.4, 7.5 and 7.6) was pooled and a statistical evaluation was carried out in order to obtain a universal regression equation in the expectation that it might be possible to predict the UMF value of manuka honeys using this equation.

The stepwise multiple regression analyses for the 60 pooled samples were performed using the UMF values of commercially determined UMF values for the 23 samples supplied by Comvita New Zealand Ltd. and University-determined UMF values for other samples.

7.7.1. Multiple Regression Analysis for the Pooled Samples

The UMF values were regressed against the levels of the same group of 11 compounds utilized in the 20/20 survey (Section 7.4.1), namely phenylacetic acid, 2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, octanedioic acid, *cis*-cinnamic acid, *trans*-cinnamic acid, nonanedioic acid, 4-methoxyphenyllactic acid, decanedioic acid and methyl syringate and subsequently backwards selection multiple regression analysis was performed as described in Section 7.4.3. This analysis showed that only *cis*-cinnamic acid (p = 0.90) was poorly correlated and it was therefore eliminated from the data set.

Overall Regression Output

The best-fit regression equation for the data set showed a normal distribution. The normal probability plots and the residual plots, after elimination of *cis*-cinammic acid, are given in Figure 7.21 and 7.22 respectively.

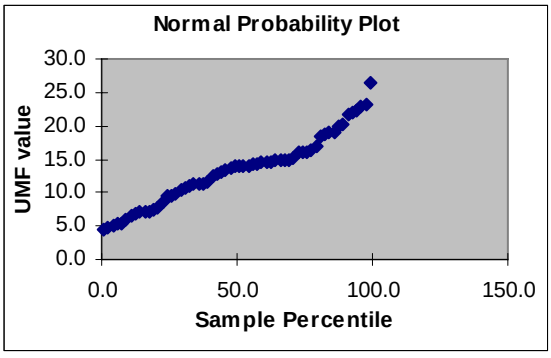


Figure 7.21. The normal probability plot in the best-fit regression equation for the pooled 60 samples.

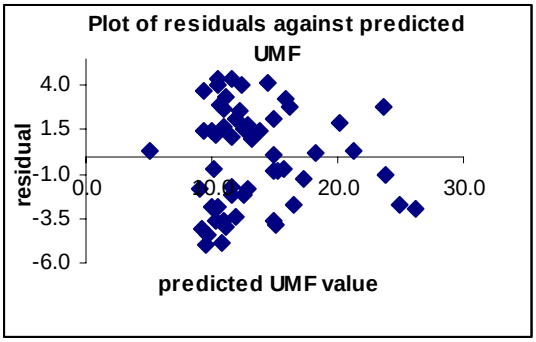


Figure 7.22. Plot of residuals against predicted UMF values in the best-fit regression equation for the pooled 60 samples.

The best-fit regression equation determined for the pooled 60 samples data set retained contributions from phenylacetic acid, 2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, octanedioic acid, *trans*-cinnamic acid, nonanedioic acid, 4-methoxyphenyllactic acid, decanedioc acid and methyl syringate.

The generated best-fit regression equations, after eliminating *cis*-cinnamic acid (with highest the R^2 value and lowest standard error) was:

UMF value = 8.88 – 1.30 (phenylacetic acid) – 0.08 (2-methoxyacetophenone) + 0.10 (2-methoxybenzoic acid) + 0.01 (phenyllactic acid) + 1.55 (octanedioic acid) – 0.19 (*trans*-cinnamic acid) – 0.84 (nonanedioic acid) – 0.02 (4-methoxyphenyllactic acid) – 0.21 (decanedioic acid) + 0.08 (methyl syringate).

R values and regression *p*-value determined for the best-fit regression equation for the pooled 60 active honey samples are given in Table 7.33. The R values (R = 0.81 and adjusted R = 0.77) and the regression *p*-value (*p*-value = 0.000) for the data set showed that there was a moderate significant linear relationship between the levels of the retained marker compounds and the UMF activity of the 60 manuka samples.

Table 7.33. R values and regression *p*-value determined for the best-fit regression equation for the pooled 60 active honey samples.

regression statistics	step 1
multiple R	0.81
multiple R^2	0.66
adjusted R	0.77
adjusted R^2	0.59
regression *p*-value[a]	2.0E-08

[a]*p*-value for the total regression output.

Relative Contributions of Retained Marker Compounds

Coefficients, standard errors and *p*-values for the individual marker compounds for the best-fit regression equation performed for pooled sample data sets (after elimination of *cis*-cinnamic acid) are given in Tables 7.34.

Table 7.34. Coefficients, standard errors and *p*-values for the variables of the best-fit regression equation (step 1) for the pooled 60 active honey samples.

	coefficient	**standard error**	***p*-value**
intercept	8.88	1.01	1.0E-11
phenylacetic acid	-1.30	0.70	0.07
2-methoxyacetophenone	-0.08	0.06	0.16
2-methoxybenzoic acid	0.10	0.03	0.0003
phenyllactic acid	0.01	0.00	0.008
octanedioic acid	1.55	1.03	0.14
trans-cinnamic acid	-0.19	0.10	0.07
nonanedioic acid	-0.84	0.40	0.04
4-methoxyphenyllactic acid	-0.02	0.01	0.05
decanedioic acid	-0.21	0.24	0.38
methyl syringate	0.08	0.02	0.003

Regression Coefficients

2-Methoxybenzoic acid, phenyllactic acid, octanedioic acid, 4-methoxyphenyllactic acid and methyl syringate showed positive regression coefficients (Table 7.34) while negative regression coefficients were found for phenylacetic acid, 2-methoxyacetophenone, *trans*-cinnamic acid, nonanedioic acid and decanedioic acid.

The regression coefficients for phenyllactic and 4-methoxyphenyllactic acid (0.01 and -0.02 respectively) were very low. Since the level of phenyllactic acid was typically 17.5 times greater than the level of 4-methoxyphenylllactic acid (the average levels are 541 and 31 mg/kg respectively) it can be anticipated that phenyllactic acid levels will contribute to the predicted UMF values to a greater extent than is the case for 4-methoxyphenyllactic acid levels.

p-Values

Low *p*-values were observed for 2-methoxybenzoic acid, phenylacetic acid, phenyllactic acid, *trans*-cinnamic acid, nonanedioic acid and methyl syringate (Table 7.34). These values imply that there were significant relationships between the predicted UMF values and the levels of these compounds.

Observed and Predicted UMF values

Observed and predicted UMF values determined for the pooled 60 samples are presented in Table 7.35.

Table 7.35. Observed and predicted UMF values for honey samples using the regression equation derived for the pooled 60 samples.

sample no.	observed UMF	predicted UMF[a]	difference[b]	sample no.	observed UMF	predicted UMF[a]	difference[b]
1	21.7	21.5	-0.2	**31**	4.6	9.4	+4.8
2	22	20	-2.0	**32**	4.8	11.7	+6.9
3	22.8	22.3	-0.5	**33**	13.7	10.8	-2.9
4	23.3	27.1	+3.8	**34**	8.5	11.6	+3.1
5	14.3	15	+0.7	**35**	7.4	10.5	+3.1
6	11.2	12.6	+1.4	**36**	9.5	10	+0.5
7	15	15.9	+0.9	**37**	11.1	12.7	+1.6
8	15	14.4	-0.6	**38**	19.9	15.6	-4.3
9	11.3	15.7	+4.4	**39**	19.1	16.6	-2.5
10	13.9	15.5	+1.6	**40**	18.9	16.5	-2.4
11	22.2	23.6	+1.4	**41**	20.1	14.8	-5.3
12	14.1	15.9	+1.8	**42**	18.6	14.2	-4.4
13	16	17.9	+1.9	**43**	14.5	10.4	-4.1
14	15.9	12.4	-3.5	**44**	14.8	10.3	-4.5
15	26.5	25.2	-1.3	**45**	16.4	12.6	-3.8
16	13	9.3	-3.7	**46**	13.5	10.6	-2.9
17	18.5	18.1	-0.4	**47**	14.5	11.2	-3.3
18	17	16.1	-0.9	**48**	14.8	12.3	-2.5
19	10.3	12.6	+2.3	**49**	11.4	9.9	-1.5
20	12.7	11	-1.7	**50**	11.5	10.3	-1.2
21	14.2	13.4	-0.8	**51**	9.9	11.6	+1.7
22	14.1	12.8	-1.3	**52**	9.5	11.5	+2.0
23	12.6	11.4	-1.2	**53**	7.1	8.9	+1.8
24	14.1	11.8	-2.3	**54**	5.8	10.8	+5.0
25	14.6	12.8	-1.8	**55**	7.2	11	+3.8
26	15.2	14.0	-1.2	**56**	6.4	13	+6.6
27	7.7	10.3	+2.6	**57**	6.7	10.1	+3.4
28	10.8	9.3	-1.5	**58**	5.1	8.6	+3.5
29	16.1	9.2	-6.9	**59**	5.3	9.7	+4.4
30	5.3	5.0	-0.3	**60**	7.1	9.7	+2.6

[a]predicted levels based on intercept value (8.88) plus individual compound contributions (Section 2.13.1);
[b]difference = (predicted UMF value - observed UMF value).

Most of the UMF values (> 96%) predicted for high and medium activity samples were comparable to that of observed UMF values (Table 7.35). As seen in Sections 7.5.3 and 7.6.6, considerable variances between the observed and the predicted UMF values were observed for low activity samples (samples 31, 32, 54, 55, 56, 57, 58 and 59).

7.7.2. Statistical Analysis for Low and High Activity Honey Samples

Since the determination of UMF values less than 8 is difficult, accurate UMF values cannot always be obtained for the low activity samples. Thus, the generation of regression equation to calculate the UMF values for moderate to high activity samples is likely to be more reliable compared to an equation derived from a set of samples which includes a number of low activity samples.

The set of pooled 60 samples set used in this investigation can be divided into two groups according to their UMF values. 30 samples had UMF value ranges in the 5.0-13.9 and while another 30 samples had UMF values in the range 14.1-26.5 respectively. Multiple regression analysis was performed for these 2 subset samples as described in Section 2.13.1.

7.7.3. Backwards Stepwise Regression Analysis

Backwards stepwise regression analysis was performed on the high and low subsets to obtain the optimum regression equations as described in Section 7.4.3.

The best-fit regression equation for the high activity subset was obtained in the 4^{th} step after eliminating phenylacetic acid, octanedioic acid and 4-methoxyphenyllactic acid as weakly correlated compounds (*p*-values =0.93, 0.91 and 0.69 respectively).

Step 7 gave the best-fit regression equation for the low activity subset after eliminating methyl syringate, decanedioic acid, phenylacetic acid, nonanedioic acid, 4-methoxyphenyllactic acid and phenyllactic acid (*p*-values = 0.96, 0.95, 0.89, 0.77, 0.60 and 0.50 respectively).

Overall Regression Output

The best-fit regression equations for the high and low activity subsets showed a normal distribution. The normal probability plot and the residual plot for the high and low subgroups are given in Figure 7.23 and 7.24 respectively.

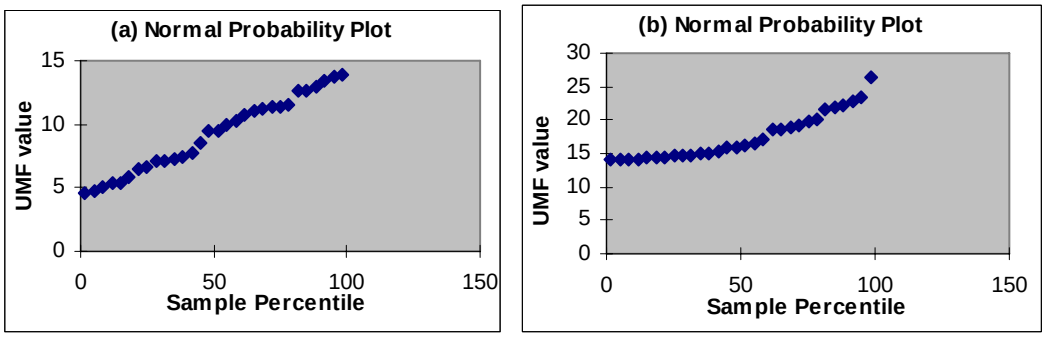


Figure 7.23. The normal probability plots in the best-fit regression equations determined for the (a) low and (b) high activity honeys.

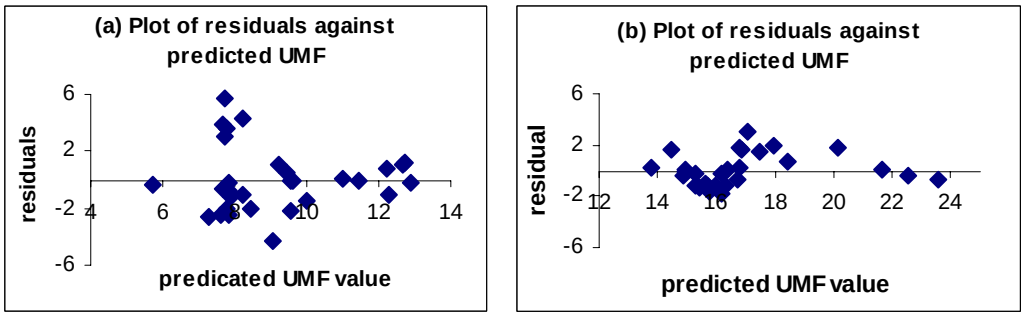


Figure 7.24. Plots of residuals against predicted UMF values in the best-fit regression equations for the (a) low and (b) high activity honeys.

Each of the reduced data sets showed a normal distribution (Figure 7.23). The plot of residuals against predicted UMF values (Figure 7.24) for the high activity subset indicated that the spread of the residuals around the predicted UMF values was even along the length of the line.The residual values for the low activity subset were spread along the length of the line over the range –6 to 6 values, whereas the high activity subset showed a lesser range (-2 to 3). This is consistent with the greaster R value determined for the high UMF activity subset.

The best-fit regression equation (with the highest R^2 value and lowest standard error) for the low activity sub data set was:

UMF value = 7.53 – 0.04 (2-methoxyacetophenone) + 0.20 (2-methoxybenzoic acid) + 1.46 (octanedioic acid) – 0.64 (*cis*-cinnamic acid) + 0.42 (*trans*-cinnamic acid).

Only 5 marker compounds (2-methoxyacetophenone, 2-methoxybenzoic acid, octanedioic acid, *cis* and *trans*-cinnamic acids) were retained in the best-fit regression equation performed for the low activity sub data set.

The generated best-fit regression equation (with the highest R^2 value and lowest standard error) for high activity sub data set was:

UMF value = 14.43– 0.18 (2-methoxyacetophenone) + 0.06 (2-methoxybenzoic acid) + 0.00 (phenyllactic acid) + 0.16 (*cis*-cinnamic acid) – 0.22 (*trans*-cinnamic acid) – 0.35 (nonanedioic acid) – 0.13 (decanedioic acid) + 0.03 (methyl syringate).

Eight marker compounds (phenylacetic acid, 2-methoxyacetophenone, 2-methoxybenzoic acid, phenyllactic acid, octanedioic acid, *trans*-cinnamic acid, nonanedioic acid, 4-methoxyphenyllactic acid, decanedioc acid and methyl syringate) were retained in the best-fit regression equation determined for the high activity set.

R values, standard errors and regression *p*-values determined for the best-fit regression equation for the low and high activity sub groups are given in Table 7.36.

Table 7.36. R values, standard errors and regression *p*-values determined for the best-fit regression equations in steps 7 and 4 for low and high activity samples (n = 30).

regression statistics	low activity samples	high activity samples
multiple R	0.64	0.92
multiple R^2	0.41	0.85
adjusted R	0.54	0.89
adjusted R^2	0.29	0.80
standard error	2.51	1.55
regression *p*-value[a]	0.018	2.0-E11

[a]*p*-value for the total regression output.

The value of the multiple correlation coefficient (R = 0.92; R^2 = 0.85), adjusted multiple correlation coefficient (R = 0.89; R^2 = 0.80) and the regression *p*-value (0.000) for the high activity sub data set showed that there was a significant

positive linear relationship between UMF values and levels of the retained compounds than the case for the low activity sub data set (R = 0.64; R^2 = 0.41; adjusted R = 0.54; adjusted R^2 = 0.29; and regression *p*-value = 0.018) (Table 7.36).

7.7.4. Observed *versus* Predicted UMF values

The observed and predicted UMF values for high activity and low activity samples determined using the best-fit regression equations are listed in Table 7.37 and depicted graphically in Figure 7.25. The UMF values were predicted based on intercept value plus individual marker compound contributions (Section 2.13.1).

Table 7.37. Observed and predicted UMF values for the low activity and high activity sub data sets.

	low activity			high activity		
sample no.	observed UMF	predicted UMF[a]	difference[b]	observed UMF	predicted UMF[a]	difference[b]
1	11.2	12.3	+1.1	21.7	21.6	-0.1
2	11.3	11.4	+0.1	22	20.2	-1.8
3	13.9	12.7	-1.2	22.8	23.6	+0.8
4	13	12.2	-0.8	23.3	25.7	+2.4
5	10.3	9.2	-1.1	14.3	16.2	+1.9
6	12.7	12.9	+0.2	15.0	14.9	-0.1
7	12.6	8.2	-4.4	15.0	15.3	+0.3
8	7.7	7.9	+0.2	22.2	22.5	+0.3
9	10.8	7.7	-3.1	14.1	13.8	-0.3
10	5.3	5.7	+0.4	16	16.7	+0.7
11	4.6	7.3	+2.7	15.9	16.2	+0.3
12	4.8	9.1	+4.3	26.5	25.3	-1.2
13	13.7	12.7	-1.0	18.5	16.9	-1.6
14	8.5	10	+1.5	17.0	16.8	-0.2
15	7.4	9.6	+2.2	14.2	15.8	+1.6
16	9.5	9.6	+0.1	14.1	15.4	+1.3
17	11.1	11	-0.1	14.1	15.3	+1.2
18	13.5	7.7	-5.8	14.6	15.6	+1.0
19	11.4	7.8	-3.6	15.2	16.3	+1.1
20	11.5	7.7	-3.8	16.1	14.5	-1.6
21	9.9	9.5	-0.4	19.9	18	-1.9
22	9.5	9.6	+0.1	19.1	18.4	-0.7
23	7.1	7.8	+0.7	18.9	17.5	-1.4
24	5.8	7.8	+2.0	20.1	17.1	-3.0
25	7.2	8.2	+1.0	18.6	16.8	-1.8
26	6.4	8.4	+2.0	14.5	14.9	+0.4
27	6.7	7.9	+1.2	14.8	14.9	+0.1
28	5.1	7.6	+2.5	16.4	16.4	0.0
29	5.3	7.8	+2.5	14.5	15.7	+1.2
30	7.1	7.7	+0.6	14.8	16.0	+1.2

[a]UMF values were predicted using the best-fit regression equation of the sub data set of low activity; [b]UMF values were predicted using the best-fit regression equation of the sub data set of high activity.

The predicted UMF values using the best-fit regression equation for high activity samples showed that there was a higher degree of correlation (with no outliers) between the predicted and the observed UMF values (Table 7.37 and Figure 7.25).

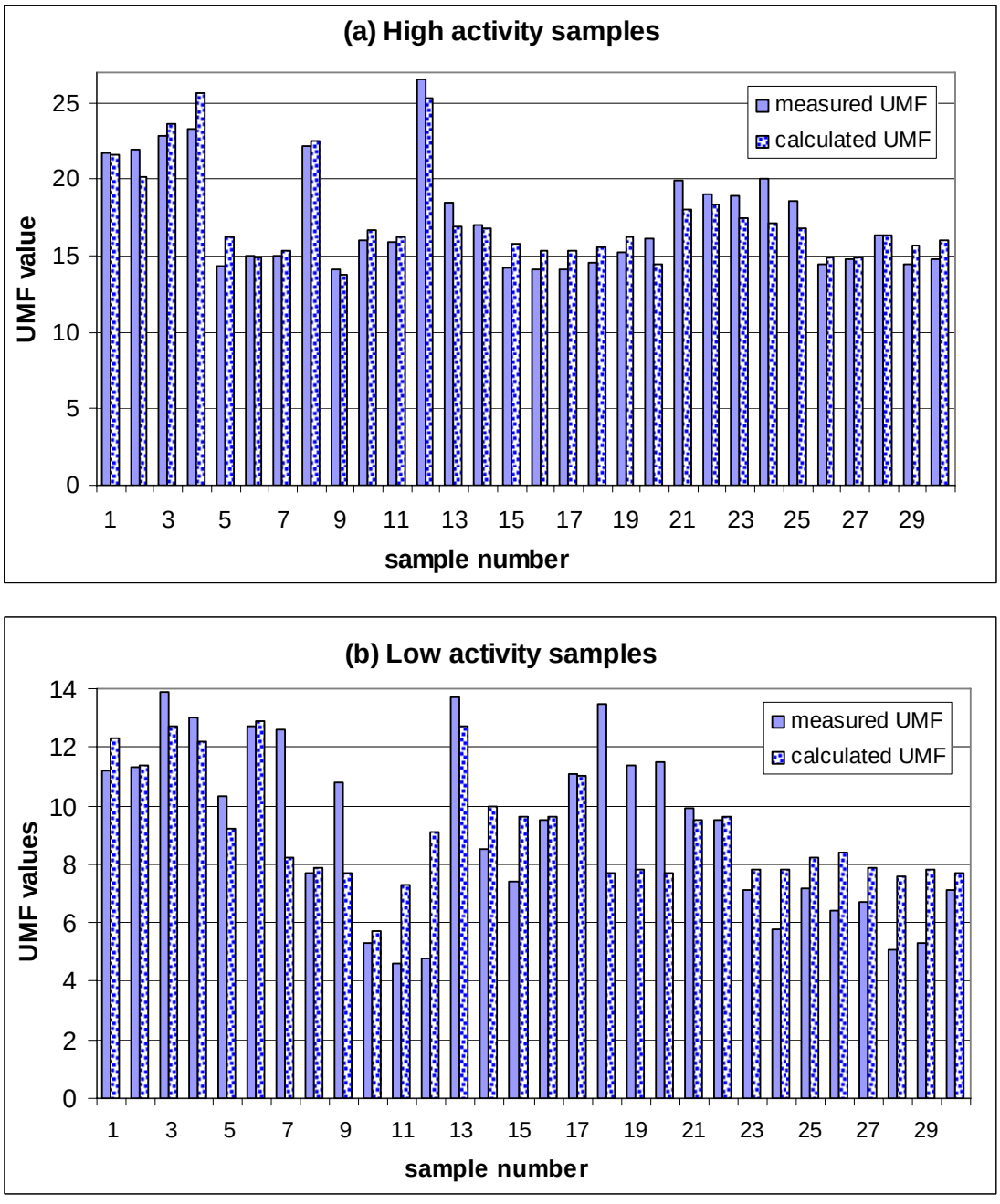


Figure 7.25. The observed and predicted UMF values for (a) high activity and (b) low activity samples determined using the best-fit regression equations.

It is apparent that regression equations derived from marker compound data determined for only high activity manuka samples is more reliable (and affords a higher R value) than is the case for data derived from both high and low activity 60 pooled manuka honey samples (Section 7.7.1). Given the difficulty in measuring low activity UMF values (particularly values < 8), it is not surprising that exclusion of samples with low UMF values leads to a better overall R value.

7.8. An Application of the Best Regression Equation

The validity of the equation derived in Section 7.7.2 can be evaluated by applying it to manuka honeys which were not part of the pooled data set, and for which UMF values are known.

During the course of the investigations reported in this thesis 29 manuka honey samples (which were considered to be inactive) were examined (see Sections 7.2, 7.5 and 7.6). These samples generally exhibited UMF values of the order 8-10 (or less). Typically this is the lowest measurable value using the Agar well diffusion assay. Practically however, it is difficult to reliably determine UMF values less than 8 since there is very high uncertainty in estimating the very small (sometimes marginally visible) zone of inhibition (*ca* 0.5 mm) associated with UMF values in the range 8-10.

If the marker compound regression equation derived in Section 7.7.2 has general validity, it can be anticipated that predicted UMF for these samples should, in general, be of the order 10 or less.

UMF values were predicted for the 29 samples using the best-fit regression equation generated from the set of 30 low activity samples (Section 7.7.2). The predicted UMF values using the selected best-fit regression equation, and the levels of marker compounds reported in Tables 7.2, 7.13 and 7.21 for the 29 samples (none of which were used to derive the regression equations) are presented in Table 7.38.

Table 7.38. The predicted UMF values for 29 low activity manuka honey samples using the best-fit regression equation of set of 30 low activity samples.

sample no	reported UMF	predicted UMF[c]
1[a]	< 10	11.2
2[a]	< 10	9.1
3[a]	< 10	7.1
4[a]	< 10	12.9
5[a]	< 10	13.1
6[a]	< 10	11.4
7[a]	< 10	11.6
8[a]	< 10	12.4
9[a]	< 10	14.4
10[a]	< 10	12.4
11[a]	< 10	8.1
12[a]	< 10	7.9
13[a]	< 10	19.1
14[a]	< 10	8.0
15[a]	< 10	6.6
16[a]	< 10	7.6
17[a]	< 10	7.2
18[a]	< 10	7.7
19[a]	< 10	6.1
20[a]	< 10	7.2
21[b]	< 10	8.2
22[b]	< 10	7.6
23[b]	< 10	7.6
24[b]	< 10	7.8
25[b]	< 10	8.0
26[b]	< 10	8.2
27[b]	< 10	7.7
28[b]	< 10	7.6
29[b]	< 10	7.7

[a]samples had been stored for various times (*e.g.* 1-10 years); [b]freshly harvested samples (*e.g.* 1 year); [c]levels predicted using the regression equation derived from the 30 low UMF manuka samples (Section 7.7.2).

Table 7.38 showed that the predicted UMF values for 20 of the 29 samples were less than 10. The other 9 samples (1, 4, 5, 6, 7, 8, 9, 10 and 13) which exhibited the predicted UMF value of > 10 had been stored for various times (1-10 years) and it could be suggested that these samples might (at least after storage for some years) exhibit a moderate levels of activity if they were retested (since the UMF activity increases slowly with the storage time (see Section 7.6.7)). Thus, overall there was a considerable level of agreement between reported UMF values and predicted UMF values, based on selected marker compound levels.

7.8.1. Predicted UMF values for Kanuka Honey Samples

It is generally accepted that kanuka honey samples do not exhibit UMF activity, whereas manuka honey samples may or may not exhibit UMF activity. It was therefore of interest to determine if the marker compound regression equation derived in Section 7.7.2 for the 30 pooled manuka honey samples with only low UMF activity, might also be applicable to kanuka samples. If so, the predicted UMF values for the inactive kanuka should be of the order 10 or less.

The levels of 17 dominant compounds identified in the methylated diethyl ether extracts of the 7 kanuka honey samples provided by Comvita New Zealand Ltd. are given in Table 7.39. The mean concentrations, standard deviations and coefficients of variances of these organic compounds present in 7 kanuka and 7 manuka honey samples collected from the same region where the kanuka samples had been collected (East Coast) and harvested in the same season (2002 season) are given in Table 7.40 (The results of Table 7.40 indicated that the extractable organic profiles of kanuka honeys cannot be distinguished from the manuka honey profiles).

Table 7.39. Concentrations (mg/kg) of 17 components detected in the methylated diethyl ether extracts of 7 commercial-grade kanuka honey samples (inactive) provided by Comvita New Zealand Ltd. Acids were quantified as the corresponding methyl esters.

		sample number						
		1	2	3	4	5	6	7
1	phenylacetic acid	0.2	0.1	0.2	-	1.1	-	-
2	2-methoxyacetophenone	2.4	1.8	1.8	1.7	1.3	0.3	0.1
3	2-methoxybenzoic acid	0.6	0.6	0.4	0.5	0.8	-	1.0
4	phenyllactic acid	259	247	346	351	131	281	79
5	3,5-dimethoxybenzoic acid	0.7	0.5	0.4	0.4	-	-	-
6	3,4-dimethoxybenzoic acid	0.8	0.8	0.6	0.6	0.5	8.7	-
7	*cis*-cinnamic acid	0.1	0.1	0.2	-	-	-	-
8	*trans*-cinnamic acid	-	0.3	-	0.5	-	-	-
9	4-methoxyphenyllactic acid	167	170	256	260	190	264	16
10	decanedioic acid	-	-	-	-	6.8	-	2.6
11	tetradecanoic acid	1.4	2.1	1.2	2.0	1.9	1.1	1.4
12	methyl syringate	63	59	99	96	110	87	21
13	palmitic acid	7.2	5.9	5.1	5.3	18	22	17
14	8-octadecenoic acid	24	20	20	19	56	43	49
15	stearic acid	2.1	1.9	1.5	1.3	11	15	12
16	ricinoleic acid (C: 18 unsat + OH)	5.9	5.1	5.2	6.4	6.0	4.1	3.3
17	unknown (*m/z* 91, 162)[a]	0.4	0.4	0.5	0.3	-	-	-
UMF		**< 8.2**[b]	**< 8.2**	**< 8.2**	**< 8.2**	**< 8.2**	**< 8.2**	**< 8.2**

[a]prominent ions observed in the mass spectra of unknown compound are given in brackets; [b]UMF values were measured by Gribbles Ltd. for Comvita New Zealand Ltd.

Table 7.40. Mean concentration (mg/kg), standard deviation (stdev), and coefficient of variance (CV%) determined for 17compounds identified in the diethyl ether extracts of 7 commercial-grade kanuka and 7 manuka[a] honey samples produced in the East Coast region. Acids were detected and quantified as the corresponding methyl ester.

		kanuka				**manuka**[a]			
	name of compound	**mean**	**stdev**	**n**	**CV (%)**	**mean**	**stdev**	**n**	**CV (%)**
1	phenylacetic acid	0.4	0.5	4	106	0.3	0.1	4	45
2	2-methoxyacetophenone	1.4	0.8	7	61	2.3	1.2	7	54
3	2-methoxybenzoic acid	0.6	0.2	6	35	1.5	0.7	7	45
4	phenyllactic acid	242	103	7	42	156	111	7	71
5	3,5-dimethoxybenzoic acid	0.5	0.2	4	30	0.6	0.3	7	48
6	3,4-dimethoxybenzoic acid	2.0	3.3	6	164	0.7	0.4	6	57
7	*cis*-cinnamic acid	0.1	0.05	3	34	0.2	0.1	2	45
8	*trans*-cinnamic acid	0.4	0.1	2	37	0.2	0.1	4	50
9	4-methoxyphenyllactic acid	189	87	7	46	74	78	7	106
10	decanedioic acid	4.7	3.0	2	64	1.7	1.2	5	73
11	tetradecanoic acid	1.6	0.4	7	26	1.9	0.6	7	35
12	methyl syringate	77	31	7	40	28	17	7	59
13	palmitic acid	12	7.3	7	63	9.0	5.3	7	59
14	8-octadecenoic acid	33	16	7	48	23	5.3	7	23
15	stearic acid	6.3	5.9	7	93	3.1	1.8	7	57
16	ricinoleic acid	5.2	1.1	7	21	8.3	3.6	7	44
17	unknown (*m/z* 91, 162)[b]	0.4	0.1	4	23	0.5	0.3	5	56

[a]manuka honey samples 13, 14, 17, 18, 21, 22 and 23 analysed in Section 7.6 ; [b]prominent ions observed in the mass spectra of unknown compound are given in brackets.

The results presented in Table 7.41, predicted using the levels of marker compounds presented in Table 7.39 and the regression equation derived in Section 7.7.2 shows that the seven kanuka honey samples are not likely to exhibit UMF activity. Lesser predicted UMF values (of the order 7-8) were predicted using the regression equation derived from low activity manuka samples.

Table 7.41. Observed and predicted UMF values for the 7 kanuka honey samples.

sample	observed[a] UMF	predicted[b] UMF
1	< 8.2	7.5
2	< 8.2	7.7
3	< 8.2	7.5
4	< 8.2	7.8
5	< 8.2	7.7
6	< 8.2	7.5
7	< 8.2	7.7

[a]UMF values measured by Gribbles Ltd. for Comvita New Zealand Ltd;
[b]UMF values predicted using the best-fit regression equation for the 30 low activity samples.

7.9. Conclusions and Summary

The results presented in this chapter support the view that the marker compound regression equations are capable of predicting the approximate UMF activity in both active and inactive manuka and kanuka honey samples. It can be anticipated that further investigations, utilizing a greater number of both high and low activity samples, with strict attention to analytical issues in both GC/MS analyses and the agar well diffusion assay, will afford more reliable marker compound regression equations which, will in turn, improve the prediction of UMF value of a kanuka or manuka samples.

Given the inherent difficulty in measuring low activity UMF values (the minimum reliably measurable value is 10, anything less is equated to the absence of activity), there is a need to construct a UMF activity test which can reliably measure values over the range 3-30, rather than only the range 8-20 (with a high uncertainty in the region 8-10). The low UMF activity (< 10) could reliably be measured by testing honey at different concentrations (*e.g.* 50% solution) in the agar well diffusion assay (Molan, personal communication, 2005). Thus, a more reliable marker compound regression equation ($R > 0.95$) derived by multiple regression analysis using reliable observed UMF values and the levels of marker compounds could be achieved.

A significant limitation of the regression equations presented in Sections 7.7.1 and 7.7.2 is the comparatively large intercept value included in these equations as a consequence of the lowest measurable UMF value currently being around 8.

A possible advantage of the regression equation approach, over the agar well diffusion method is that (if it was accepted in the market place) it could be used as a faster (within a day) and economical method to measure the UMF activity approximately since the Agar well diffusion method takes a longer time (1-2 weeks) to assay the UMF activity. A modern rapid screening chemical analysis method should however be developed for to use in the market place (*e.g.* HPLC-APCI-MS/MS) since GC/MS method, which was used in this study, is a more labour intensive techniques.

The principal conclusions of the work reported in this chapter are:

(1). There are statistically significant differences between the levels of selected marker compounds determined by GC/MS in extracts of active and inactive manuka honeys.

(2). There is a statistically significant correlation between observed UMF values and predicted UMF values derived from the levels of selected marker compounds.

(3). The best-fit regression equation was obtained from a set of pooled 30 moderate to high activity samples (UMF > 14) with 8 retained marker compounds (R = 0.92).

(4). Application of the derived regression equation for the manuka and kanuka honey samples gave a considerable level of agreement between reported UMF values and predicted UMF values.

Further Discussion

Some of the marker compounds (*e.g.* phenyllactic acid, 4-methoxyphenyllactic acid and methyl syringate) were also present in the other unifloral honeys (*e.g.* kamahi, ling-heather, Erica and honeydew honeys etc.) (Sections 3.3.11, 4.3.2-3, 5.3.8 and 6.4.7) and they were, however, detected in much lower concentrations in these honeys than those found in manuka honey. This appears to be suggestive that there might be a minor input of these marker compounds in manuka honey from other floral sources but, due to their lower concentration levels, these would be unlikely to

interfere with the prediction of UMF values of active manuka honeys using marker compound equation.

While the type of analysis used in this study (GC/MS) to determine the levels of marker compounds can only detect the derivatized materials, it cannot determine the high polar compounds (*e.g.* glucosides, flavonoids) present in honey. Thus, there is a need to develop a simple, more rapid screening method for analysis of the manuka honey compounds (*e.g.* HPLC/MS/MS and MALDI-TOF) and these techniques would be useful to determine qualitatively and quantitatively the highly polar and underivatized materials present in honeys.

Finally, further evaluation of series of active, inactive manuka and kanuka honeys needs to be carried out to ascertain whether the levels of marker compounds would change with the storage time.

Chapter Eight

Leaf Oil Profiles of Manuka Plants Contributing to Active and Inactive Manuka Honeys

Chapter Eight

Leaf Oil Profiles of Manuka Plants Contributing to Active and Inactive Manuka Honeys

8.1. Introduction

It is known that there are several chemotypes of manuka (*L. scoparium)* leaf oil produced from what appear to be genetically distinct populations of manuka plants. These chemotypes are characterized by variations in their steam distilled essential oil composition (Perry *et al*., 1997; Porter and Wilkins, 1998; Christoph, 1999). For example, Perry *et al*. (1997) have reported three chemotypes: namely (i) pinene rich, (ii) sesquiterpene rich and (iii) leptospermone rich oils.

The East Cape (East Coast) chemotype which affords an oil with elevated levels of leptospermone, has appreciable antimicrobial activity (Porter and Wilkins, 1998). Molan (1995) has reported that manuka honeys collected from the East Coast possessed non-peroxide antibacterial activity (Figure 1.8) (Chapter 1).

In an endeavour to link the antimicrobial activity of East Cape (East Coast) manuka honey and the leptospermone-rich manuka oils, Tan *et al*. (1988), Wilkins *et al*. (1993a) and Weston *et al*. (2000) explored the possibility that leptospermone might be present in active manuka honeys, but they were unable to detect leptospermone in the manuka honey samples which they examined (Section 1.4.1).

It was of interest in the present investigation to ascertain if active manuka honeys were derived predominantly from a single manuka chemotype, or from multiple chemotype sources.

Hitherto a limiting factor in investigating this issue has been requirement to collect a substantial amount of manuka leaf (*ca* 5 kg) in order to obtain a quantity of oil sufficient for GC/MS analyses. It was envisaged that this difficulty could be overcome if the micro-scale GC/FID or GC/MS, technique which Brophy *et al*.

(1989) developed for leaves from tea tree plants could also be applied to manuka leaves. There are no reports in the literature of the Brophy technique (which requires only 20 leaves as opposed to *ca* 5 kg for pilot scale steam distillation) being used to determine manuka oil chemotype fingerprints.

The principal objectives of this investigation were to:

- determine the oil chemotype profiles of New Zealand manuka (*L. scoparium*) leaf using the micro-scale technique developed by Brophy *et al*. (1989) and compare them to conventional steam distillate data,

- determine the leaf oil profiles of manuka plants collected from areas which afforded active and inactive honeys,

- explore the possibility that for manuka honeys there might be correlation between leaf oil profiles and the UMF activity.

In this investigation, the leaf oil extract profiles of manuka are assessed within and between populations of manuka plants throughout the North Island (Section 8.2.1) while the honey samples were obtained from beekeepers with hives kept in the North Island region where the plant leaves were collected.

8.2. Experimental

8.2.1. Leaf Samples

125 Leaf samples from manuka (*L. scoparium*) populations in Northland, Waikato, Coromandel, Central North Island and the East Coast district of the North Island were provided by Mr. Jon Stephens, Biology Department, The University of Waikato, New Zealand. Leaf samples were collected in the vicinity of hives producing unifloral grade manuka honeys.

At each site five plants at least 100 m apart were sampled to minimize the possibility of collecting closely related individuals. One-year old apical branches 2 m

from the ground were selected to minimize within-tree variation. In order to avoid age dependant variability within manuka populations (Porter *et al*., 1998), and to minimize the cyclical and environmental variation that might depend on the oil production (Butcher *et al*. 1994), mature leaves were selected from one-year old wood. The leaves were bagged and stored at 4 °C prior to analysis. The leaf sample descriptions are listed in Table 8.1.

Table 8.1. Regions, sites, location and site descriptions of *L. scoparium* populations sampled[a].

region	site no.	site	latitude, longitude	habitat description	altitude (m)
Northland	1	Omanaia	35°270 N, 173°310 E	Lowland scrub	20-40
	2	Takehe	35°280 N, 173° 400 E	Lowland scrub	40
	3	Ngawha Springs	35°250 N, 173°510 E	Hill scrub	100
	4	Maromaku	35°290 N, 174°050 E	Hill scrub	150
	5	Kawakawa	35°270 N, 174°040 E	Hill scrub	100
Coromandel	6	Waikawau	36°570 N, 175°290 E	Coastal hill scrub	20-100
	7	Colville	36°390 N, 175°290 E	Coastal scrub	20
	8	Kuaotunu	36°430 N, 175°450 E	Coastal hill scrub	150
	9	Whenuakite	36°570 N, 175°480 E	Hill scrub	100-200
	10	Hikuai	37°060 N, 175°470 E	Coastal hill scrub	20-200
Waikato	11	Meremere	37°200 N, 175°100 E	Lowland swamp	20
	12	Whangamarino	37°200 N, 175°100 E	Lowland swamp	20
	13	Te Kauwhata	37°230 N, 175°80 E	Lowland swamp	20
	14	Orini	37°340 N, 175°160 E	Lowland swamp	20
	15	Torehape	37°200 N, 175°280 E	Lowland swamp	20
Central North Island	16	Rotorua	38°270 N, 176°030 E	Highland scrub	500
	17	Rangipo	39°120 N, 175°450 E	Montane scrub	900
	18	Raetihi	39°230 N, 175°150 E	Highland scrub	600
	19	Kuripapango	39°250 N, 176°200 E	Montane scrub	800
	20	Esk Valley	39°160 N, 176°420 E	Hill scrub	400
East Coast	21	Ruatoria	37°520 N, 178°120 E	Hill scrub	120
	22	Te Araroa	37°390 N, 178°200 E	Coastal scrub	10
	23	Hicks Bay	37°340 N, 178°190 E	Coastal hill scrub	100
	24	Whangaparoa	37°350 N, 178°020 E	Coastal scrub	20
	25	Whanarua Bay	37°410 N, 177°470 E	Coastal hill scrub	100

[a]data supplied by Mr. Jon Stephens, Department of Biological Sciences, The University of Waikato.

8.2.2. Honey Samples

Twenty honey samples (2003 Season) obtained from beekeepers with beehives kept in the North Island (New Zealand) region where the plant leaves were collected

were provided by Mr. Jon Stephens (The University of Waikato). GC/MS data for these samples are given in Section 7.5.

8.2.3. Extraction and Analyses of Leaf Samples

An adaptation of the micro-scale technique developed by Brophy *et al*. (1989) was used to characterize leaf oil constituents of the oils from *L. scoparium* leaves. Leaves (20-40) were soaked in ethanol:dichloromethane (1:1) (~ 2 mL) in glass vials (18-20 h) at room temperature. Two mL portions of the extracts were transferred to GC vials and analysed using the GC/MS method described below.

GC/MS Analyses

GC/MS analyses were performed using He as the carrier gas (column inlet pressure 16 psi; carrier gas flow, 1.5 mL/min) and a 30 m x 0.25 mm id ZB-5 column (Phenomex) installed in a HP6890 (Hewlett Packard) GC coupled to a HP5973 mass selective detector (MSD). The GC injector, MS interface and MS ion source were maintained at 250 ^{o}C, 280 ^{o}C and 200 ^{o}C respectively. The mass spectral data were acquired in total ion chromatrogram (TIC) mode, scanning the range *m*/*z* 42 – 500 Daltons. Aliquots of the extractive solutions (typically 1-2 µL) were injected with using a HP7683 auto-sampler and the Grob split/splitless technique (splitless time = 6 sec). The GC/MS oven temperature was programmed from 45 ^{o}C (20 sec hold) to 65 ^{o}C at 20 ^{o}C/min and then to 285 ^{o}C at 6 ^{o}C/min. The final temperature was maintained for 10 min to elute flavonoids, wax hydrocarbons and other higher boiling point components from the column. Compounds were identified using a combination of retention time and mass spectral data determined for manuka oils previously characterized in our laboratory (Porter and Wilkins, 1998). Integration of peaks was performed using Hewlett Packard ChemStation software (G1701BA Version B.01.00).

8.2.4. % TIC Calculations

The leaf oil chemotypes were distinguished by the % contribution of selected classes of essential oil components, rather than considering the contributions of a complex series of individual compounds. Integrated peak areas for classes of compounds, expressed as % total ion current (TIC) contributions, were calculated as follows:

$$\%\ \text{group A contribution} = \left(\frac{\text{peak area sum (group A)}}{\text{total peak area sum (groups A - F)}} \right) \times 100$$

where: peak area sum (group A) = peak area sum for class A compounds

total peak area (A - F) = total peak area sum for all groups

8.2.5. Statistical Analyses

Data were assumed to be normally distributed and statistical analyses were performed after logarithmic transformation of data. Cluster analyses were performed Mr. Jon Stephens (The University of Waikato) using StatSoft Statistica Version 6 software.

8.3. Results and Discussions

8.3.1. Characterization of Leaf Oil Classes Using Brophy's Technique

Leaf oil components were characterized using an adaptation of the micro-scale leaf extraction and GD/FID or GC/MS analysis technique developed by Brophy *et al*. (1989). This technique has, in the case of tea tree leaves, been found to afford a GC/FID or GC/MS profile that closely matches the profile of commercial steam distilled oils.

Six major groups of volatile (steam distillable) compounds; namely

Group A: monoterpenes

Group B: sesquiterpene hydrocarbons

Group C: oxygenated sesquiterpenes excluding eudesmols

Group D: eudesmols

Group E: triketones

Group F: nor-triketones

were recognized in the leaf oil components, based on GC peak retention time windows and the presence or absence of specific compounds (Table 8.2). Although trace levels of cinnamic acid were also found in some of leaf extracts, this compound was not considered to be a significant component of the extracts since the peak area of this compound was negligible compared to other groups of compounds.

In addition, the solvent extraction technique also recovered a range of higher molecular weight, non-volatile compounds including flavonoids and the $-CH_2CH_2-C_6H_5$ analogues of the triketones and nor-triketones which have M_r = 314 and 300 Daltons respectively. These compounds are however not found in commercial steam distilled manuka oils due to the non-volatile nature of these components.

The GC/MS retention time windows and the groups of principal components of the extractable volatile and semi-volatile compounds identified in this investigation are presented in Table 8.2. The GC/MS profile of a representative manuka leaf sample extract is presented in Figure 8.1.

The proportions of monoterpenes, sesquiterpenes, oxy-sesquiterpenes, eudesmols, triketones, nor-triketones, flavonoids, phenyl triketone and phenyl nor-triketone were calculated as % of TIC (see Section 8.2.4). The average % contributions for these classes of compounds for 5 independent collections of leaf material from each site are given Table 8.3.

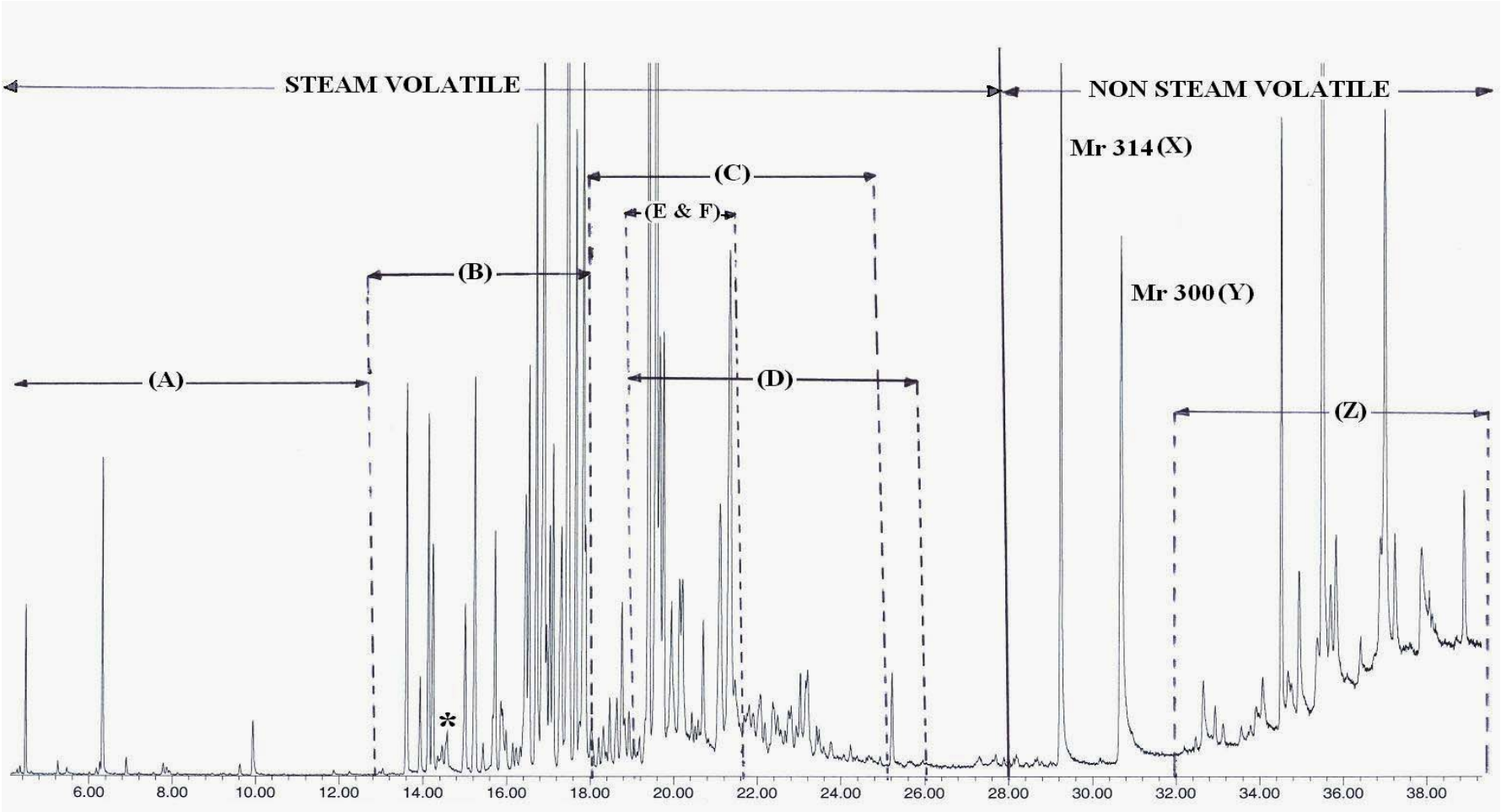


Figure 8.1. The GC/MS profile of a representative manuka leaf sample extract. Compound class identifications are given in Table 8.2. * = cinnamic acid.

Table 8.2. Major components of classes of compounds identified in extracts of *L. scoparium* leaves and their (approximate) GC/MS time windows.

Group	group	major constituents	retention time (min)
A[a]	monoterpenes	α-pinene, β-pinene, myrcene	3.0-13.6
B	sesquiterpenes	α-ylangene, α-copaene, β-caryophyllene, α-humulene, α-muurolene	13.7-17.8
C	oxygenated sesquiterpenes[b]	β-nerolidol, spathulenol, caryophyllene--epoxide, virifloral, ledol	17.9-25.0
D	eudesmols	α-eudesmol, β-eudesmol, γ-eudesmol	17.9-26.0
E	triketones	leptospermone, isoleptospermone, flavesone	17.9, 19.5, 19.7
F	nor-triketones	nor-leptospermone, nor-isoleptospermone, nor-flavesone	19.8, 21.3 21.5
(X)[c]	phenyl-triketone	grandiflorone (M_r 314)	30.8
(Y)	phenyl nor-triketone	nor-grandiflorone (M_r 300)	32.3
(Z)	flavonoids	flavonoids	32-40

[a]A-F steam volatile compounds; [b]excluding eudesmols; [c](X), (Y) and (Z) non-steam volatile compounds.

8.3.2. Groups A-D (Mono and Sesquiterpenes)

Group A compounds consisted principally of monoterpene hydrocarbons such as α- and β-pinene, myrcene and some oxygenated analogues (*e.g.* α-terpineol and 1,8-cineole). Group B was comprised of sesquiterpene hydrocarbons, including α-ylangene, α-copaene, β-caryophyllene, α-humulene and α-muurolene while group C was comprised of oxygenated sesquiterpenes, including β-nerolidol, spathulenol, caryophyllene epoxide**,** viridiflorol**,** ledol, but excluding eudesmol isomers which classified as group D compounds.

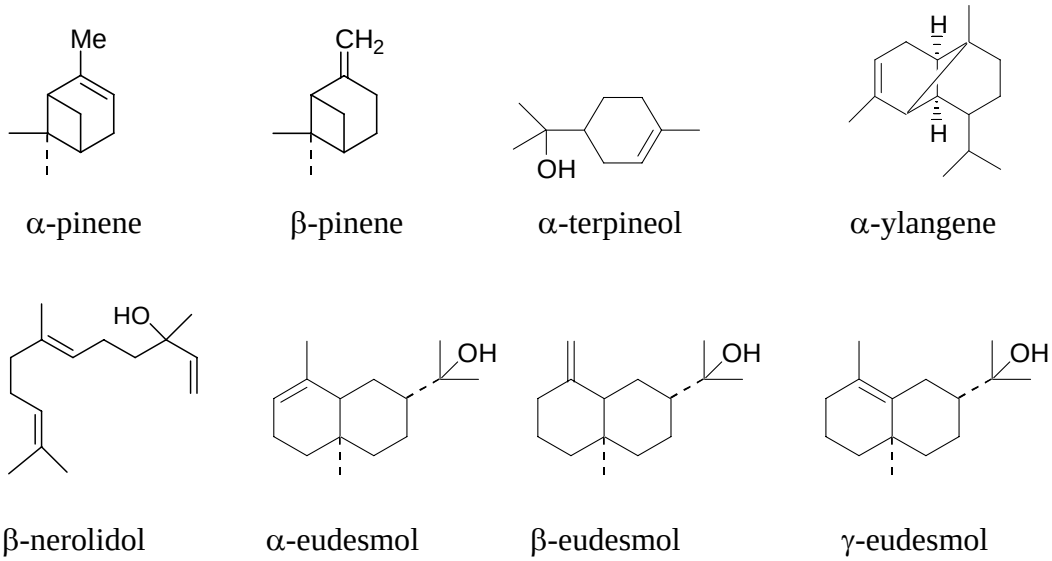


Figure 8.2. Chemical structures of some mono and sesquiterpenes identified in manuka leaf extracts.

The distribution in the extracts of eudesmols (principally α-, β- and γ-isomers) was of particular interest since in previous studies these sesquiterpene alcohols were often present in oils produced from manuka plants growing in specific geographic locations (*e.g.* Northland and Nelson districts) (Porter and Wilkins, 1998).

Table 8. 3. % Contributions of selected classes of compounds identified in micro-scale manuka leaf extracts.

			classes of compounds[a]									
site	region	n	%A[a]	%B	%C	%D	%E	%F	%X	%Y	%Z	%cinnamic acid
Ruatoria	East Coast	5	0.6	36	22	0	27	0	15	0	0	tr
Te Araroa	East Coast	5	0.2	32	25	0	28	0	14	0.3	0	-
Hicks Bay	East Coast	5	0.5	30	25	0.1	26	tr	17	1.6	0	-
Whangaparoa	East Coast	5	0.5	29	22	0	24	1.8	14	8.4	0	tr
Whanarua Bay	East Coast	5	0.2	32	17	0	24	tr	22	4.3	0	-
Omanaia	Northland	5	3.7	24	14	11	0	0	5.6	26	16	tr
Takehe	Northland	5	6.6	25	13	22	0	0	0	6.2	27	-
Ngawha	Northland	5	6.2	29	14	8.6	0	0	7.8	8	26	tr
Maromaku	Northland	5	11	20	11	22	0	0	0	5.8	31	tr
Kawakawa	Northland	5	15	18	15	8.2	0	0.1	0	6.6	36	-
Rangipo	Central	5	0.3	35	21	0	1.3	19	0	2.6	22	tr
Raetihi	Central	5	1.1	33	23	0	0	22	0	2.5	18	tr
Rotorua	Central	5	0.3	25	12	3.3	0.4	15	0.1	11	34	-
Kuripapango	Central	5	1	30	22	3.1	0	13	0	4.3	27	tr
Esk Valley	Central	5	1.5	22	21	4.2	0.1	10	0	1.8	39	tr
Waikawau	Coromandel	5	1.8	32	16	0	16	5.4	5.1	6.5	17	-
Colville	Coromandel	5	1.8	31	20	0.3	4.2	8.9	1.9	15	17	-
Kuaotunu	Coromandel	5	1.3	30	21	10	7.3	10	1.8	8	17	tr
Whenuakite	Coromandel	5	1	24	22	7.6	0.2	5.3	0	7.4	32	tr
Hikuai	Coromandel	5	2.1	33	25	13	0	5.7	0	5.3	18	tr
Meremere	Waikato	5	1.9	22	28	20	0	0	0	0.5	27	tr
Whangamarino	Waikato	5	2.2	16	24	22	0	0	0	1.1	35	tr
Te Kauwhata	Waikato	5	1.8	16	27	22	0	0	0	0.6	33	tr
Orini	Waikato	5	2	18	34	20	0	0	0	0	26	tr
Torehape	Waikato	5	2.5	16	30	17	0	0	0	0	35	tr

[a]A = monoterpenes; B = sesquiterpenes; C = oxygenated sesquiterpenes; D = eudesmols; E = triketones; F = nor-triketones; X = phenyl-triketone; Y = phenyl nor-triketone; Z = flavonoids.

8.3.3. Groups E and F (Triketones and Nor-triketones)

The detection of three of the triketones, leptospermone (**1**), isoleptospermone (**2**) and flavesone (**3**), and three nor-triketone compounds in some of the leaf oil extracts was unexpected. The three nor-triketones with molecular weights of 252, 252 and 238 Daltons respectively are reported here for the first time here from New Zealand *L. scoparium* essential oil extracts.

The nor-triketones, were designated as nor-leptospermone (**4**), nor-isoleptospermone (**5**) and nor-flavesone (**6**) since mass spectral fragmentation data indicated that that they were the nor-methyl analogues of leptospermone (**1**), isoleptospermone (**2**) and flavesone (**3**) respectively. The mass spectra of the triketones and nor-triketones are shown in Figure 8.4.

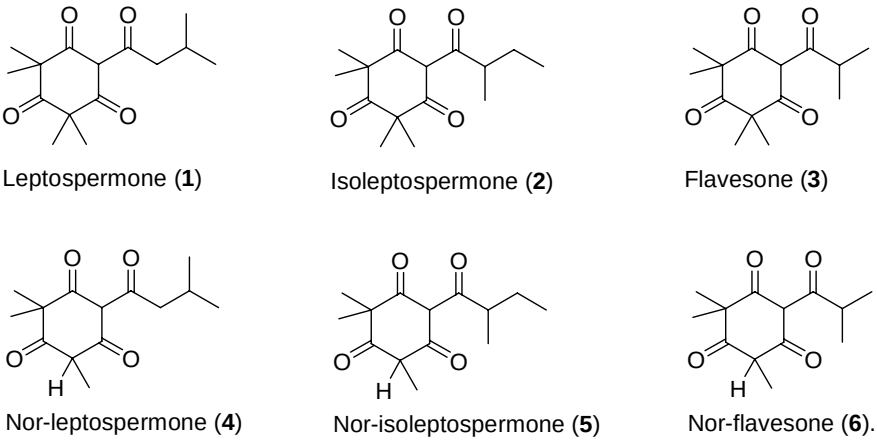


Figure 8.3. Chemical structures of leptospermone (**1**), isoleptospermone (**2**), flavesone (**3**), nor-leptospermone (**4)**, nor-isoleptospermone (**5)** and nor-flavesone (**6**).

The nor-triketones (**4**, **5** and **6)** were eluted from the GC column after the corresponding triketone peaks (**1**, **2** and **3**), presumably because in the solution and the vapour phase of the triketones are present in tautomeric (interconverting) triketo-enol forms, whereas the nor-triketones are present in tautomeric diketo-diene-diol forms, as illustrated in (Figure 8.5) for leptospermone and iso-leptospermone.

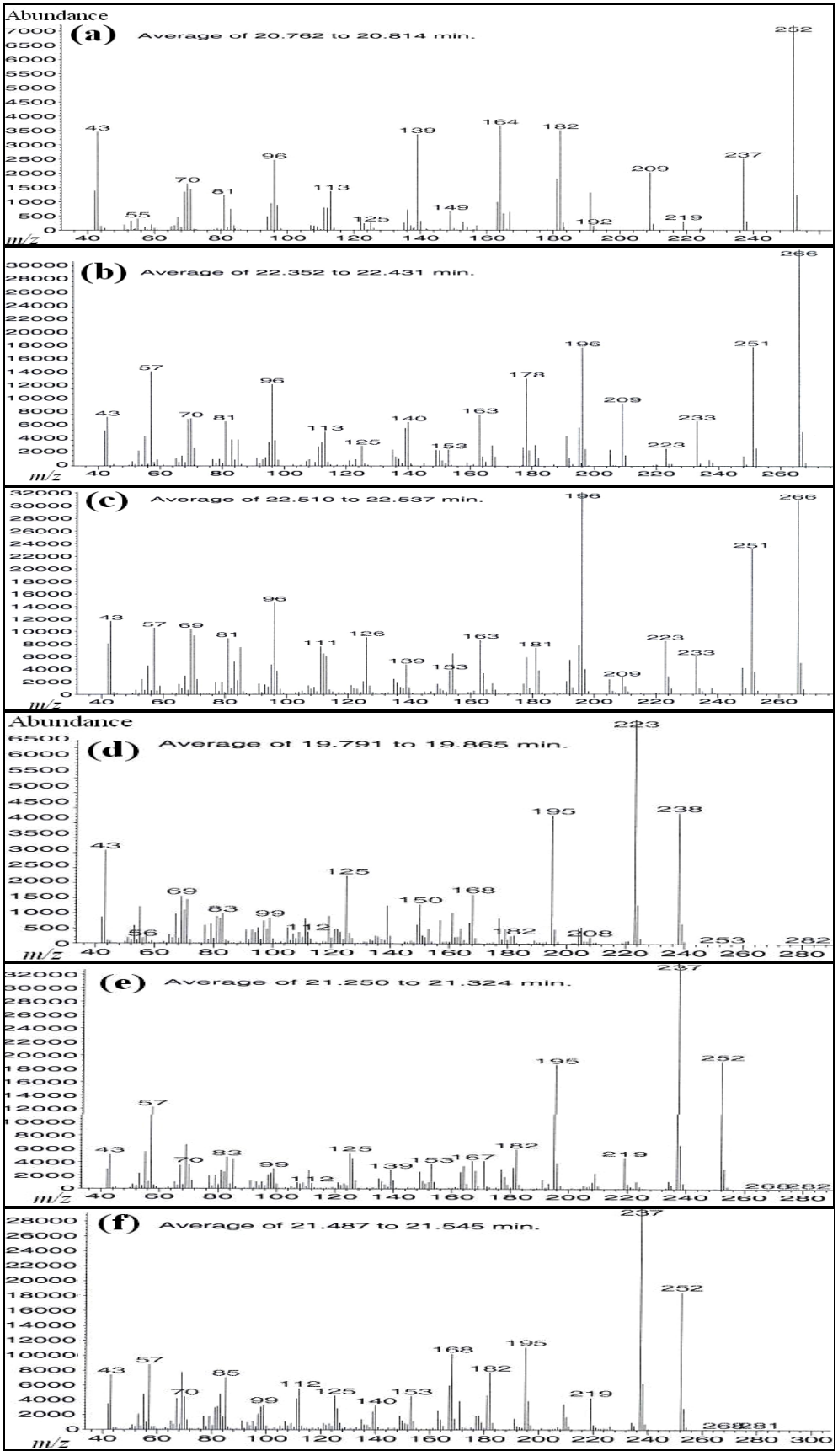


Figure 8.4. Mass spectra of (**a**) leptospermone, (**b**) isoleptospermone, (**c**) flavesone, (**d**) nor-leptospermone, (**e**) nor-isoleptospermone and (**f**), nor-flavesone.

Diketo-dienol analogues would be expected to elute from a GC column later than the corresponding triketo-enol analogues, due to their increased polarity and presumably also higher boiling points.

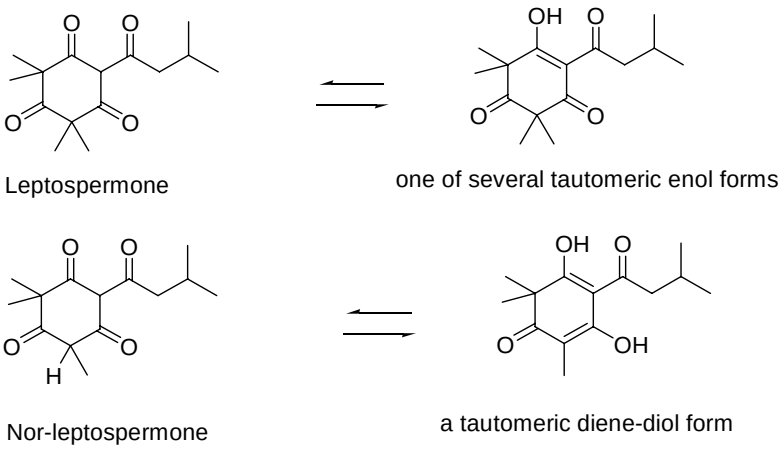


Figure 8.5. A tautomeric enol forms of leptosperomone and nor-leptosperomone.

The presence of triketones in *Myrtaceae* and *Eucalyptus* species has been reported previously (Hellyer *et al*., 1968; Ghisalberti *et al*., 1996; Van Klink *et al*., 1999). The synthesis of triketones has been achieved by C-methylation of phloroglucinol (Bick *et al*., 1965). Perry *et al*. (1997) has reported the synthesis of **1**, **2** and **3**, and like Hellyer *et al.* (1966), has showed that enol tauomers are the dominant solution forms of these compounds.

Since the biosynthetic route to triketones involves the successive addition of four methyl groups to an aromatic precursor (Bick *et al*., 1965), it can be hypothesised that the nor-triketones arise as a result of a genetically regulated blockage of the 4th methylation step.

8.3.4. Groups X and Y (Grandiflorone and Nor-Grandiflorone)

The phenyl analogues of the foregoing triketones and nor-triketones were also detected in the leaf extracts. Grandiflorone (M_r = 314 Daltons, $C_{19}H_{22}O_4$) (**7**) has been detected previously as a minor component of some in New Zealand manuka oils (Porter and Wilkins, pers commun) and in extracts of some Australian plants (Hellyer *et al*., 1966). The nor-analogue of grandiflorone (M_r = 300 Daltons, $C_{18}H_{20}O_4$) (**8**) (designated in this thesis as nor-grandiflorone) was also detected in extracts which

contained nor-triketones. The mass spectra of these compounds are depicted in Figure 8.7.

Grandiflorone (**7**) Nor-grandiflorone (**8**)

Figure 8.6. Chemical structures of grandiflorone (**7**) and nor-grandiflorone (**8**).

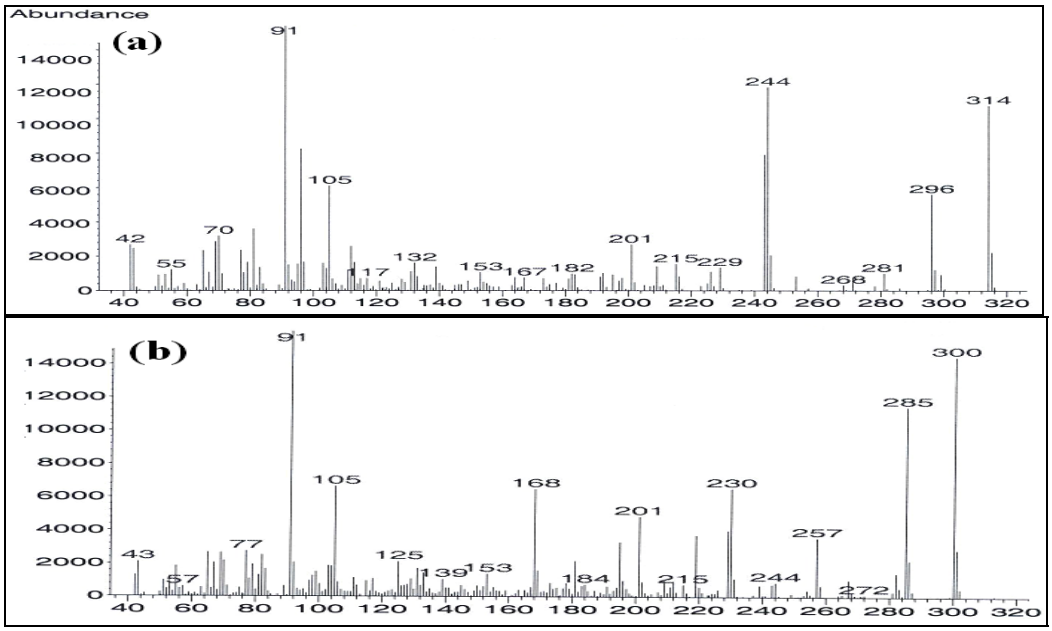


Figure 8.7. The mass spectra of grandiflorone (M_r = 314) and nor-grandiflorone (M_r = 300).

Grandiflorone and nor-grandiflorone, while readily detectable in solvent extracts, are rarely found in steam distilled essential oils due to their low volatility. They were therefore not included in the % TIC contribution of leaf oil compounds presented below.

8.3.5. Group Z (Flavonoids)

Unlike steam distillation techniques, the Brophy micro-scale leaf extraction technique also recovers a range of relatively polar semi-volatile compounds including flavonoid (see Figure 8.1) and fatty acids. Since flavonoids are not essential oil constituents they were excluded from the % TIC contribution of leaf oil compounds presented below.

Häberlein *et al.* (1994) identified a new methylated flavonoid as 5,7-dimethoxy-6-methylflavone in the dichloromethane extract of manuka (*L. scoparium*) leaf samples. In a recent study, Häberlein *et al*. (1998) also found different compositions and amounts of methylated and methoxylated flavonoids (*e.g.* 5,7-dimethoxyflavone, 5-hydroxy-7-methoxy-6-methylflavone, and 5-hydroxy-7-methoxy-6,8-dimethylflavan-3-one) in the dichloromethane extracts of manuka (*L. scoparium*) leaf samples collected from Auckland, Coromandel, Whangaruru North, and Rawhiti regions of New Zealand. They reported that these methylated and methoxylated flavonoids possess a high level of pharmacological activity.

8.4. Cluster Analysis

Since compounds observed in commercial steam distilled manuka oils are comprised essentially of monoterpenes, sesquiterpenes, oxy-sesquiterpenes, eudesmols, triketones and possibly nor-triketones (if suitable plant material is utilised commercially in the future) only groupings of these compounds (Groups A-F, see Section 8.3.1) were considered in the cluster analysis presented below. As noted in Section 8.3.1, flavonoid, grandiflorone and nor-grandiflorone were excluded for cluster analyses since they are not typically present in commercial steam distilled *L. scoparium* oils.

Consideration of the TIC contributions of the Group A-F compounds only (Table 8.3) showed that while generally similar levels of sesquiterpenes hydrocarbons (~ 40 % TIC) and oxygenated sequiterpenes (~ 30% TIC) and low to moderate levels of monoterpenes were present in all of the leaf extracts, there were appreciable variations in the levels of eudesmols, triketones, and nor-triketones.

This leads to the formulation of three chemotype classifications (Table 8.4) based on considerations the distribution of eudesmols, triketones, and nor-triketones (putting aside variations in monoterpenes and sesquiterpenes other than eudesmols) namely

Type 1: eudesmol rich;

Type 2: triketone rich;

Type 3: nor-triketone rich.

Table 8.4. The UMF values for the honey samples collected from the region where the plant leaves were collected and essential oil profiles of *L. scoparium* sites, the proportion of monoterpenes, sesquiterpenes, oxy-sesquiterpenes, eudesmol, triketones, and nor-triketones are shown as % of TIC (excluding non-volatile components).

region	site	UMF[a]	%mono-terpenes	%sesqui-terpenes	%oxy-sesqui-terpenes	%eudes-mols	%triket-ones	%nor-triket-ones
Type 1: eudesmol rich								
Waikato	Meremere	15.2	2.6%	30.6%	39.3%	**27.5%**	0.0%	0.0%
Waikato	Whangamarino	-	3.4%	25.7%	37.4%	**33.5%**	0.0%	0.0%
Waikato	Te Kauwhata	-	2.7%	23.7%	40.7%	**32.9%**	0.0%	0.0%
Waikato	Orini	-	2.7%	24.4%	45.6%	**27.3%**	0.0%	0.0%
Waikato	Torehape	-	3.3%	24.1%	46.7%	**25.3%**	0.0%	0.0%
Northland	Takehe	14.1	9.9%	37.8%	19.2%	**33.1%**	0.0%	0.0%
Northland	Maromaku	14.1	16.6%	30.8%	17.8%	**34.7%**	0.0%	0.0%
Northland	Kawakawa	14.6	26.7%	32.1%	26.7%	**14.4%**	0.0%	0.2%
Northland	Omanaia	14.2	7.1%	45.1%	26.4%	**21.4%**	0.0%	0.0%
Northland	Ngawha	12.6	10.7%	50.8%	23.7%	**14.9%**	0.0%	0.0%
Type 2: triketone rich								
East Coast	Whanarua Bay	11.1	0.6%	37.9%	28.3%	0.1%	**30.8%**	2.3%
East Coast	Hicks Bay	7.4	0.6%	37.3%	30.3%	0.1%	**31.7%**	tr
East Coast	Ruatoria	13.7	0.7%	41.5%	25.7%	0.1%	**32.1%**	0.0%
East Coast	Whangaparoa	9.5	0.3%	43.5%	23.2%	0.1%	**33.0%**	tr
East Coast	Te Araroa	8.5	0.3%	37.3%	29.7%	0.0%	**32.7%**	0.0%
Coromandel	Waikawau	7.7	2.5%	44.7%	22.5%	0.0%	**22.7%**	7.6%
Type 3: nor-triketone rich								
Coromandel	Colville	10.8	2.8%	46.7%	30.2%	0.4%	6.4%	**13.5%**
Coromandel	Kuaotunu	16.1	1.6%	37.5%	26.9%	13.0%	9.1%	**11.9%**
Coromandel	Whenuakite	5.3	1.6%	39.9%	36.8%	12.6%	0.3%	**8.8%**
Coromandel	Hikuai	0.0	2.7%	41.4%	31.6%	17.1%	0.0%	**7.2%**
Central	Rotorua	-	0.5%	44.2%	21.4%	5.9%	0.7%	**27.1%**
Central	Rangipo	-	0.4%	46.0%	27.5%	0.0%	1.7%	**24.4%**
Central	Raetihi	4.6	1.3%	41.3%	29.4%	0.0%	0.0%	**27.9%**
Central	Kuripapango	4.8	1.0%	43.0%	32.0%	4.0%	0.0%	**19.0%**
Central	Esk Valley	0.0	2.0%	37.0%	36.0%	7.0%	0.0%	**17.0%**

[a]UMF values for the honey samples were measured at The University of Waikato.

These classifications should be not confused with those described by Perry *et al*. (1997) and Douglas *et al*. (2004), although there are some common features (*e.g.* a triketone rich grouping).

Since the cluster analysis detects natural groupings in data, the cluster analysis constructed from the matrix of the %TIC contributions of the Group A-F compounds

data (Figure 8.8) confirmed the validity of this analyse by verifying the existence of three principal chemotypes (Type 1, 2 and 3).

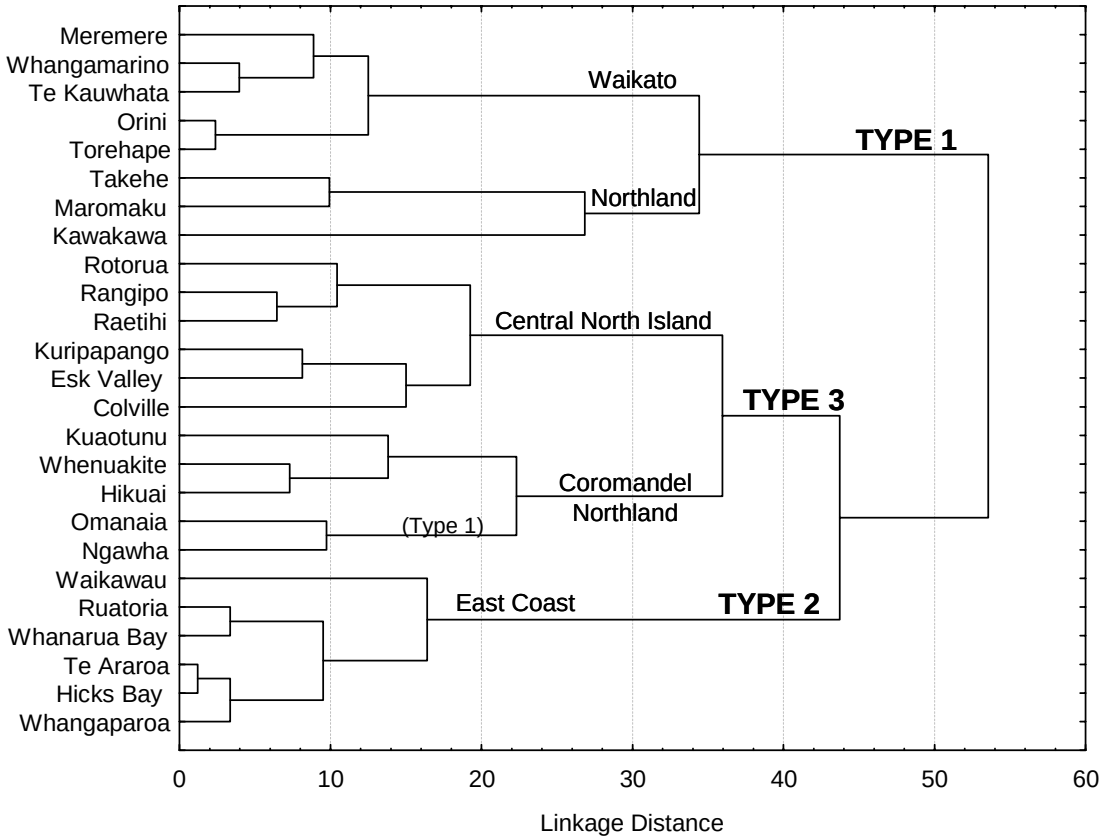
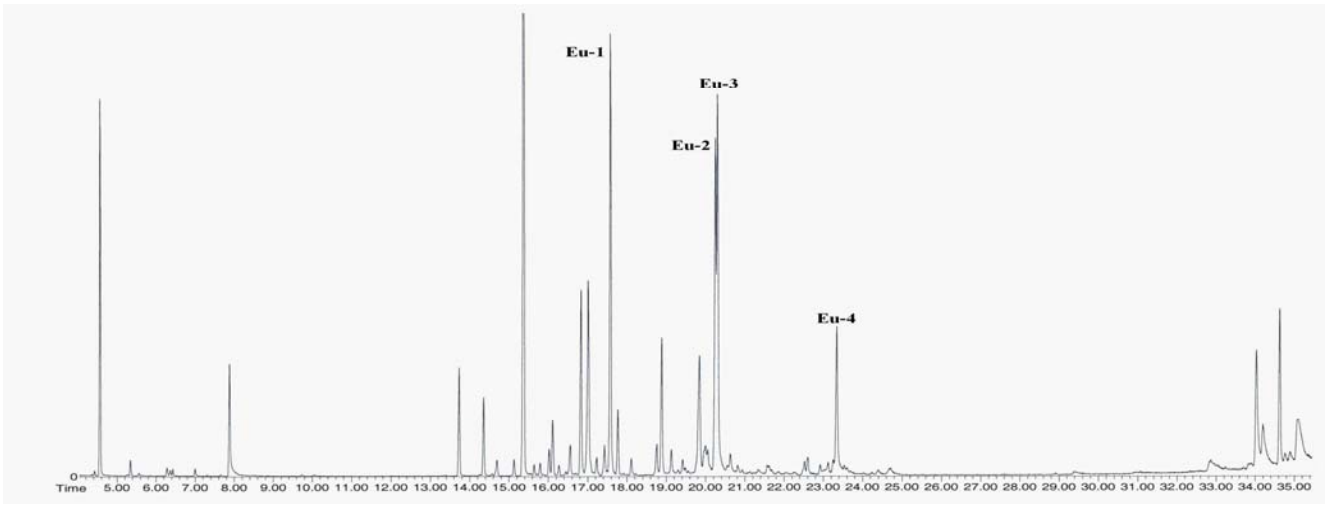


Figure 8.8. Cluster analysis for *L. scoparium* essential oil profiles, utilizing Euclidean distance and complete linkage.

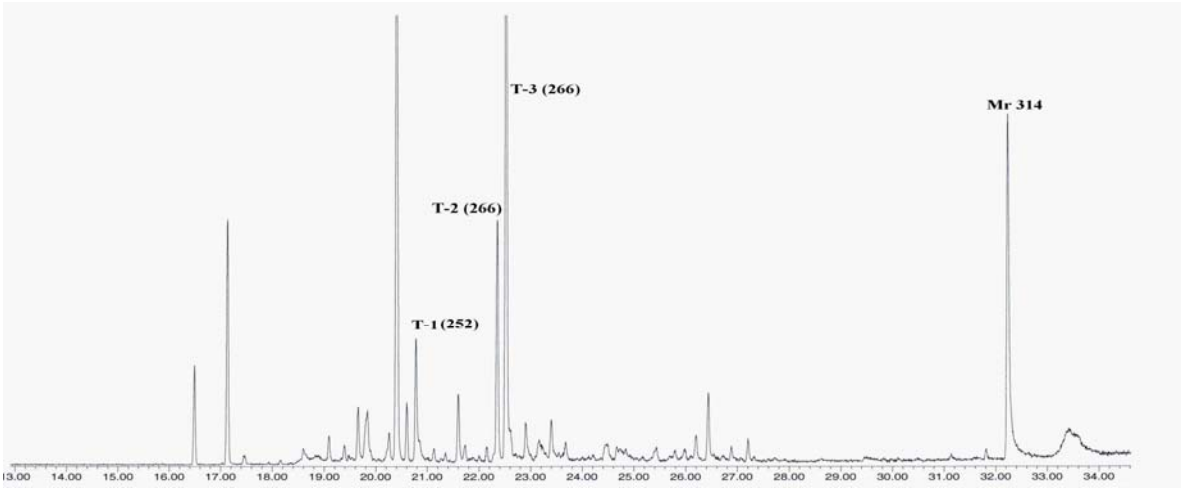
GC/MS profiles of representative leaf oil types (type 1: eudesmol rich); type 2: triketone rich; and (c) type 3: nor-triketone rich) are given in Figure 8.9.

Populations from sites showing Type 1 (eudesmol rich) profiles branched from the other sites and later split into Waikato and Northland sites. The other major branch divided into Type 2 (triketone rich) East Coast and Coromandel Waikawau populations, and the Type 3 (nor-triketone rich) contained the Central North Island and remaining four Coromandel sites. The Coromandel sites division between Type 2 and 3 patterns are driven by a preponderance of triketones in the Waikawau population, whilst the other four populations contain considerably more nor-triketones.

(**a**)



(**b**)



(**c**)

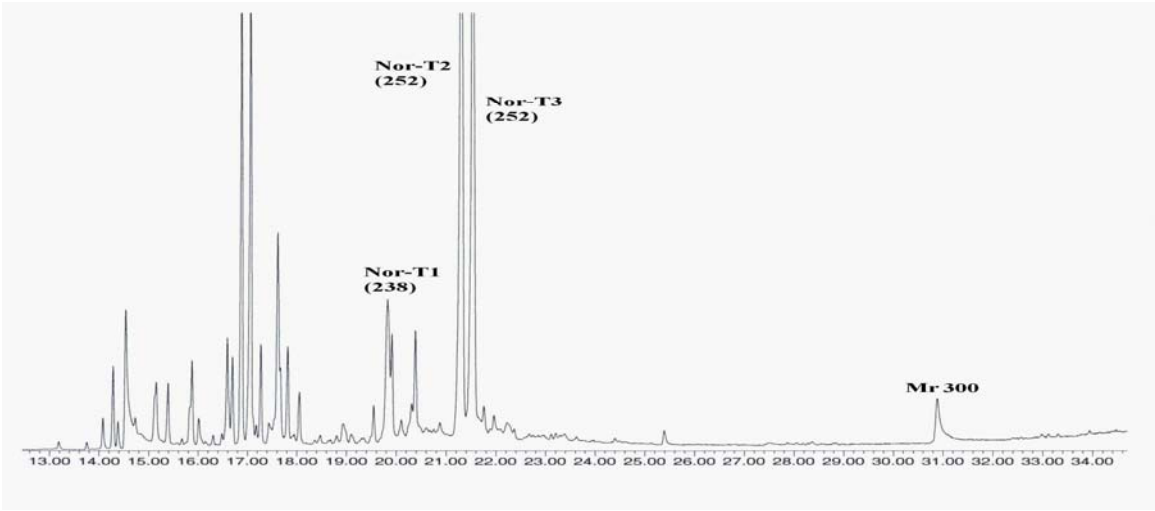


Figure 8.9. GC/MS profiles of representative leaf oil type identified from the extractives of manuka leaf samples (**a**) Type 1 (eudesmol rich, *e.g.* Northland) (**b**) Type 2 (triketone rich, *e.g.* East Coast) (**c**) Type 3 (nor-triketone rich, *e.g.* Central North Island).

8.5. Chemical Groupings and the UMF Values

A significant relationship between UMF values of the 20 honey samples, collected from the same regions where the leaf samples were collected, and the relative % TIC level of the nor-triketones recovered from the leaf samples using the Brophy micro-scale technique was observed (Figure 8.10). The Pearson's correlation coefficient determined between the UMF value of the honey samples and the % TIC level of the nor-triketones recovered from the leaf samples showed that there was a significant negative relationship between these two variables (coefficient = -0.65; $p < 0.01$).

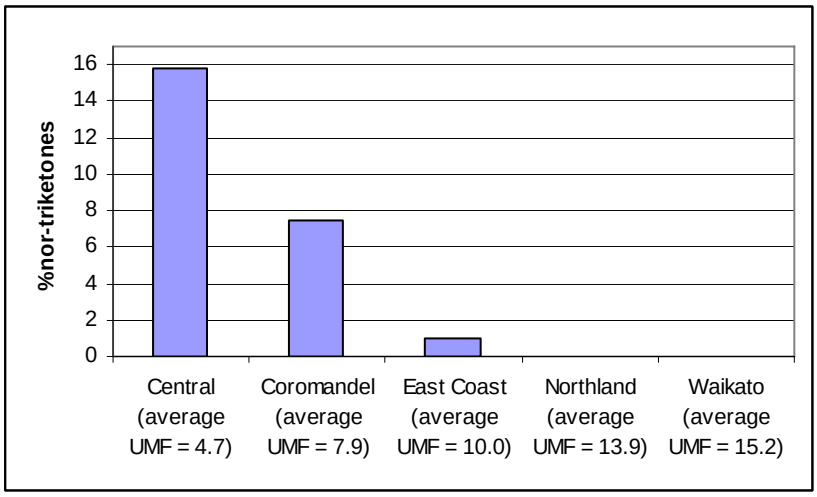


Figure 8.10. The average % TIC levels of nor-triketones recovered in micro-scale extracts for leaf samples collected from Waikato, Northland, East Coast, Coromandel and Central North Island regions. Average UMF values of the honey samples collected in each region are shown in the brackets

The Figure 8.10 shows that nor-triketone % TIC contributions might be negatively related to the UMF values determined active honeys from those regions.

The leaf samples collected from Waikato and Northland regions which afforded moderate activity honey samples (average UMF values = 15.2 and 13.9 respectively) contained elevated levels of eudesmols and did not contain triketones or nor-triketones. On the other hand, leaf samples collected from the East Coast region which afforded low or moderate activity honeys (UMF values in the range 7.4-13.7) contained elevated levels of triketones, but not eudesmols.

Variable levels nor-triketones (%TIC's of 7.2%-27.9%) were present in the leaf samples collected from Coromandel and Central North Island regions which afforded honeys which were essentially inactive, or showed low to moderate activity (see Table 8.4)

A significant constraint in interpreting the data presented in Table 8.4 is the lack of highly active samples with UMF scores >20. Notwithstanding this constraint, it appears that while UMF activity may be negatively correlated with nor-triketone levels (Figure 8.10), the UMF levels of active manuka honeys is not predictably correlated to the essential oil of manuka plants growing in a particular region.

8.6. Conclusions

Conclusions that can be drawn for data presented in this Chapter are:

(1). The leaf oil components of manuka (*L. scoparium*) plant can be characterized using an adaption of the micro-scale extraction and GC/FID or GC/MS, technique developed by Brophy *et al*. (1989).

(2). Six major groups of volatile (steam distillable) compounds (monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes (excluding eudesmols), eudesmols, triketones, and nor-triketones) and 3 groups of non-volatile or semi-volatile compounds (flavonoids, grandiflorone and nor-grandiflorone) were recognized in the leaf oil components. Four of these groupings (monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes and triketones) correspond to those found in conventional steam distilled oils.

(3). After putting aside monoterpene and sesquiterpene contributions, other than eudesmols, three chemotype patterns (eudesmol rich, triketone rich and nor-triketone rich) were found in the leaf oil extract profiles of manuka leaves collected within and between population of manuka plant throughout the North Island.

(4). The active manuka honeys do not appear to be derived uniquely, or predominantly, from a single leaf oil chemotype.

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