Indicators of Bioactivity and Floral Origin of New Zealand Honeys

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Abstract

The hypothesis that NIR might be capable of discriminating one floral source from another was explored. No prior analysis of NIR for New Zealand honeys has been reported. A visual inspection of the NIR spectra of ten New Zealand honey types indicates that beech honeydew honey is significantly different from nectar honeys. Rata honey is the most unique nectar honey with very little variability seen in the NIR spectra compared to other honey types. Both beech honeydew and rata honey can be distinguished from other floral types using Linear Discriminant analysis (LDA) on selected wavelengths. A degree of clustering within other honey types is achieved, however none of these are fully resolved.

A Partial Least Squares (PLS) model successfully classified all main New Zealand unifloral honeys with an average correct classification of 93%. 100% of all beech honeydew honeys were correctly classified with close to 100% achieved for rata, kamahi, manuka, rewarewa and clover honeys. Honeys with a clover contribution: tawari, thyme, nodding thistle and vipers' bugloss displayed reduced performance in this model with a proportion of samples misclassified as clover honey. These results indicate that the NIR spectra evaluated using a PLS model would be an effective industry classification method for the identification of New Zealand unifloral honeys with the exception of nodding thistle and vipers' bugloss honeys. A multi-technique classification model incorporating NIR classification results with conductivity, colour and sugar analysis has been proposed.

A series of compounds in manuka honey were examined in respect to UMF™ activity. The carbohydrate profiles of 38 manuka honeys of varying UMF™ activity were determined using a combination of HPLC, GC-FID and GC-MS. A method was developed to determine the proportion of nigerose, turanose, maltose and maltulose in reduced and silylated honey using the ratio of *m/z* 307 to *m/z* 308 ion responses as determined by GC-MS-SIM.

An examination of the glucose and fructose concentrations in manuka honey revealed a moderate correlation between the glucose/fructose ratio and UMF™ activity. Due to an improvement in chromatographic resolution, the peak assignment of three disaccharides (cellobiose, laminaribiose and gentibiose) differed from that of a previous investigation. Despite the retention time of palatinose being identical to the corresponding peak in honey, an examination of the mass spectra provided strong evidence to suggest that the corresponding honey disaccharide is α-1→2 linked as opposed to β-1→6 linked and that it was therefore unlikely that this peak arose from palatinose. The mono and disaccharide composition of manuka honey was evaluated with respect to the level of UMF™ activity. Linear Discriminant analysis successfully distinguished between high, moderate and low UMF™ activity honeys. Glucose was identified as the single most important compound in the discriminant model. The connection between glucose concentration and UMF™ activity was not unexpected as a significant proportion of UMF™ activity has been attributed to the presence of methyl glyoxal, a degradation product of glucose.

The existences of indicator compounds in honeys from various floral origins were examined. The extractable organic substances of five New Zealand honeys: beech honeydew honey, kamahi, pohutukawa, rata and tawari were determined by GC-MS of methylated extracts. This survey confirmed the results of a previous investigation and established ranges for marker compounds. Due to difficulties in obtaining sufficient certified unifloral honeys, previous studies on these honey types were exploratory only and not published. Statistical analysis of the extractable organic substances showed that each honey contains a unique fingerprint of compounds. Agglomerative clustering successfully separated all honeys into the correct floral group with the exception of two samples. Well separated clusters were produced in the score plot of the first and second Linear Discriminants. 4-Hydroxyphenylacetic acid, salicylic acid, indole-3-acetic acid and an unknown compound (identified by characteristic ions in the mass spectra) were identified as being the most important discriminants, all of which were present in a single floral source.

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Abbreviations

a_w	water activity
CVA	Canonical Variates Analysis
d.w.	dry weight
f	furanosyl
FID	Flame Ionisation Detector
FTIR	Fourier Transform Infrared
g	gram
g/f ratio	glucose/fructose ratio
Gal	galacto
GC	Gas Chromatography
Glc	gluco
GO	glyoxal
GSH	reduced glutathione
HMF	hydroxy methyl furfuraldehyde
HPLC	High Performance Liquid Chromatography
HPAE	High Performance Anion Exchange Chromatography
kg	kilogram
LDA	Linear Discriminant Analysis
MGO	methyl glyoxal
mL	millilitre
MIC	Minimum Inhibitory Concentration
min	minutes
mm	millimetre
MS	Mass Spectrometry
NIR	Near Infrared
NPA	Non-Peroxide Activity
p	pyranosyl
PAD	Pulsed Amperometric Detector
PCA	Principal Component Analysis
PLS	Partial Least Squares
psi	pounds per square inch
R	Statistical software package

RF	Response Factor
SIM	Selected Ion Mode
TIC	Total Ion Chromatogram
TMS	trimethylsilyl
UMF™	Unique Manuka Factor
USA	United States of America
vs	versus

Chapter 1

Introduction and Review

1.1 Honey Sources

Nectar or honeydew is collected by bees and taken back to the hive where it is processed into honey, which serves as a food for larvae. Nectar is a mixture of glucose, fructose and sucrose with small amounts of amino acids, minerals, organic acids, vitamins, aromatics and enzymes. Some nectars may also contain minor quantities of maltose, melibiose and raffinose.[1]

Ripened honey is predominantly a mixture of glucose and fructose with lesser amounts of sucrose, and maltose; a number of other oligosaccharides have also been identified in honey. The enzymes glucose oxidase, invertase, diastase and catalase are present in honey togeather with various amino acids, aromatics, diacids, degraded carotenoids, aliphatic fatty acids and hydrocarbons.

The quantity of honey produced in New Zealand and honey exports has been steadily increasing over the last five years (Table 1.1).[2] This in part can be attributed to an increase in hive numbers. New Zealand is the largest consumer of honey per capita in the world with each person consuming an average of 2 kg of honey per year.[3]

Table 1.1 New Zealand honey production[2]

Year	Honey Production (tonnes)	Honey Exports (tonnes)
2004	8888	2767
2005	9689	3631
2006	10423	4134
2007	9666	4871
2008	12375	-

The good management of hives has become crucial since the discovery of the varroa mite (*Varroa destructor*), which is an external parasite of honey bees. Adult female mites are found on adult bees and are reddish brown in colour (1.1 x 1.6 mm).[4] The mites reproduce by laying eggs in a brood cell, the juvenile mites feed on the haemolymph (blood analogue of insects) and go through two juvenile stages before becoming adults. The health of the adult bees is also compromised by mites feeding off them and leaving open wounds thus making them more susceptible to infections. If uncontrolled, varroa mite can destroy the whole colony. Since the initial discovery of the varroa mite in New Zealand in 2000, the mite has spread throughout the North Island and into the South Island as far as Canterbury.[5]

The floral origin of honey has a significant impact on the market value (Table 1.2). It is now widely accepted that some New Zealand manuka honeys exhibit significant antibacterial activity, otherwise known as Unique Manuka Factor (UMF™). Knowledge of the existence of this activity has lead to both an increase in demand, and an increased price for both active and non-active manuka honey. The bulk price for active manuka honey increases by between $0.90 and $1.25/kg per UMF™ point (UMF™ 10 is normally the lower limit). An active manuka honey of UMF™ 25 will achieve a bulk price between $27.50 - $38.50/kg. There is also an increase in demand for certified organic honeys which achieve $1.20 - $1.50/kg over non-organic honeys.

Table 1.2 Bulk honey prices for New Zealand (2006 season)[3]

Floral origin	**Bulk price ($/kg)**
Beech honeydew	2.90-3.30
Kamahi	3.25
Rewarewa	3.50-3.90
Clover	3.50-4.10
Rata	4.20
Blue borage (Vipers' bugloss)	4.25
Thyme	5.30-6.40
Manuka (non active)	5.00-8.30

1.2 Floral Source Identification Techniques

The accurate identification of floral origin is important in order to guarantee a level of predictability in organoleptic properties for the consumer. The inaccurate labelling of honey produced from the mixing of honey varieties either in the hive or subsequent to extraction is in violation of the Codex Alimentarius.

Fraudulent honey is produced either by the deliberate incorrect labelling of monofloral honey or the blending of high with low value honeys to increase profit. In New Zealand the highest value honey is manuka honey. Manuka nectar flow can occur at the same time as rewarewa and clover and consequently will often have an element of rewarewa and clover floral origin. As it is not yet mandatory for marketers in New Zealand to verify the floral origin of their honey; honey which would not otherwise be accepted as being truly unifloral may be sold as such.

The international standards for honey are laid out in the Codex Alimentarius to which New Zealand is an active signatory. According to the Codex Standard for Honey, honey may be designated according to floral or plant source if it comes wholly or mainly from a particular source and has the organoleptic, physiochemical and microscopic properties corresponding with that origin.[6] The main organoleptic property is colour which is measured on a Phund Grader. Physiochemical properties include conductivity and analysis of sugar content. Microscopic properties refer to pollen analysis of honey which is the principal technique in floral source identification. Unifloral honey produced in New Zealand display a diverse range of organoleptic and physiochemical properties unique to each nectar source. The general properties of the most common New Zealand honey types are listed in Table 1.3.

Table 1.3 Characteristics of common New Zealand honeys[2]

Honey	Geographical Location	Nectar production	Pollen[7]	Colour (mm)	Conductivity (ohms/cm x 10^{-4})	Carbohydrate
Clover	Pasture throughout NZ, particularly Canterbury, Otago and Southland	Nov - Feb, main flow often lasts only a few days	>50% clover, may contain lotus, manuka, nodding thistle or vipers' bugloss	Graded extra light: 0-9, light: 10-19, medium: 20-34, dark: 35+	Low (<1.5)	
Beech honeydew	Beech forests, West coast SI		May contain some clover, manuka, matagouri. Spores from sooty mould often present	Dark, 87 ± 11	High (12.6 ± 2.5) (10 x normal honey)	High oligosaccharides, very slow crystallising
Kamahi	Regenerating and virgin bush from Thames southwards	Nov - Jan	>60% kamahi as well as manuka, rata, clover, lotus, quintinia or willow	Yellow hue, 39 ± 9	Low (<1.5)	Low fructose, fast crystallising
Manuka (kanuka)	Lowland scrub in both islands	Sept - Feb	High manuka pollen content (>70%), lotus, clover, kamahi or vipers' bugloss may also be present	Dark, 84 ± 12	Higher than normal (5.8 ± 1.5)	
Nodding thistle	Canterbury, Otago and Hawkes bay	Late summer	Extremely low nodding thistle pollen content (usually <10%, see Section 1.2.1), clover common	Very light, 17 ± 10	Low (<1.5)	High sucrose, slow crystallising
Rata	Northern rata found in lowland and sub alpine forests as far south as Greymouth, southern rata (main source) common south of Greymouth	Northern rata: Nov - Jan. Southern rata Dec - April	>45% Rata pollen, kamahi and quintinia most predominant secondary pollen source. Some clover, lotus or manuka	Very light, 24 ± 18	Low (<1.5)	High glucose, fast crystallising, very low oligosaccharides
Rewarewa	Lowland forests from Northland to Marlborough	Nov - Jan	Low rewarewa pollen content (>10%), moderate levels of lotus, clover or kamahi pollen	Amber (dark) with red brown tint, 93 ± 10	Low (<1.5)	Slow crystallising
Tawari	Forests from Northland to Waikato	Oct - Jan	Low tawari pollen, flowers same time as manuka and rewarewa. May contain moderate quantities of lotus, clover or kamahi	Light, 35 ± 14	Low (<1.5)	High fructose
Thyme	Central Otago	Mid Oct - Nov	Low pollen (>20%) with often substantial clover, matagouri, vipers' bugloss or kamahi	Dark, 75 ± 24	Low (<1.5)	
Vipers' bugloss	Dry areas in South Island, common in Marlborough and Otago	Dec - March	>45% vipers' bugloss with minor clover, matagouri, lotus or manuka	Light with brown tint, 25 ± 9,	Low (<1.5)	High fructose, slow crystallising

1.2.1 Pollen Analysis

Pollen grains are introduced into honey by either falling from the flower into nectar which bees then collect or by subsequent contamination during the extraction process when pollen in the frames are removed with honey. The quantity of pollen grains in the nectar is affected by the structure of the flower resulting in some honeys being under or over represented in pollen. Some flowers have anthers which are separated by some distance from the nectaries. Consequently pollen grains on the anthers are only moderately distributed by bees when collecting nectar and therefore pollen grains are under represented in honey from such flowers.

A detailed analysis of the pollen content of New Zealand honeys has been reported by Moar.[7] The methods and procedures described by Moar form the basis for commercial pollen analysis of New Zealand honeys.

Most New Zealand honeys are normally represented in terms of pollen content and contain 20,000 - 100,000 grains of pollen in a 10 g sample.[7] The frequency of pollen found in each contributing nectar source must be taken into account when determining the predominant floral origin. Manuka honey contains over 100,000 grains of pollen and is therefore over represented in pollen and requires a minimum frequency of 70% manuka pollen. There are two dominant *Leptospermum* varieties in New Zealand, *Leptospermum scoparum* (manuka) and *Kunzea ericoides* (kanuka). The pollen from manuka and kanuka can not be distinguished[7] hence the majority of kanuka honey is sold as manuka. No distinction appears to exist between the pollen content (grain appearance or frequency) of active and non-active UMF™ manuka honeys.

Rewarewa honey is under represented in pollen (as well as nodding thistle and tawari honey), and requires a minimum frequency of 10% rewarewa pollen.[7] Other nectar sources such as manuka are often found growing in the same areas as rewarewa and consequently rewarewa honey often contains a large proportion of other pollen types. For reasons such as this, it is imperative that organoleptic and physiochemical properties are taken into account when determining the floral source of honey.

1.2.2 Colour

The colour of honey is measured using a Pfund grader. The scale is a metric ruler measuring the point along a calibrated amber glass wedge where the sample matches the amber wedge. The colour of the honey is affected by the proportion of honey from a particular floral source. If a typically light coloured honey such as rata contains a small proportion of a dark coloured honey, the colour of the final product may not be typical of rata honeys.

1.2.3 Conductivity

The conductivity is an indirect way of measuring the mineral content of honey. Most floral honeys have a conductivity less than 1.5 ohms/cm x 10^{-4} while honeydew honey has a conductivity greater than 8.5 ohms/cm x 10^{-4}.[2] Honeydew honey has a higher mineral content which is directly related to conductivity compared to floral honeys as it is sourced from sap excreted by insects on trees.[8] Manuka honey has an intermediary conductivity of 5.8 ohms/cm x 10^{-4}. This is most likely due to a contribution of honeydew produced by the scale insect *Eriococcus orariensis* which is found on manuka.[2]

1.2.4 Sugar Content

The relative proportions of glucose and fructose can be used to distinguish between some floral sources including rata honey which has a characteristically high glucose content.[2] The adulteration of honey can also be detected by the analysis of sugar content.

1.2.5 Chemical Methods

Numerous studies have revealed compounds which can be attributed to a particular honey source. Early studies on honey composition focused on the main components of honey which were present in all honeys; however the development of GC methods in particular enabled the rapid analysis of minor honey constituents. An investigation of the volatile components in several unifloral Australian honeys[9] lead to a series of papers on the extractable organic substances in New Zealand honeys.[10-16] A summary of the characteristic compounds found in New Zealand honeys is given in Section 5.1.

The development of improved extraction techniques combined with more sensitive detectors has enabled HPLC methods to feature along with GC techniques in the identification of floral markers. A summary of floral markers found in various honeys is given in Table 1.4.

Table 1.4 Proposed floral markers in unifloral honey

Honey	Country of Origin	Proposed Floral Marker(s)	Concentration (mg/kg)	Method
Acacia (*Robinia pseudacacia*)[17]	Italy, Slovakia	kaempferol glycosides	1 - 8	HPLC-DAD-MS-MS
Almond tree (*Prunus dulcis*)[18]	Spain	2,6,6-trimethyl-2,4-cycloheptadien-1-one	64[a]	GC-MS
Avocado (*Persea americana*)[18]	Spain	perseitol	0.0075	GC-MS
Caraway (*Carum carvi.* spp.)[19]	Lithuania	vitexin	41.9	HPLC-DAD-MS
Chestnut (*Castanea sativa*)[20]	France, Italy	1-phenylethanol 2-aminoacetophenone	0.09 - 0.22 0.15 - 0.54	GC-FID, GC-MS
Citrus[21]	Spain	methyl anthranilate hesperetin	1.4 - 3.6 0.28 - 0.84	GC HPLC
Citrus[22]	Spain	sinensal (*isomer I*) sinensal (*isomer II*)	0.0673 - 0.252 0.118 - 0.391	SDE-GC-MS
Eucalyptus (*Eucalyptus* spp.)[23]	Australia	gallic acid	3.4 - 66.2	HPLC
Eucalyptus (*Eucalyptus* spp.)[24]	Spain	2-hydroxy-5-methyl-3-hexanone 3-hydroxy-5-methyl-2-hexanone	0.25 - 10[a]	SPME-GC-MS
Lime (*Tilia* spp.)[19]	Lithuania	vitexin	102.7	HPLC-DAD-MS

Honey	Country of Origin	Proposed Floral Marker(s)	Concentration (mg/kg)	Method
Lime (*Tilia* spp.)[20]	France	ethylmethylphenol estragole carvacrol	0.03 - 0.15 0.05 - 0.24 0.08 - 0.39	GC-FID, GC-MS
Evergreen Oak (*Quercus ilex*)[18]	Spain	quercitol	0.0036	GC-MS
Oak honeydew honey[25]	Spain	*trans*-oak lactone	0.074	SDE-GC-MS
Rosemary (*Rosmarinus officinalis*)[26]	Spain	kaempferol	0.66 - 1.18	HPLC
Strawberry tree (*Arbutus unedo*)[27]	Sardinia	homogentisic acid	197 - 540	HPLC
Strawberry tree (*Arbutus unedo* spp.)[28]	Sardinia	α-isophorone β-isophorone 4-oxoisophorone	all 3 compounds must be present	DHS-GC-MS
Strawberry tree (*Arbutus unedo*)[18]	Spain	isophorone	76 - 81[a]	GC-MS
Thyme (*Lamiaceae* spp.)[29]	Turkey	3,4,5-trimethoxybenzaldehyde	2.86[a]	SPME-GC-MS
Willow (*Salix* spp.)[18]	Spain	methyl salycilate	11[a]	GC-MS
Willow (*Salix alba* spp., *Salix caprea* spp.)[19]	Lithuania	hyperoside	0.74 - 1.79	HPLC-DAD-MS

[a] = % of volatiles, DAD = diode array detector, DHS = dynamic headspace, SDE = simultaneous distillation extraction, SPME = solid phase micro extraction

Despite the growing number of studies conducted in this field, chemical markers are not yet used in the industry to aid in the determination of floral origin. Some honeys such as clover contain very low levels of volatile substances and are devoid of any obvious marker compounds.[10] This means that the presence of a marker compound can only confirm the presence of a floral source and cannot rule out a significant contribution of another nectar source.

1.3 Adulteration

Honey adulteration occurs when honey syrups are either mixed with extracted honey or fed to bees to increase honey production. There are several different methods which can detect adulteration in honey, however no single method has been developed for the rapid, cost-effective accurate detection of all forms of honey adulteration.

Pollen analysis can detect the addition of cane sugar annuli, parenchyma or starch grains. However, this is not effective if the honey has undergone ultrafiltration, a process which is becoming more common in order to make honey more visually appealing to consumers.

Carbon isotope ratios are commonly used to detect adulteration.[30] The carbon isotope ratio is dependant upon the origin of the plant. Products from the Calvin cycle of photosynthesis (C_3) produce a different carbon isotopic ratio than those from the Hatch and Slack cycle (C_4). Most honey nectar sources are from C_3 plants and detection of adulteration with corn or cane syrup (C_4) is possible at levels as low as 7%. It is very difficult to detect adulteration with C_3 syrups such as those derived from beets.

Carbohydrate profiles can be used to detect adulteration of honey with sugar syrups as the syrups contain a unique carbohydrate fingerprint (Section 3.1.2), however these methods often require specialised equipment or are very time consuming.

1.4 Antibacterial properties of honey

Honey has been used as traditional folk remedy to treat a wide variety of ailments such as sore throats, burns and dyspepsia. Over the last few years the medicinal use of honey has undergone a resurgence due to the discovery of the antibacterial properties of honey.

1.4.1 Osmotic Effect

Honey is predominantly a mixture of glucose and fructose (~80%) in water (~20%). Sugar molecules interact strongly with water molecules and as a result supersaturated solutions of sugar have very few water molecules available to microorganisms. The percentage of free water molecules is measured as the water activity (a_w) which in honey ranges from 0.56 - 0.62.[31] Inhibition by the osmotic effect is dependent on the species of bacteria. The growth of bacteria is typically inhibited by an a_w of 0.94 - 0.22 however not all bacteria are inhibited under these conditions. *Staphylococcus aureus* has a high tolerance of low a_w, complete inhibition is achieved only when the a_w is less than or equal to 0.86, equivalent to an aqueous solution containing 29% honey[32] Dilute solutions of honey may not be effective against *S. aureus* unless other antibacterial substances are present in the honey.

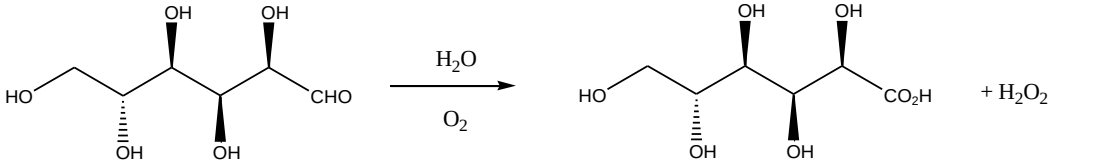
1.4.2 Acidity

Honey is characteristically quite acidic with the pH ranging from 3.42 - 6.10 with an average of 3.91.[33] This acidity is due to gluconolactone/gluconic acid produced enzymatically during the ripening of the honey. Inhibition of some common wound infecting bacteria such as *Escherichia coli*, *Salmonella* spp., *Pseudomonas aeruginosa*, and *Streptococcus pyogenes* occurs between pH 4.0 - 4.5. The acidity of some honeys is significant enough to cause the inhibition of these bacteria.

1.4.3 Hydrogen Peroxide

All honeys have some degree of antibacterial activity due to the formation of hydrogen peroxide by the enzyme glucose oxidase (Scheme 1.1).[31] The glucose oxidase enzyme is introduced to nectar by the bee and acts as a preservative while the honey is ripening. Ripe honey is acidic which causes the hydrogen peroxide to undergo decomposition to form H_2O and O_2.

Scheme 1.1 Formation of hydrogen peroxide from glucose in honey



glucose gluconic acid hydrogen peroxide

The therapeutic potential of honey containing only peroxide activity is limited. Full strength honey contains negligible quantities of hydrogen peroxide, it is only when honey is diluted that the level of hydrogen peroxide will be significant enough to have any therapeutic effect.[31] The addition of catalase to honey breaks down the hydrogen peroxide formed by glucose oxidase. Catalase is present in serum, a fluid excreted in open wounds and is also found in some honeys. Honey containing hydrogen peroxide as the only form of antibacterial activity may not have a therapeutic effect on healing wounds because only diluted honey contains significant levels of hydrogen peroxide. Although honey may become diluted by serum if a wound is weeping, the catalase which is present in serum will break down any hydrogen peroxide formed.

1.4.4 Non-peroxide Activity of New Zealand Manuka Honey

It has been discovered that some honeys contain antibacterial activity additional to hydrogen peroxide activity. This activity measured after the addition of catalase is known as non-peroxide activity or UMF™ activity. Non-peroxide activity is found in significant quantities in manuka (*Leptospermum scoparium*) honey and to a lesser degree vipers' bugloss (*Echium vulgare*) honey.[34] Not all manuka honeys possess significant non-peroxide activity. High UMF™ activity honey is generally only produced in Northland, Coromandel, East Cape and Marlborough.[35] This is similar to the area which produces antibacterial essential oils from manuka.[36, 37] The antibacterial components of the oils have been identified as β-triketones which are only produced by one chemotype of *Leptospermum scoparium*.[36-38]

Active manuka honey has been found to be effective against many common wound infecting bacteria such as *Escherichia coli, Proteus mirabilis, Pseudomonas*

aeruginosa, Salmonella typhimurium, Serratia marcescens, Staphylococcus aureus and *Streptococcus pyogenes*.[39] For optimal healing, wounds should be kept moist, however this also provides optimal conditions for bacteria to grow. Traditional antibiotics cause tissue damage which slows down the healing process. The application of active manuka honey to wounds allows the wound to be kept moist and bacteria free without damaging tissue. *Staphylococcus aureus* is notorious for developing resistance to antibiotics. Active manuka honey has been tested against the collection of Methicillin-resistant *Staphylococcus aureus* held at Waikato Hospital and was found to inhibit growth of all strains.[40]

The reliable determination of the non-peroxide activity (NPA) of honey is problematic. The current method and alternatives are discussed below.

1.4.4.1 Well Diffusion Assay

NPA is currently measured using a well diffusion assay which, after destruction of hydrogen peroxide (addition of catalase), determines the Unique Manuka Factor (UMF™) value of the honey relative to the activity of phenol. This assay is discussed in more detail in Section 2.1.11. The well diffusion assay is far from ideal, it is time consuming (4 days from inoculation of broth to reading of plates) and inherently unreliable.

The minimum measurable UMF™ value is 8 for a 25% dilution, and 6 for a 50% dilution if the minimum measurable zone of inhibition is 9 mm. These values are calculated with the assumption that for the first millimetre beyond the well diameter, a reading can not be accurately measured.

Honeys which display low to moderate activity (up to a UMF™ of 15) are often seen to exhibit partial inhibition, this is where a cloudy zone is seen around the well in which not all bacteria are dead. This makes the accurate reading of zones of inhibition much more difficult.[41]

It has been found during work undertaken at The University of Waikato that there is a significant variation between results obtained for the same honey on different days and by different practitioners.[41] This variation is not as significant for high to moderately active honeys, however honeys with low activity (especially those which display partial inhibition) are much more variable.

A further complication is that replicates frequently afford differing results, and the way in which zero (no observed clearance) versus 9 - 10 mm (effectively 1 - 2 mm taking into account an 8 mm well is used) results for low activity honeys are handled can vary between different testing laboratories. In a study of a number of manuka honeys, several honeys consistently displayed either no inhibition or a small ring of partial inhibition which would change from day to day.[41] This raises the question of what should be done with zero results; should these be recorded as a zone of inhibition of 8 mm (the size of the well) and conceivably produce a UMF™ value of 6.5 or as zero which would then give a UMF™ value lower than the detectable limit.

A commercial laboratory which carries out UMF™ testing of manuka honey tests each honey sample in 4 wells on one plate either at 25% or 50% dilution (depending on how active the honey is expected to be). Results of honeys tested at 50% dilution are calculated as per the 25% dilution method but are divided by a factor of 2. As only a single analysis is undertaken, the same honey could conceivably be tested and deemed inactive (if tested at 25% dilution) or give a 1 - 2 mm result (UMF™ of 8 - 9 at 50% dilution) which will have a significant influence on the price.

1.4.4.2 Alternative Methods

A disk diffusion assay has been used to determine the non-peroxide activity. This assay is very similar to the well diffusion assay except honey soaked disks are placed on the agar instead of directly in a well. This assay suffers from the same problems as the well diffusion assay but is more insensitive. This is because the quantity of honey used is limited by the size and absorbance of the disk.

A spectrophotometric assay of bacterial growth in broth has been used to determine the antimicrobial activity of manuka honey.[42] This spectrophotometric assay is proposed to be a faster, cheaper and more reliable alternative to the traditional well diffusion assay. Spectrophotometric results were compared to the well diffusion assay and disk diffusion assay results. No catalase was added to the honey solutions prior to incubation, therefore reported results refer to total activity (peroxide + non-peroxide activity). The spectrophotometric assay was found to be significantly more sensitive than the well diffusion assay obtaining an MIC_0 (highest concentration of test material which results in no inhibition of growth) for *Staphylococcus aureus* of 0.05% and compared to 3.7% (v/v) for the well diffusion assay. The spectrophotometric assay is potentially more reliable than the well diffusion assay as the readings can be automated which removes the possibility of subjective observations when reading inhibition zones (a common problem with the well diffusion assay).

1.4.5 Methyl Glyoxal

During the course of the investigations reported in this thesis, methyl glyoxal (MGO) has been implicated as the compound primarily responsible for the non-peroxide activity in manuka honey.[43, 44] The formation of MGO in food and the impact on biological systems is discussed in the following sections.

1.4.5.1 Formation in Food

During heating or prolonged storage, carbohydrates in food can undergo a series of degradation reactions to form dicarbonyl compounds via caramelisation or Maillard reactions. The dicarbonyl compound methyl glyoxal (MGO) is found in a variety of different foods, many of which contain a high proportion of carbohydrates (Table 1.5). The concentration of MGO in food is of interest due to concerns over the toxicity of MGO.

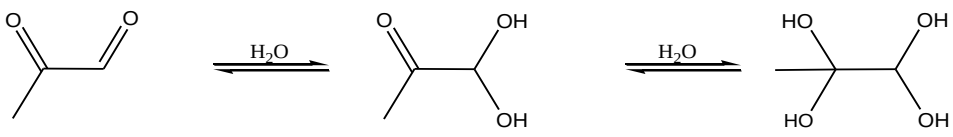
Table 1.5 Concentration of methyl glyoxal in food and beverages

Food	Methyl glyoxal (mg/L)	Reference
Apple juice	0.26	[45]
Beer	0.08	[45]
Root beer	0.76	[45]
Brandy	1.9	[46]
Cocoa	1.2	[45]
Brewed coffee	25	[45]
Decaffeinated brewed coffee	47	[45]
Honey	0.4 - 5.4	[47]
Instant coffee	23	[45]
Cola	0.23	[45]
Maple syrup	2.5	[45]
Nonfat dry milk	1.4	[45]
Orange juice	0.04	[45]
Soy bean paste	0.7	[45]
Soy sauce	7.6, 3.0	[45]
Instant tea	2.4	[45]
Tomato juice	0.06	[45]
Wine (white)	0.11	[45]

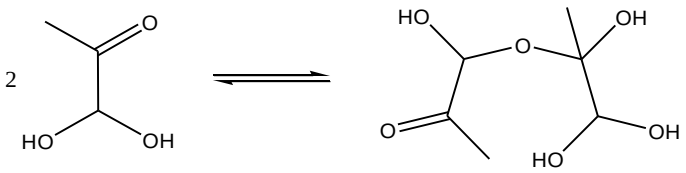
The formation of MGO in food may be accelerated when heated for prolonged periods of time. The formation of MGO upon heat treatment or storage appears to be matrix dependant. Heating fish oil has been found to produce more MGO than vegetable oil, this may be due to higher levels of long chain polyunsaturated fatty acids in fish oils.[48]

1.4.5.2 Speciation

The speciation of MGO is temperature and matrix dependant.[49] In aqueous solutions an equilibrium forms between the monohydrate and dihydrate of MGO (Scheme 1.2). Upon evaporation, hydrates can react to form dimers (Scheme 1.3) and higher oligomers.



Scheme 1.2 Formation of mono and dihydrates from MGO in aqueous solutions



Scheme 1.3 Formation of a dimer from hydrated MGO

1.4.5.3 Biological Systems

MGO is produced in animals by three kinds of enzymes: methylglyoxal synthase; cytochrome P450 IIE1 isozyme and amine oxidase.[50] The degradation of MGO predominantly occurs by the glyoxalase system. The glyoxalase system utilises glyoxalase I, glyoxalase II and reduced glutathione (GSH) as a catalyst.

There are numerous reports on the potential toxicological effects of MGO. Excess MGO has been found to deplete thiols, particularly glutathione (GSH) through covalent bonding. Chronic exposure of mice to MGO has resulted in a significant decrease in blood GSH levels which in turn was linked to a decrease in the capacity of red blood cells to combat oxidative stress.[51] The capacity of MGO-exposed mice to regulate excess glucose was also reduced. It is suggested that the chronic consumption of food which contains high levels of MGO may be detrimental to health particularly for those with impaired GSH regeneration.

These finding were supported by a separate study which found that the enzymes involved in antioxidant function, as well as GSH levels in mice, were adversely affected by the administration of MGO.[52] It is thought that MGO undergoes a redox cycle and generates free radicals which increase the oxidative stress of the animal and can lead to peroxidation of the liver.

Elevated levels of MGO have been implicated in the development of diabetic long term complications. The reaction of MGO with amino groups of proteins form advanced glycation end products (AGEs).[53] These MGO derived AGEs have been found to accumulate in corneal collagen at a rate which increases with age and severity of diabetes. Increased kidney collagen and thickening of the glomerular basement membrane was also seen in mice after the oral administration of MGO.[54]

This is significant as thickening of the glomerulus is the first stage of diabetic nephropathy; a progressive kidney disease. Diet derived AGEs can exert significant damage as the elimination of AGEs in urine is suppressed in diabetic nephropathy patients.[55] AGEs are formed inside the body through normal metabolism and ageing or by ingestion of food which either contains AGEs or by cooking sugars with fats or proteins.

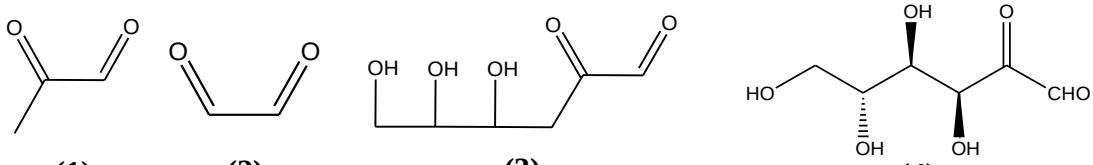
MGO has been found to be mutagenic. Guanine residues in DNA are acetylated by methyl glyoxal at a rate which is markedly enhanced in the presence of hydrogen peroxide.[56] This may have significant implications as hydrogen peroxide is found in many of the same foods as MGO.

While many studies have focused on the toxicity of MGO, some have focused more on the curative effects. The *in vivo* toxicity of MGO treated mice, rats, dogs and rabbits has been assessed.[57] No adverse effects were observed on the behavioural pattern, fertility or teratogenicity of MGO treated animals. Several biochemical and haematological parameters were tested along with histological studies of selected organs of MGO treated animals; all studies found there were no deleterious effects. Complete inhibition of cell proliferation in cancer-bearing mice was observed with mice treated with a combination of MGO, ascorbic acid and creatine.

The reported toxicity and curative effects of MGO appears to be quite variable. This may be due to the reactive nature of MGO. When MGO levels rise above the detoxifying capacity of a cell, irreversible damage can occur. The capacity of glyoxalase enzymes which metabolises MGO can vary significantly between various cancerous and non-cancerous tumour tissues. Until more research is undertaken outlining safe dosage levels of MGO, it is unlikely to be accepted as a potential treatment agent.

1.4.5.4 Manuka Honey

MGO **(1)** has been detected in multifloral honeys from Germany along with glyoxal **(2)** 3-deoxyglucosulose **(3)** and glucosone **(4)**.[47] The concentration of MGO in these honeys was 0.4 - 5.4 mg/kg. No significant increase in MGO was observed upon storage at elevated temperatures.



(1) **(2)** **(3)** **(4)**

A fortuitous discovery by Henle[43] implicated MGO as the principle active component of manuka honey. MGO was found in concentrations up to 700 mg/kg in manuka honey which is more than 10 times the amount found in any other food.

Independently, a group at the University of Waikato isolated MGO as the active component of manuka honey by HPLC.[44] The quantitation of MGO by direct measurement using HPLC was compared to the standard *o*-phenylenediamine derivatisation method. Concentrations of MGO determined using both methods were similar and ranged from 38 - 828 mg/kg.

It is likely that MGO is formed in honey by the degradation of glucose[58]. The reason why this occurs in manuka honey much more than any other honey is not yet known.

The effectiveness of manuka honey products in treating a variety of conditions is well established (Section 1.4.4). Manuka honey formulations recently have been launched commercially as a wound care product. The presence of such high concentrations of MGO in manuka honey may have serious implications for the industry. Manuka honey wound care products are used in the treatment of a variety of wounds which include wounds and ulcers on diabetic patients. Comvita has recently received marketing clearance from the US Food and Drug Administration (FDA) for a wound care dressing.[59] Elevated levels of MGO have been implicated in the onset of diabetic long term complications (Section 1.4.5.3) which is a concern as manuka honey is becoming widely used in the treatment of diabetic ulcers.[60] The mutagenicity of MGO is also increased in the presence of hydrogen peroxide, a well known

constituent of all honeys, however peroxide activity is a very small proportion of the total activity in manuka honey.[34]

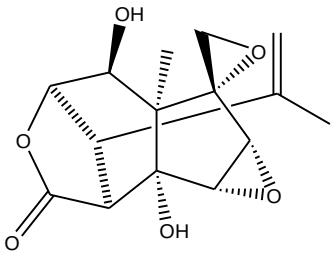
Much work needs to be done to verify the safety of manuka honey health products. Safe levels need to be established for both the oral and dermal administration of manuka honey products. The speciation of MGO may have a significant affect on toxicity and has not yet been determined in a honey matrix.

The discovery of MGO as the active component in manuka honey has raised many more questions than it has answered. While much work needs to be done in this area to clarify the beneficial and possible adverse affects of manuka honey products, this falls outside the scope of the present investigation.

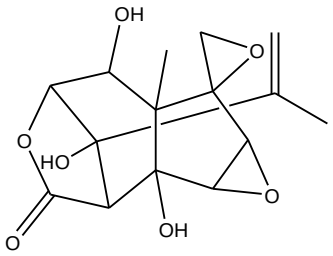
There is currently a division within the industry on the merits of using UMF™, MGO or both values in the marketing of manuka honey. A series of legal wrangles within the manuka honey industry has increased the division between UMF™ and MGO supporters and has impacted the profitability of the industry.[61, 62]

1.5 Toxic Honey

The importance of determining the floral origin of honey was highlighted recently after several cases of poisoning were reported after the consumption of toxic honey.[63] Toxic honey is produced when bees collect honeydew from the tutu shrub (*Coriaria arborea*).[64] The honeydew is produced by the passion-vine hopper (*Scolypopa australis*) which ingests tutin naturally present in the sap and metabolises a portion to hyenanchin. Both tutin **(5)** and hyenanchin **(6)** are found in honey produced from honeydew collected on tutu and are toxic to humans.



(5)



(6)

Toxic honey is produced regularly in the Coromandel Peninsula, Eastern Bay of Plenty and the Marlborough sounds.[65] Toxic honey is only produced during a season where there are high numbers of vine hoppers, hot dry weather and an absence of more attractive food sources for bees. Both comb and extracted honey are poisonous, however comb honey poses the greatest risk as an individual cell can contain concentrated levels of tutin and hyenanchin as bees deposit honeydew directly into a limited number of cells. The risk period extends from late December to the end of April. It is left up to individual beekeepers to either remove hives before the risk period or to closely monitor the tutu, vine hopper and foraging conditions within a 3 km radius of the apiary while honey is being produced.

In the recent honey poisoning cases tutin was found between 30 - 50 mg/kg and hyenanchin between 180 - 300 mg/kg.[63] As yet no safe levels have been determined for either tutin or hyenanchin, therefore no honey can be sold or exported containing trace amounts of either compound.

1.6 Objectives of the Present Investigation

The principle objectives of the investigations reported in this thesis were:

1) Investigate the possibility that oligosaccharide profiles might serve as a chemical ‘marker method’ for the determination of non-peroxide activity of manuka honey,

2) Determine to a publishable standard the extractable organic substances of Beech honeydew honey, kamahi, pohutukawa, rata and tawari honeys,

3) Develop a rapid method for the determination of floral origin of New Zealand honeys.

Objective 1

An original objective of this work was to develop a non-biological method for the accurate evaluation of the non-peroxide activity of New Zealand manuka honey. There are many shortfalls of the currently accepted bioassay including poor resolution and reproducibility, particularly for honeys containing low levels of non-peroxide activity.

A chemical method based on compositional data would remove aspects of uncertainty arising from the use of biological media. The carbohydrate composition of manuka honey will be evaluated to determine if a correlation with non-peroxide activity exists.

An additional objective arising during the course of this investigation was the identification of linkage position of *O*-trimethylsilyl disaccharides. Identification of disaccharides is routinely obtained by the comparison of retention time to standards. Due to difficulties in obtaining disaccharide standards and the prevalence of co-elution, additional structural information will provide a higher degree of certainty.

Following the discovery, while the oligosaccharide investigations were in progress, that MGO was the dominant compound responsible for the non-peroxide activity of manuka honey, the significance of these investigations was reduced and increased emphasis was placed on the NIR floral source investigations.

Objective 2

A detailed account of the extractable organic substances of many New Zealand unifloral honeys has been published. There are five notable omissions from this record: beech honeydew honey, kamahi, pohutukawa, rata and tawari honey.

Preliminary surveys of some of these honey types have been undertaken however due to either insufficient samples, the use of non certified honeys for which pollen data was not available and/or the presence of contaminants such as phthalates, solvent stabilizers and anti-oxidants, unintentionally introduced during the extraction process, results from the earlier studies were not considered to be reliable or publishable in peer reviewed journals.

In order to address the shortcomings of previous investigations it was proposed that a series of 2005-2007 season certified beech honeydew honey, kamahi, pohutukawa, rata and tawari honeys should be obtained and analysed using the diethyl ether extraction and GC-MS analyses previously applied to other New Zealand unifloral types (including clover, ling-heather, manuka, nodding thistle, rewarewa, thyme, vipers' bugloss and willow). It was also anticipated that the use of multivariate statistical analyses would facilitate the identification of floral source marker compounds in these honeys.

Objective 3

Currently, the floral source of honey is determined using information supplied from the apiarist along with organoleptic (colour), physiochemical (conductivity, sugar profile) and microscopic (pollen analysis) results. Pollen analyses is an integral part of the floral source verification process however it is very time consuming and requires highly skilled personnel.

The development of a rapid, low cost method such as NIR for the determination of floral origin would enable suppliers to choose which honeys were most suitable for further verification analyses. Such methods could also be used as a screening tool to ensure that honeys sold commercially are labelled correctly.

The initial phase of this work involved an investigation of the ability of NIR data, aided by multivariate statistical analyses to distinguish between honey types.

The second phase of the NIR investigations was directed towards the development of a commercially viable classification model.

Chapter 2

Methods and Materials

2.1 Samples, Methods and Derivatisation Procedure for the Analysis of Carbohydrate Composition of Manuka Honey

2.1.1 General Reagents

Hypersolv grade *n*-heptane (Riedel-de Haën) was used. Methanol and diethyl ether were drum grade.

2.1.2 Carbohydrate Standards

Xylitol, cellobiose, gentiobiose, isomaltose, laminaribiose, maltose monohydrate, palatinose, nigerose, α,α-trehalose dehydrate, turanose, melezitose, maltotriose and panose were supplied by Sigma. Glucose and fructose were supplied by BDH and maltulose monohydrate by CMS. Kojibiose was a gift from Dr Vince Pozsgay and Kestose gifted by Dr Ted Christian. Erlose was isolated from honeydew by Duncan Kerr.

2.1.3 Honey Samples

A set of 38 manuka honeys of varying antibacterial activity were supplied by Comvita.

2.1.4 Preparation of Super Dry Methanol

Super dry methanol was used in the reduction of sugars.

Approximately 300 mL of distilled methanol, 0.5 g iodine and 25 g of oven dried magnesium turnings were placed in a dry 5 L three necked round bottom flask. A reflux condenser and $CaCl_2$ drying tube was attached. The mixture was gently refluxed for 1 hour in which time the magnesium dissolved producing a cloudy white solution. Once all the magnesium had reacted to form the methylate, 3 L of drum grade methanol was added. The mixture was then refluxed for a further 2 hours. The reaction mixture was distilled using a double walled condenser connected to a receiver adaptor with a $CaCl_2$ drying tube attached to the vent. The first 100 mL of distillate was discarded and the remaining distillate collected in a second three necked five litre flask containing molecular sieve type 4A, equipped with a second $CaCl_2$ drying tube.

2.1.5 Extraction of Honey Samples

2.1.5.1 Ether Extraction

Honey samples were extracted using a procedure previously described by Tan[10] and modified as described below. This was done to remove organic material which may interfere with the analyses of sugars. Approximately 1 g of honey was added to a beaker containing 100 mL of distilled water and stirred to dissolve. The honey solution was transferred into a 100 mL continuous liquid-liquid extractor. Diethyl ether (250 mL) was added to the round bottom flask attached to the extractor. The extraction was carried out for 15 hours. The diethyl ether fraction was discarded.

2.1.5.2 Freeze Drying

The aqueous fraction from the diethyl ether extraction was isolated using a separating funnel and reduced under vacuum until 20 mL of solution remained. The concentrated honey extract was then freeze dried for 48 hours. The freeze dried honey extract was then transferred to a vial and stored in a desiccator at 5˚C until used for analysis by HPLC or GC-FID/MS.

2.1.6 Quantitation of Monosaccharides in Honey by HPLC

2.1.6.1 Preparation of Standards and Samples

A series of mixed glucose and fructose standards were prepared at 2.5, 5, 7.5, 10 and 12 mg/mL. Standards were transferred into vials and stored in a freezer until analysis. A fresh set of standards were thawed to room temperature and analysed prior to honey samples at the beginning of each day; thawed standards were discarded at the end of the day. Ether extracted freeze dried honey (Section 2.1.5) was diluted to 15 mg/mL solutions.

2.1.6.2 Instrumentation

Shodex KS-801 and KS-802 sugar columns (8.0 mm x 300 mL) were linked in series and heated to 50˚C. The eluent was Milli-Q water with a flow rate of 1 mL/min. Carbohydrates were detected using a Waters 2410 Refractive Index detector.

2.1.6.3 Calibration

The peak area of glucose and fructose in each standard mixture were used to construct a calibration graph using Millenium software. Calibration graphs were constructed daily prior to the analysis of honey samples.

The concentration of glucose and fructose in ether extracted freeze-dried honey was calculated using the following equations:

$$\text{Conc. M}_{(\text{sol.})} = \frac{\text{A}}{\text{G}}$$

$\text{Conc. M}_{(\text{sol.})}$ = concentration of monosaccharide in solution (mg/mL)

A = integrated peak area

G = gradient of standard curve

$$\%\ \text{M}_{\text{dw}} = \frac{100}{\text{W}_{(\text{h})}} \text{ x Conc. M}_{(\text{sol.})}$$

$\%\ M_{dw}$ = percent of monosaccharide in freeze-dried ether extracted honey

$W_{(h)}$ = weight of freeze-dried ether extracted honey (mg)

The g/f ratio was calculated using:

$$\mathrm{g/f\ ratio} = \frac{\%G_{dw}}{\%F_{dw}}$$

$\mathrm{g/f\ ratio}$ = glucose/fructose ratio

$\%G_{dw}$ = glucose (% freeze dried ether extracted honey)

$\%F_{dw}$ = fructose (% of freeze dried ether extracted honey)

2.1.7 Reduction and Silylation

Standards were prepared by accurately weighing 1 mg each of carbohydrate standard and xylitol in a 10 mL glass vial. A 1 mL aliquot of 10 mg/mL solution of freshly prepared sodium borohydride in ammonium hydroxide (1 M) was added to the carbohydrate mixture and sonicated until dissolved. The reaction mixture was then heated in a heating block for 3 hours at 50˚C. The solution was cooled before the addition of rinsed Amberlite 120-H resin (approximately 2 mL) until a neutral pH obtained. The solution was transferred into a separate vial and the resin washed with (4 x 1 mL) distilled water. The combined solution and washings were blown down under a stream of nitrogen at 40˚C in a heated block. Superdry methanol was added to the dried mixture, sonicated to dissolve the residue and then evaporated under a stream of nitrogen. The addition of superdry methanol was repeated until the white powder was replaced by a clear film on the inside of the vial.

Standards were silylated by the addition of 0.3 mL of TriSil reagent to the dried reduction mixture. Samples were sonicated and heated at 50˚C for one hour before drying under a stream of nitrogen. The carbohydrate fraction was extracted into 1 mL of *n*-heptane and centrifuged for 5 min. The supernatant was transferred into a GC vial and stored in the freezer until analysis.

The ether extracted honey samples were reduced using the same method described above with the following modifications; 15 mg of freeze dried ether extracted honey was added to 200 µL of a 1.25 mg/mL aqueous solution of xylitol in an 18 mL glass vial. Reduction was achieved by the addition of 3 mL of a 50 mg/mL sodium borohydride solution. Neutralisation of the reduction mixture would typically require 10 mL of resin.

Silylation of the reduced honey sample was achieved by the method described above using 1.5mL of TriSil reagent or by a TMSI method. Silylation with TMSI was achieved by the addition of 1 mL of pyridine and 400 µL of TMSI to the dried reduced honey. The mixture was sonicated and heated at 50˚C for 1 hour. Once cooled, the mixture was then transferred into a GC vial and stored in the freezer until analysed.

2.1.8 Quantitation of Myo-Inositol

The myo-inositol concentration in manuka honey was calculated from the GC-FID chromatogram of each manuka honey as described in Section 2.1.9. Mixed xylitol and myo-inositol silylated standards were used to calculate the relative response factor of myo-inositol (Figure 2.1).

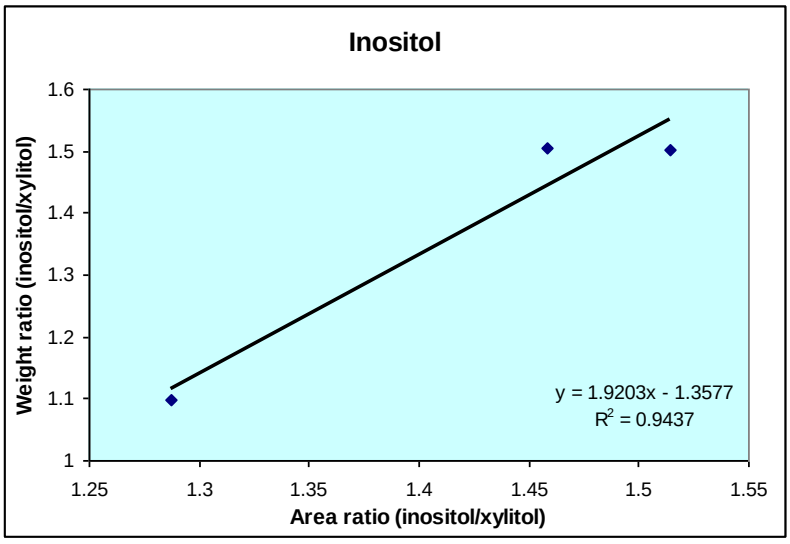


Figure 2.1 Calibration graph of *O*-trimethylsilyl inositol relative to xylitol

2.1.9 Quantitation of Disaccharides by GC-FID

2.1.9.1 Instrumentation

Gas Chromatography was performed using an Agilent 6890N network GC system with a 30 m x 0.30 mm id ZB-5 column (Phenomenex) with FID detection. Gas pressures and flow rates used were:

Hydrogen carrier gas	6.23 psi (43 KPa)
Hydrogen column gas	2.3 mL/min
Dry air FID gas	350 mL/min
Hydrogen FID gas	35 mL/min
Nitrogen make-up gas	20.2 mL/min
Total make-up + hydrogen gas	27.5 mL/min

The oven temperature was programmed as follows: 0.5 min isothermal hold at 90˚C, increasing to 180˚C at 30˚C/min then rising at 4˚C/min to 270˚C with a final ramp to 300˚C at 10˚C/min which was held for 20 min. A cool on-column injector was used.

2.1.9.2 Calculation of Response Factors

A set of standards was made using known quantities of a carbohydrate standard with xylitol as the internal standard. Use of the internal standard removes the possibility of instrumental variation affecting peak area and the effect of variability during the derivatisation process, for example losses during transfer or small variations in the quantity of *n*-heptane added. These standards were analysed by GC-FID and the Response Factors (RF) calculated relative to the internal standard (xylitol).

$$RF_{(o)} = \frac{A_{(o)}/A_{(x)}}{W_{(o)}/W_{(x)}}$$

$RF_{(o)}$ = Response factor of oligosaccharide

$A_{(o)}$ = Integrated peak area of oligosaccharide

$A_{(x)}$ = Integrated peak area of xylitol

$W_{(o)}$ = Weight of oligosaccharide

$W_{(x)}$ = Weight of xylitol

The response factors are shown in Table 2.1 with full results reported in Appendix A1.3. Kestoses reduce to form two products (for full explanation see Section 3.3.2), the first eluting product was defined as the front peak and the second eluting product as the rear peak.

Table 2.1 Calculated disaccharide response factors

Disaccharide	RF
Sucrose	0.25
α,α-Trehalose	1.2
Cellobiose	1.1
Laminaribiose	1.0
Nigerose	1.0
Turanose (front)	3.7
Turanose (rear)	7.6
Maltulose (front)	3.5
Maltulose (rear)	2.3
Maltose	1.4
Kojibiose	1.4
Gentiobiose	1.2
Isomaltose	0.9

The concentration of unknown disaccharides were calculated using an average response factor for aldohexoses ($RF_{(unknowns)} = 1.2$).

2.1.9.3 Quantitation of Nigerose, Turanose, Maltulose and Maltose in Honey

The reduction of mixtures of ketoses such as turanose and maltulose forms multiple products, some of which can not be separated by GC under any conditions. When other alditols such as nigerose and maltose are present, the quantitation of these disaccharides is not possible by direct methods as a series of three peaks (referred to as A, B and C where A = reduction product of turanose and nigerose, B = reduction product of turanose and maltulose and C = reduction product of maltulose and maltose (Figure 3.8 - Figure 3.9) were detected. It was proposed by Wu that the indirect quantitation of these disaccharides could be achieved by first calculating the proportion of nigerose and turanose in peak B by measuring the relative abundance of the *m/*z 307 and 308 ions in borodeuteride reductions (for full explanation see Section 3.3.2.1).[66]

The relationship between the proportion of turanose by weight and the abundance of the *m/z* 307 ion relative to *m/z* 308 ion measured by GC-MS-SIM in standard mixtures containing turanose and maltulose (reduced with borodeuteride) was established using the following equations:

$$\%\ \mathrm{T} = \frac{100}{\left(\mathrm{W}_{(\mathrm{T})} + \mathrm{W}_{(\mathrm{M})}\right)} \times \mathrm{W}_{(\mathrm{T})}$$

$$\%\ 307\ \mathrm{ion} = \frac{100}{\left(\mathrm{A}_{(\mathrm{T})} + \mathrm{A}_{(\mathrm{M})}\right)} \times \mathrm{A}_{(\mathrm{T})}$$

T = turanose

M = maltulose

W = weight

$A_{(T)}$ = area *m/z* 307 ion

$A_{(M)}$ = area *m/z* 308 ion

The relationship between % T and the % *m/z* 307 ion was used to calculate the percentage of turanose in unknown mixtures:

$$\%\mathrm{T}_{(\text{rear peak})} = \frac{(\%307\ \mathrm{ion} - \mathrm{c})}{\mathrm{m}}$$

c = intercept of calibration graph = 24.5

m = slope of calibration graph = 0.45

(Both c and m were calculated using Figure 3.11)

Once the percentage of turanose in peak B is known, the proportion of maltulose can be calculated by:

$$\text{Calc. } \mathrm{A}_{\text{turanose}} = \mathrm{A}_{(\text{turanose + maltulose})} \times \%\mathrm{T}_{(\text{honey})}$$

$$\text{Calc. } \mathrm{A}_{\text{maltulose}} = \mathrm{A}_{(\text{turanose + maltulose})} - \text{Calc. } \mathrm{A}_{\text{turanose}}$$

Calc. A = calculated area

The proportion of turanose in peak A and B was calculated by first measuring the area of peak A and B in a series of reduced and silylated turanose standards. The relationship between the area of peak A and peak B was determined (Figure 3.12) and used to calculate the area of turanose in peak A in a mixture from the calculated area of peak B. The remaining area was then assigned to nigerose. The proportion of maltulose in peak C was determined in the same fashion by determining the relative contribution of peak B and C in maltulose standards (Figure 3.13) and thus an area estimate of maltose in peak C can be calculated.

$$A_{\text{turanose(peak A)}} = m \times \text{Calc. } A_{\text{turanose(peak B)}}$$
$$A_{\text{maltulose(peak C)}} = m \times \text{Calc. } A_{\text{maltulose(peak B)}} + c$$

m = slope ($m_{turanose}$ = 2.16, $m_{maltulose}$ = 0.95)

c = intercept ($c_{maltulose}$ = 103.7)

$$\text{Calc. } A_{\text{nigerose}} = A_{\text{(nigerose + turanose)}} - A_{\text{turanose(peak A)}}$$
$$\text{Calc. } A_{\text{maltose}} = A_{\text{(maltulose + maltose)}} - A_{\text{maltulose(peak C)}}$$

2.1.9.4 Quantitation of Disaccharides

The percentage of each oligosaccharide (with the exception of those described above) in freeze-dried ether extracted honey was calculated using the equations:

$$W_{(d)} = \frac{W_{(x)} \times A_{(d)}}{A_{(x)} \times RF}$$

$W_{(d)}$ = weight of disaccharide

$W_{(x)}$ = weight of xylitol

$A_{(d)}$ = area of disaccharide

$A_{(x)}$ = area of xylitol

RF = response factor of disaccharide

$$\%D_{fh} = \frac{W_{(d)}}{W_{(s)}} \times 100$$

$\%D_{fh}$ = percent of disaccharide in ether extracted freeze-dried honey

W_s = weight of ether extracted freeze-dried honey

2.1.10 Identification of Disaccharides in Manuka Honey by GC-MS

GC-MS analyses were performed using a HP6890 (Hewlett Packard) GC coupled to a HP5973 mass selective detector. He was used as the carrier gas with the pressure set at 9 psi. A 30 m x 0.25 mm id ZB-5 column (Phenomenex) was used with a split/splitless injector. The injector temperature was maintained at 265˚C and the MS source at 230˚C. The oven temperature was programmed as follows: 0.5 min isothermal hold at 90˚C, increasing to 160˚C at 35˚C/min then rising at 4˚C/min to 265˚C with a final ramp up 300˚C at 10˚C/min which was held for 5 min. Mass spectral data was originally acquired in total ion chromatogram (TIC), scanning from *m/z* 42 - 800 Daltons. Ion ratios were calculated by repeating the analysis in single ion chromatogram (SIM) mode (maximum of 17 ions).

The relative ion intensity ratio was calculated as follows:

$$I_R = \frac{T_A}{R_A} \times 100$$

I_R = Relative ion intensity ratio
T_A = Target ion area
R_A = Relative ion area

2.1.11 Determination of UMF™

The non-peroxide activity of 38 manuka honeys was measured by an agar well diffusion assay in quadruplicate on a minimum of eight plates on different days. The method used in this study was modified from that published by Allen.[34] This modified method was established by the Honey Research Unit at the University of Waikato and has been used to determine UMF™ activity in previous studies.[41, 67, 68]

2.1.11.1 Inoculum Preparation

A freeze-dried culture of *Staphylococcus aureus* (ATCC 9144) obtained from ESR was reconstituted in Trypticase Soy broth according to the instructions supplied, and incubated at 37ºC for 18 hours. A loopful of the broth culture was subcultured onto blood agar plates incubated for 24 hours at 37ºC and used to inoculate 7 x Microbank vials for long term storage at -70ºC. Working cultures were obtained by placing one bead from the preserver ampoule stock into 10 mL of Trypticase Soy broth (TS) and incubating for 18 hours at 37ºC. A further working culture was prepared by inoculating a 200 µL volume of the prepared culture from the previous day, into another vial containing 10 mL of TS broth. This was incubated for approximately 5 hours at 37ºC. This culture was then adjusted to an absorbance of 0.5 by dilution in a cuvette which was measured at 540 nm using sterile TS broth as a blank with a 1 cm pathway. A volume of 100 µL of the culture adjusted to 0.5 absorbance was used to seed 150 mL of nutrient agar to make the assay plates.

A new freeze dried culture was obtained from 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.

2.1.11.2 Plate Preparation

To prepare the assay plates, 150 mL of nutrient agar (23 g/L) was sterilised then held at 50ºC for 30 min before seeding with 100µL of *S. aureus* culture. The agar was swirled to mix thoroughly and poured into a large square assay plate placed on a level surface. As soon as the agar was set the plates were stored upside down at 4ºC overnight before use the following day.

The autoclaved agar was prepared once a week; daily requirements were steamed in a saucepan of boiling water for 30 min then cooled in a 50ºC water bath for 30 min.

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.

2.1.11.3 Catalase Solution

A 2 mg/mL solution of catalase from bovine liver in distilled water was prepared fresh each day.

2.1.11.4 Sample Preparation

A primary honey solution was prepared by adding 10 g of well mixed honey to 10 mL of distilled water in universal vials and held at 37ºC for 30 min to aid mixing. To prepare secondary solutions, 1 mL of the primary honey solution was added to 1 mL of catalase solution for non peroxide activity testing. This produced a 25% honey solution. The density of honey (1.35 g/mL) was factored into the final calculations.

Each sample was tested by adding 100 µL to each of 4 wells with the same allocated number in the assay plate.

2.1.11.5 Preparation of Standards

Standards of 2%, 3%, 4%, 5%, 6% and 7% were prepared from a 10% w/v solution of phenol in water. These solutions were kept at 4ºC for a maximum of one month and brought to room temperature in the dark before use. Each standard was tested in duplicate in each plate.

After application of samples and standards the plates were incubated on individual racks for 18 hours at 37ºC.

2.1.11.6 Zone Measurement

The plates were placed back over the quasi-Latin square template to measure the zone of inhibition with digital callipers using the points of the prongs to measure the inside diameter of the clear zone.

2.1.11.7 Calculation of Antibacterial Activity of Honey

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 and the equation of this line used to calculate the activity of each diluted honey from the square of the mean diameter of the clear zone. To calculate a theoretical value for whole honey (a 25% honey solution was used in the assay) and adjust for the density of honey (1.35) the equivalent phenol value was multiplied by 4.69. The activity was expressed as the equivalent phenol concentration (% w/v).

In the present investigation, when no zone of inhibition was observed, a diameter of 9 mm was assigned as this was deemed the minimum value which could be obtained with 8 mm wells.

2.1.12 Statistical Analysis of Carbohydrate Profile as an Indicator for UMF™ Activity

A data set was constructed containing the concentration of mono and disaccharides of the 38 manuka honeys. The log of the carbohydrate data set was used in all subsequent analysis. Each honey was classified as high (H), medium (M) or low (L) UMF™ activity as outlined in Table 2.2. A value of 0.002 (% freeze-dried ether extract) was assigned to compounds not detected in a given sample where 0.002 was half the lowest recorded concentration.

Table 2.2 UMF™ classification of manuka honeys

Sample No.	UMF™ Activity	UMF™ Classification
1	25.4	H
2	28	H
3	24.3	H
4	22.9	H
5	20.9	H
6	29.8	H
7	17	M
8	17.8	M
9	19.4	M
10	16.8	M

Sample No.	UMF™ Activity	UMF™ Classification
11	17.7	M
12	17.7	M
13	15.7	M
14	16	M
15	17.9	M
16	18.2	M
17	15.9	M
18	15.6	M
19	14.8	M
20	13	M
21	10	L
22	10.4	L
23	10.6	L
24	9.2	L
25	16.1	L
26	9	L
27	8.6	L
28	7.6	L
29	8.2	L
30	11.9	L
31	8.5	L
32	8	L
33	7.6	L
34	10.2	L
35	9.8	L
36	8	L
37	9.9	L
38	8.2	L

H = high, M = medium, L = low

The resulting carbohydrate matrix was analysed using various statistical programmes as outlined in Section 2.4.

2.2 Extraction Methods and Procedures for Extractive Organic Substances in Honey

2.2.1 Honey Samples

Ten honey samples from beech honeydew, kamahi, rata and tawari were supplied by Airborne Honey Ltd. Airborne samples were supplied with pollen, moisture, colour,

conductivity and carbohydrate data where available. Pohutukawa honey (ten samples) was supplied by Waitemata Honey Ltd.

2.2.2 Extraction of Honey

Samples were extracted using a modified extraction procedure developed by Tan.[10] Approximately 2.5 g of accurately weighed honey was dissolved in 50 mL of distilled water in a beaker and stirred with a magnetic stirrer at room temperature for 10 min. The resulting solution was transferred into a 125 mL continuous liquid-liquid extractor containing 100 μL of a 0.5 mg/mL solution of *n*-heptadecanoic acid (17:0) fatty acid in dichloromethane. The beaker was washed with 50 mL and then 25 mL of distilled water and the washings added to the extractor. A further two beaker washings of 50 mL of diethyl ether (AR grade (Univar) stabilised with 0.5 - 1.5 mg/L BHT purified on a Pure Solv^{TM} Solvent Purification System) was added to the extractor. Diethyl ether was added directly to the extractor until the attached 250 mL round bottom flask was half full. Honey samples were extracted for a total of 24 h.

Following the commencement of the extraction, the two solvent phases were separated using a separating funnel. A 100 mL aliquot of a 0.5 mg/mL solution of *n*-heptadecanoic acid ethyl ester solution made up in dichloromethane was added to the diethyl ether solution.

The diethyl ether extract was dried over anhydrous Na_2SO_4 and filtered through cotton wool prior to concentration by rotary evaporation (≈2 mL). The concentrated solution was transferred into a 5 mL glass vial and stored in at 5˚C.

2.2.3 Methylation and Ethylation Procedures

Samples were methylated with an ethereal solution of diazomethane prior to analysis by GC-MS. Approximately 0.3 g of *N*-nitrosomethyl urea was added to a one-piece diazomethane distillation apparatus containing 25% sodium hydroxide (30 mL) and

diethyl ether (AR grade (Univar) stabilised with 0.5 - 1.5 mg/L BHT purified on a Pure Solv^{TM} Solvent Purification System). The solution was heated in a water bath (~ 60˚C) and the resulting yellow distillate collected in a 30 mL glass vial. Excess etheral diazomethane (≈2 mL) was added to each honey extract and stored at 5˚C overnight. Methylated samples were concentrated to 250 μL over a stream of nitrogen and transferred into GC vials containing low volume inserts. Samples were stored at 5˚C until analysis.

Ethylation of the internal standard *n*-heptadecanoic acid was achieved by the method described above using 0.4 g of *N*-nitrosoethyl urea and adding all the resulting etheral diazoethane solution to a 100 mL volumetric flask containing 25 mg of *n*-heptadecanoic acid.

2.2.4 Analysis of Extracted Honeys by GC-MS

The analysis of methylated ether extracted honeys was conducted using a HP6890 GC coupled to a HP5973 mass spectrometer. Separation was achieved using a 30 m x 0.25 mm ZB-5 column (Phenomenex) with He as the carrier gas (column inlet pressure 62 kPa, carrier gas 1.1 mL/min) under the following conditions: 0.3 min isothermal at 50˚C rising to 75˚C at 30˚C/min then increasing to 290˚C at 8˚C/min which was held for 25 min to elute wax components. Samples were injected (3 μL) using an HP7683 auto-sampler into the injection port (265˚C) with a splitless time of 0.1 min. The MS ion source was maintained at 250˚C with data acquired in total ion chromatogram (TIC) mode from *m/z* 42 - 450 Daltons.

2.2.5 Quantitation Procedure

2.2.5.1 Instrumentation

TIC ion profiles were manually integrated using Hewlett Packard MSD ChemStation D.03.00.611. The recovery of the internal standard *n*-heptadecanoic acid ethyl ester was measured relative to the recovery standard *n*-heptadecanoic acid methyl ester. Quantification of all compounds was performed relative to the internal standard *n*-

heptadecanoic acid ethyl ester. Relative response factors were determined for the methylated analogues of acetophenone, benzoic acid, phenlyllactic acid, palmitic acid and pimelic acid. Identified compounds were quantified relative to the appropriate standard for the compound class where appropriate (Table 5.3). All other compounds (including unknowns) were calculated relative to the internal standard *n*-heptadecanoic acid ethyl ester. Results were reported as mg/kg (ppm) of honey (fresh weight).

2.2.5.2 Calculation of Response Factors

Relative response factors (RRF) of class standards were calculated relative to the internal standard heptadecanoic acid ethyl ester. A calibration graph of the weight ratio versus the area ratio was used to determine the RRF where:

$$\text{Weight ratio} = \frac{\text{weight}_{(a)}}{\text{weight}_{(std)}}$$

$$\text{Area ratio} = \frac{\text{peak area}_{(a)}}{\text{peak area}_{(std)}}$$

$\text{peak area}_{(a)}$ = integrated peak area of analyte

$\text{weight}_{(a)}$ = weight of analyte (mg)

$\text{peak area}_{(std)}$ = integrated peak area of internal standard (17:0 Et ester)

$\text{weight}_{(std)}$ = weight of internal standard (17:0 Et ester (mg))

The concentration (mg/kg) of each compound was calculated as follows:

$$\text{Conc}_{(a)} = \left\{ \frac{\left(\left(\text{area}_{(a)} / \text{RRF}_{(a)} \right) - \text{I} \right) \times \left(\text{weight}_{(std)} / \text{area}_{(std)} \right)}{\text{weight}_{(honey)}} \right\} \times 1000$$

$\text{Conc}_{(a)}$ = concentration of analyte (mg/kg)

$\text{area}_{(a)}$ = integrated peak area of analyte

$\text{RRF}_{(a)}$ = relative respose factor of analyte

I = intercept of relative response factor of analyte

$weight_{(std)}$ = weight of internal standard (17:0 Et ester) (mg)

$area_{(std)}$ = integrated peak area of internal standard (17:0 Et ester) (mg)

$weight_{(honey)}$ = weight of honey (g)

1000 = factor to convert from mg/g to mg/kg

Analyte recovery was calculated to determine the efficiency of the extraction process. A standard solution containing 100 µL of both *n*-heptadecanoic acid and *n*-heptadecanoic acid ethyl ester was methylated prior to analysis by GC-MS. The % recovery of *n*-heptadecanoic acid during the extraction process was determined as follows:

$$\%\ \text{recovery} = \left\{\frac{\text{area}_{(\text{Me ester(honey)})}/\text{area}_{(\text{Et ester(honey)})}}{\text{area}_{(\text{Me ester(std.sol.)})}/\text{area}_{(\text{Et ester(std.sol.)})}}\right\} \times 100$$

$area_{(Me\ ester\ (honey))}$ = integrated peak area of 17:0 Me ester in extracted sample

$area_{(Et\ ester(honey))}$ = integrated peak area of 17:0 Et ester in extracted sample

$area_{(Me\ ester(std.\ sol.))}$ = integrated peak area of 17:0 Me ester in standard solution

$area_{(Et\ ester(std.sol.))}$ = integrated peak area of 17:0 Et ester in standard solution

The final adjusted concentration was calculated by:

$$\text{Adjusted concentration} = \text{Conc}_{(a)} \times (100/\%\ \text{recovery})$$

The adjusted concentration is the concentration reported in Section 5.3.

2.2.6 Linearity of MS Detector

The linearity of the detector was determined by injecting a series of mixed standards of varying concentration. A 5 mg/mL solution each of acetophenone, benzoic acid, pimelic acid, phenyllactic acid and palmitic acid was made with chloroform as the solvent with the exception of pimelic acid which was dissolved in diethyl ether.

Aliquots of 50, 100, 200, 400, 800 and 1800 µL of each standard were added to separate vials containing 200 µL of a *n*-heptadecanoic acid ethyl ester solution (5 mg/mL). The resulting mixed standards were then methylated with an etheral solution of diazomethane before analysis by GC-MS as described in Section 2.2.4.

The ratio of the target analyte peak relative to *n*-heptadecanoic acid ethyl ester was plotted against the ratio of the corresponding volumes to determine the detector linearity (Table 2.3, Figure 2.2). Ratios of standard peak area vs heptadecanoic acid ethyl ester were used as opposed to an external single standard. This was done in order to minimise variablility which could be introduced during the derivatisation process such as losses during transfer and variability in the quantity of *n*-heptane added prior to analysis or subsequent evaporation.

Table 2.3 Peak area ratio of standards relative to heptadecanoic acid ethyl ester

Volume ratio	Peak area ratio (standard/17:0 Et)				
	Acetophenone	Benzoic acid	Pimelic acid	Phenyllactic acid	Palmitic acid
0.25	0.13	0.16	0.11	0.07	0.18
0.5	0.84	1.11	0.75	0.55	1.00
1	0.77	1.00	0.89	0.80	1.18
2	1.57	2.07	1.80	1.80	2.61
4	3.44	4.55	4.10	4.47	6.27
9	8.33	10.78	9.73	12.23	15.96

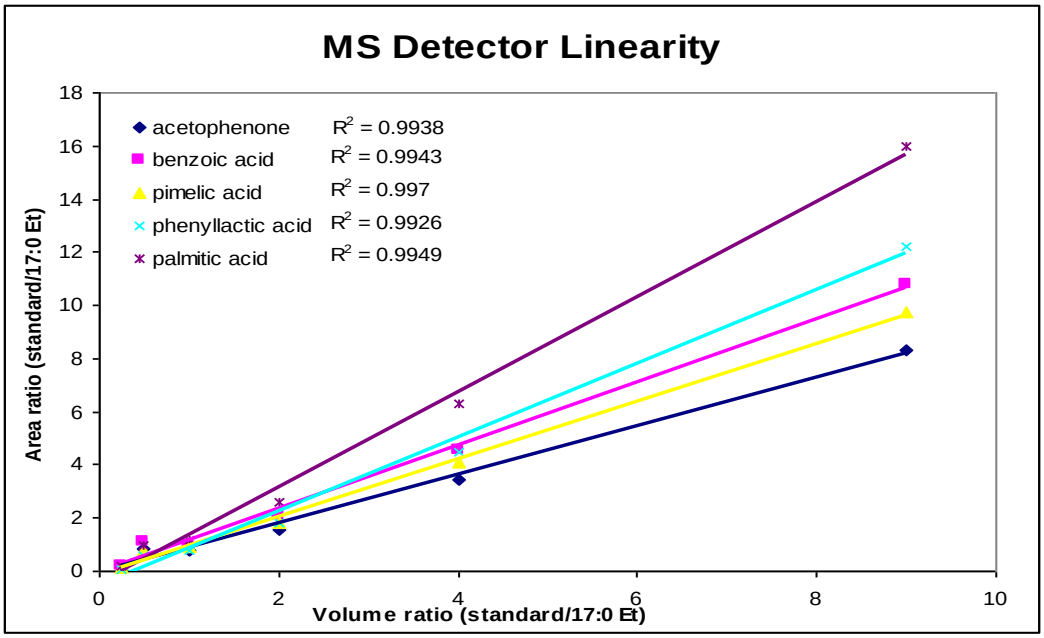


Figure 2.2 Linearity of methylated standards analysed using GC-MS

2.2.7 Detector Reproducibility

The reproducibility of the MS detector was assessed by injecting the same sample six times. The standard containing 200 μL each of acetophenone, benzoic acid, pimelic acid, phenyllactic acid, palmitic acid and *n*-heptadecanoic acid, methylated with diazomethane was analysed six times by GC-MS using the method described in Section 2.2.4.

The ratio of the peak area of the target analyte divided by the peak area for *n*-heptadecanoic acid ethyl ester was determined (Table 2.4). These results indicate a good level of reproducibility as the coefficient of variation was less than 4%.

Table 2.4 Reproducibility of GC-MS detector

	Peak area ratio (standard/17:0 Et)				
Injection	**Acetophenone**	**Benzoic acid**	**Pimelic acid**	**Phenyllactic acid**	**Palmitic acid**
1	0.77	1.00	0.89	0.80	1.18
2	0.73	0.95	0.83	0.75	1.15
3	0.72	0.95	0.82	0.74	1.15
4	0.72	0.94	0.81	0.74	1.15
5	0.71	0.94	0.81	0.74	1.15
6	0.71	0.93	0.81	0.73	1.14
μ	0.73	0.95	0.83	0.75	1.15
σ	0.02	0.02	0.03	0.03	0.01
%CV	2.8	2.4	3.8	3.4	1.2

μ = arithmetic mean, σ = standard deviation, CV = coefficient of variation

2.2.8 Reproducibility of Honey Extraction

The reproducibility of the honey extraction was determined by extracting three subsamples of beech honeydew honey. As honey is not homogeneous, three subsamples of a single honey would not be a true representation of the extraction reproducibility; therefore a honey solution was used. A honey solution is generally more homogenous than pure honey especially when limited quantities of wax or pollen are present. The beech honeydew honey used in this analysis was very clear and contained few particulates. Beech honeydew honey (7.5 g) was accurately weighed into a beaker. Water (150 mL) was added to the honey and stirred with a

magnetic stirrer for 10 min or until all honey was fully dissolved. 50 mL of the honey solution was transferred into each continuous liquid-liquid extractor using a 50 mL glass pipette. The beech honeydew honey was extracted with diethyl ether using the procedure described in Section 2.2.2. The levels of 10 compounds (phenylacetic acid, unknown (*m/z* 55, 114, 128, 158), phenyllactic acid, 4-hydroxyphenyllactic acid, lauric acid, 4-methoxyphenyllactic acid, methyl syringate, 4-hydroxy-3,5-dimethoxyhydrazidebenzoic acid, indole-3-acetic acid and palmitic acid) were quantitatively determined as described in Section 2.2.5. Results are given in Table 2.5.

Table 2.5 Concentration of compounds in diethyl ether extraction of beech honeydew honey (mg/kg). Acids are quantified as the corresponding methyl ester

		Sample					
RT	**Compound**	**1**	**2**	**3**	**μ**	**σ**	**%CV**
7.809	phenylacetic acid	14.2	14.1	14.9	14.4	0.4	2.5
8.196	unknown (*m/z* 55, 114, 128, 158)	4.0	4.0	3.9	4.0	0.1	1.7
11.288	phenyllactic acid	19.5	19.8	15.3	18.2	2.0	11.2
13.229	4-hydroxyphenylacetic acid	2.8	3.2	2.9	3.0	0.2	5.2
13.651	lauric acid	4.2	4.3	5.0	4.5	0.4	8.0
15.483	4-methoxyphenyllactic acid	18.2	17.8	15.8	17.2	1.0	6.0
16.762	methyl syringate	10.5	9.1	6.9	8.8	1.5	16.7
17.539	4-hydroxy-3,5-dimethoxyhydrazide benzoic acid	2.6	3.1	4.4	3.4	0.8	22.9
18.336	indole-3-acetic acid	2.3	2.6	2.1	2.3	0.2	8.2
19.49	palmitic acid	2.9	2.7	3.2	2.9	0.2	5.8

μ = arithmetic mean, σ = standard deviation, CV = coefficient of variation

The extraction reproducibility demonstrated for beech honeydew honey is reasonably reproducible for most compounds with the possible exception of methyl syringate and 4-hydroxy-3,5-dimethoxyhydrazidebenzoic acid. These two aromatic acids are closely related in structure and are expected to take longer to extract as they have a high water affinity. A balance must be reached between conducting a shorter extraction (>16 h) with poor recovery of some compounds and a lengthy extraction (<48 h) with almost complete recovery.[68]

2.2.9 Statistical Analysis of Extractives Data

A data set of the combined extractives concentrations was constructed with a value of 0.05 mg/kg assigned to any compound not detected in each sample (0.05 was half the minimum recorded concentration). The number of variables in the extractives data set was reduced by removing compounds which were detected in ≤ 6 samples from the same floral source. The procedures for the statistical analysis of the log extractives data set are outlined in Section 2.4.

2.3 Samples and Analysis Procedure for the Evaluation of Floral Origin by NIR Spectroscopy

2.3.1 Honey Samples

All unifloral honeys were supplied by Airborne Honey Limited. The sample set for the initial survey of 100 unifloral honeys was comprised of ten samples from each of ten floral sources: clover, beech honeydew, kamahi, manuka, nodding thistle, rata, rewarewa, tawari, thyme and vipers' bugloss. Floral origin was determined using a combination of pollen analysis, colour, moisture, conductivity, fructose, glucose, sucrose, maltose and HMF data (Appendix A4.1). This compositional data was supplied by Airborne Honey Ltd.

A second set of 428 honeys (none of which were present in the 100 unifloral honey set) was obtained from Airborne Honey Limited. The number of samples from each floral origin is listed in Table 2.6. The corresponding floral source data provided by Airborne Honey Limited for these honeys is given in Appendix A4.1.

Table 2.6 Floral origin of second honey set

ID	Description	Quantity
B	vipers' bugloss	23
CA	calluna	2
CD	clover (dark)	45
CL	clover (light)	23
CM	clover (medium)	55
CX	clover (extra light)	20

ID	Description	Quantity
HD	beech honeydew	21
K	kamahi	19
M	manuka	28
MF	manufacturing grade	4
NC	noddy clover	16
NT	nodding thistle	15
PF	polyfloral	48
R	rata	20
RW	rewarewa	15
T	thyme	10
TW	tawari	29

The polyfloral honeys were removed from the data set along with noddy clover (largely a mixture of nodding thistle and clover) which is essentially a polyfloral honey of known composition. Given the low number of samples available for manufacturing grade and calluna honey, these honeys were also excluded, giving a final set of 323 honeys.

2.3.2 Method of Analysis

A drop of honey was smeared between two 18 mm circular glass coverslips (Thomas Red Label Micro Cover Glasses) which were then pressed together so that the honey was sandwiched into a thin film. The prepared sample was then placed inside a purpose built sample holder. Samples were analysed using a Bruker MPA NIR spectrometer scanning the range from 8000 - 3850 cm^{-1} with a resolution of 4 cm^{-1} in transmission mode. Two separate sub samples of each honey were analysed.

2.3.3 Data Sets and Pre-Processing Procedures

Acquired NIR spectra were collated into a dataset using Statistica. For the visual inspection (Section 6.3.1.1), the full standardised spectra (rows mean centered, prepared as for Dataset C described below) of 10 randomly selected honeys from each floral source within the 323 honey set were used.

A subset comprising of the spectra range 6000 - 3850 cm^{-1} was used to construct datasets for statistical analyses as this region was seen to contain the most useful variation. This was determined by a visual inspection of the data. The data set was further reduced in size by decreasing the resolution by selecting every fourth wavelength. The duplicates of each honey sample were then averaged to produce a single spectrum for each sample. Vector normalisation (dividing by the mean and multiplying by the standard deviation of a row) was used to adjust for the differences in absorbance obtained when different film thicknesses of honey were analysed. The vector normalisation was carried out in Statistica by standardising rows (instances). The effect of standardising columns was also explored. Large differences in absorbance will have a greater bearing on subsequent calculations than small features; the vector normalisation of columns should reduce this effect.[69] The honey samples and type of processing for each Dataset is summarised in Table 2.7.

Table 2.7 Processing procedures used on NIR Datasets

Dataset	Honey set	Duplicates averaged	Vector normalisation (rows)	Vector normalisation (columns)
A	100	✓	✓	×
B	323	✓	×	×
C	323	✓	✓	×
D	323	✓	✓	✓
E	323	✓	✓	×

2.4 Statistical Analysis

Matrices were analysed using the statistical programme R and classification models tested using the "machine learning workbench" software WEKA.[70] The analysis procedures applied using these programmes are outlined in the following sections.

Two types of analysis were undertaken, unsupervised analysis and supervised analysis. Supervised methods take into account class membership (i.e. floral type) in the calculations whereas unsupervised methods do not.

2.4.1 Multivariate Statistical Analysis

Three different types of analysis were conducted using R, a statistical computing language that is a public domain version of S/SPlus.[71] A series of packages for R are available from the CRAN family of internet sites which enable users to undertake techniques including linear and nonlinear modelling, statistical tests, classification, clustering and graphical techniques.[71] All software and packages are available free under the GNU General Public Licence.

The data analyses carried out using R were as follows:

Hierarchical Cluster Analysis

In cluster analysis, samples are divided into groups so that there is minimal variance within groups ("clusters") and maximal variance between clusters. Two different types of algorithms are used for hierarchical clustering; agglomerative clustering and divisive clustering. Agglomerative clustering starts with individual samples and fuses them to form larger groups whereas divisive clustering starts with a single cluster containing all samples and successively divides it.[71, 72] In this work agglomerative clustering was used, and is implemented in R by calculating the between-object distance matrix and joining the smallest elements in that matrix into a single cluster. A new distance matrix is then constructed with the new cluster as an object replacing the original data points. These steps are repeated until the final two clusters have been fused. A range of methods exist which differ mainly in the object distance metric and how the clusters are joined. The finished cluster analysis is visualised using a tree structure known as a dendrogram.

Principal Component Analysis

Principal components analysis (PCA) is a common technique used to reduce the dimensionality of a data set while retaining useful information. In PCA the original data axes are rotated to create a new, orthogonal coordinate system in which maximal data variance is captured in a reduced set of computed (independent) variables (known as principal components, factors, or latent variables).[73] The class of each sample is not taken into account during the analysis. The purpose of PCA in this context is to reveal underlying structure in the data.

An important limitation of PCA is that it is a variance-based technique that relies on there being significant linear correlations between at least some of the original measurement variables. It also needs to be borne in mind that the coordinate system computed by PCA is optimal in terms of capturing variance, but this coordinate system is not likely to be optimally correlated with dependent variables (in this case, such as honey type).

Linear Discriminant Analysis

Linear discriminant analysis (LDA) is a supervised learning technique which is closely related to PCA, and in which the new coordinate system is calculated so as to maximise the class differences. The eigenvalues reflect the proportion of the between class variance which is explained by the linear combinations. The axes of the new coordinate system (equivalent to the principal components computed by PCA) are referred to as Linear Discriminants.

In order for Linear Discriminant Analysis to be effective, several fundamental requirements must be met. The first requirement is that the data must be independent. Other requirements include homoscadasticity which implies the data has an equal variance and is normally distributed. A small aspect of heteroscadasticity or skewness in the distribution is generally acceptable. By undertaking LDA on data which is grossly in contradiction with these requirements, sever overfitting can occur.

The R scripts used to carry out the various analyses are given in Appendix A4.3.

2.4.2 Machine Learning Analysis

The 323 unifloral NIR data matrix with manufacturing grade, calluna and noddy clover honeys removed (Dataset C) was examined in WEKA using a Partial Least Squares (PLS) model.

2.4.2.1 Principles of Partial Least Squares

PLS is one of a number of multivariate methods which are an extension of Multiple Linear Regression. Other methods include Discriminant Analysis and Principal Components Regression. These methods impose restrictions such that factors underlying the Y and X variables are extracted only from the Y'Y and X'X matrices, not from matrices involving both the Y and X variables.[74] A second restriction is that the number of prediction functions is limited by number of Y variables and X variables.

Partial Least Squares does not impose such restrictions as the prediction functions are represented by factors extracted from a Y'XX'Y matrix. The number of prediction functions extracted can also exceed the number of Y and X variables. This means that the PLS model is suitable for examining data where the matrix of dependent and independent measurements contains fewer observations than predictor variables.

Partial Least Squares analysis can be described as a two step procedure involving a form of principal component regression followed by Multiple Linear Regression; in practice however both steps are combined.[69] The weight covariance matrix produced in PLS takes into account the class of the predictor variables whereas principal components regression does not

2.4.2.2 Implementation of Partial Least Squares

The PLS models used to examine the NIR spectra were computed in WEKA using the following procedure:

PLS was implemented in WEKA as the base classifier for a "Classification via Regression" metaclassifier.[70] In the regression classifier, a numeric value is assigned to each class and a regression model built. This enables regression to be undertaken on datasets containing nominal (non numeric) class values.

The original data set contains a high proportion of clover samples. In order to test the PLS model with a more balanced data set, the WEKA "resampling with replacement" filter was applied.[75] Five resampled data sets were generated using different random

seeds and a bias to uniform class setting of 0.75. A bias to uniform class of zero leaves the class distribution unchanged whereas a value of 1.0 gives a uniform class distribution. A sample size of 300% was used to produce a data set of 969 instances.

By changing the random seed before generating each resampled data set, different instances were chosen. Analysing several different data sets produced in this way provided as realistic picture as possible of the performance of the classification models.

Performance of each PLS model was assessed using 10 fold cross validation. Modelling results are affected by the distribution of the instances in the training data. The cross-validation procedure splits the data set into groups ("folds", ten folds were used throughout) based on a random ordering of the data instances, nine folds then being used to train a model which can then be tested against the remaining folds. In order to produce a robust result, this cross validation procedure was repeated ten times, each time using a different seed value for randomisation of the data ordering prior to constructing the cross-validation folds.

The classification results of the various models were assessed using two tailed paired T-tests with a significance level of 0.05.[75] The test statistic considered was Root Mean Square error of cross-validation.

Confusion matrices were produced from the evaluation of each individual dataset with the PLS model. A confusion matrix is a table of actual class versus predicted class results. The number of correctly classified instances is aligned along the diagonal, with the off diagonal instances indicating the number of instances misclassified in a particular class.

An average confusion matrix for the 20 component PLS model was generated by averaging five matrices obtained using a different ordering of the instances.

The five versions of the data created by resampling with differing randomisation seeds were each tested by cross-validation five times using a different ordering of the instances to create a total of 5 x 5 confusion matrices. These 25 matrices were averaged to produce a final averaged confusion matrix.

Chapter 3

Carbohydrate Profile of Manuka (*Leptospermum scoparium*) Honey

3.1 Introduction

For many years honey was thought to be a mixture of glucose, fructose and sucrose. This remained the case until the mid 20th century when paper chromatography and charcoal columns revealed the presence of other oligosaccharides. To date 37 oligosaccharides have been isolated from honey (Table 3.1).

Table 3.1 Oligosaccharides previously reported in honey

Trivial Name	Nomenclature	Reference
Disaccharides		
Cellobiose	β-D-Glc*p*-(1→4)-D-Glc*p*	[76, 77]
Gentiobiose	β-D-Glc*p*-(1→6)-D-Glc*p*	[76-79]
Inulobiose	β-D-Fru*f*-(2→1)-β-D-fru*f*	[80]
Isomaltose	α-D-Glc*p*-(1→6)-D-Glc*p*	[76-79]
Laminaribiose	β-D-Glc*p*-(1→3)-D-Glc*p*	[76-79]
Leucrose	α-D-Glc*p*-(1→5)-D-Fru*p*	
Kojibiose	α-D-Glc*p*-(1→2)-D-Glc*p*	[76-79]
Maltose	α-D-Glc*p*-(1→4)-D-Glc*p*	[76-79]
Maltulose	α-D-Glc*p*-(1→4)-D-Fru*p*(*f*)	[77-79]
Melibiose	α-D-Gal*p*-(1→6)-D-Glc*p*	[77, 79]
Nigerose	α-D-Glc*p*-(1→3)-D-Glc*p*	[76-79]
Palatinose (isomaltulose)	α-D-Glc*p*-(1→6)-D-Fru	[76-79]
Sucrose	β-D-Fru*f*-(2↔1)-α-D-Glc*p*	[76-79]
α,α-Trehalose	α-D-Glc*p*-(1↔1)-α-D-Glc*p*	[77]
α,β-Trehalose (Neotrehalose)	α-D-Glc*p*-(1↔1)-β-D-Glc*p*	[76-79]
Trehalulose	α-D-Glc*p*-(1→1)-D-Fru*f*	[77]
Turanose	α-D-Glc*p*-(1→3)-D-Fru*f*(*p*)	[76-79]
Trisaccharides		
Erlose	α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	[76, 77, 79, 81]
Isomaltotriose	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-D-Glc*p*	[77, 79, 81]
Isopanose	α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-D-Glc*p*	[76, 79, 81]
1-Kestose	β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*	[77, 79, 81]
6-Kestose	β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2→6)-α-D-Glc*p*	[77]
Neokestose	β-D-Fru*f*-(2→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	[77]
Maltotriose	α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc*p*	[76, 77, 79, 81]
Melezitose	α-D-Glc*p*-(1→3)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*	[77, 79, 81]

Trivial Name	Nomenclature	Reference
Panose	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p*	[76, 77, 79, 81]
Raffinose	α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	[77]
Theanderose	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	[76, 79, 81]
3-α-Isomaltosylglucose	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-D-Glc*p*	[81]
4-α-Gentiobiosylglucose	β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p*	[81]
Tetrasaccharides		
Isomaltotetraose	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-D-Glc*p*	[81]
Maltotetraose	α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc*p*	[82]
α-Panosyl-β-D-fructofuranoside	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	[82]
Pentasaccharides		
Isomaltopentaose	α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc*p*	[81]
	α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	[82]
	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	[82]
Hexasaccharides		
	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	[82]

3.1.1 Methods of Analysis

GC and HPLC are the two principal methods used in the analysis of oligosaccharide composition of honey. The main limitation of both methods is the prevalence of overlapping peaks and accurate peak identification.

3.1.1.1 Gas Chromatography (GC)

GC methods require samples to be derivatised prior to analysis - an often tedious and time consuming process. Sugars in solution exist as an equilibrium of the α and β furanose and pyranose as well as the acyclic form. To reduce the number of peaks, samples are first derivatised by either reduction which forms one product for aldoses and two for ketoses, or oximation which forms two products for each reducing sugar. Hydroxylated compounds are not volatile so samples must then be further derivatised by either silylation or methylation.

Both reduction and oximation of oligosaccharides produce multiple peaks, some of which overlap - particularly in the disaccharide region. Reduction and silylation of mixtures of nigerose, turanose, maltulose, maltose, palatinose and isomaltose produce overlapping peaks which can not be separated.[66]

A simplified GC-FID method has been developed for the analysis of TMS oxime derivatives of honey.[83] This method utilises a fused silica column coated with DB-5 which enables the analysis of 11 disaccharides and 5 trisaccharides in honey. Overlapping of peaks occured between turanose (peak 1 and 2), nigerose (peak 1) and maltose (peak 1) which meant turanose could not be quantified unless nigerose was absent.

The analysis of TMS oximes in honey using a combination of GC-FID and GC-MS on two different columns (phenyl methyl silicone and methyl silicone) was found to improve the reliability of the analysis.[77] The phenyl methyl silicone column was found to be more suited to the analysis of trisaccharides whereas the methyl silicone column achieved better separation of disaccharides.

Due to the large number of peaks and the necessity to use multiple columns when analysing TMS oxime disaccharides in honey, multivariate techniques are often employed. Least squares multiple regression is often used however the presence of unidentified compounds can result in negative values. The use of an iterative method has been found to be more robust when analysing mixtures in which the presence of unidentified compounds co-eluting with known compounds can not be ruled out.[84]

GC methods have historically achieved much higher sensitivity than HPLC methods. The analysis of reduced and silylated oligosaccharides in honey by GC achieved a lower detection limit of 5 ppb for disaccharides and 40 ppb for trisaccharides.[76]

3.1.1.2 High Pressure Liquid Chromatography (HPLC)

The analysis of oligosaccharides in honey by high pressure anion exchange chromatography coupled to a pulsed amperometric detector (HPAE-PAD) is the most

widely used HPLC method and does not require derivatisation, however various sample clean-up methods are often undertaken.

One of the earliest HPAE-PAD methods required extensive sample preparation.[79] Samples were filtered through a reversed phase cartridge and anion exchange resin to remove organic acids before the removal of monosaccharides by activated charcoal. The analysis was undertaken using two anion exchange columns in series with a pulsed amperometric detector. Seventeen carbohydrates were quantified using this method however the co-elution of isomaltose/maltulose, turanose/gentibiose and maltose/1-kestose meant the direct quantitation of these disaccharides could not be achieved.

Subsequent studies have demonstrated that no sample pre-treatment is required beyond filtering for the analysis of major di and trisaccharides in honey.[85] A method which uses a single anion exchange column with PAD achieved the separation of 9 disaccharides and 3 trisaccharides with a lower detection limit ranging from 5 - 20 ppm.

An HPLC-UV method using reversed phase chromatography has been developed for the analysis of oligosaccharides in honey.[86] Pre-treatment utilises solid phase extraction to remove monosaccharides. Samples undergo derivatisation with a selective chromophoric reagent which enables the analysis to be undertaken using reversed phase chromatography with UV detection. This method achieved a lower detection limit of 50 ppm after isolation from the monosaccharide matrix.

Bonded normal phase amine columns have also been used in carbohydrate analysis. Six major disaccharides and four trisaccharides have been quantified in honey using a Lichrosphere 5-NH_2 column with a refractive index detector.[87] No sample pre-treatment was undertaken beyond dilution and filtering. One of the drawbacks of using amino columns is the large volume of acetonitrile required for the analysis and the susceptibility of reducing sugars to bond irreversibly to columns.

Higher oligosaccharides have been detected in honey using a modified activated charcoal method to fractionate honey.[88] Fractions from a column packed with charcoal were analysed by HPAE-PAD and molecular weight distributions determined using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF).

Oligosaccharides with a degree of polymerisation from 3-14 were detected using this method.

The HPAE-PAD methods have developed to such an extent that they are becoming preferable to GC methods due to the limited sample preparation required. Detection limits have also improved with the introduction of the pulsed amperometric detector to such an extent that all but the scarcest oligosaccharides can be detected. The availability of HPAE-PAD systems is still far less than GC-FID or GC-MS which are common in almost all research and commercial laboratories.

3.1.2 Detection of Fraudulence

The adulteration of honey with sugar syrups or the misrepresentation of floral origin are both fraudulent practices which the industry must be proactive in combating in order to preserve the integrity of the industry.

The most serious fraudulent practice is adulteration. Adulterating syrups are either added to honey after harvesting or fed to bees during the nectar flow to increase yield. Both methods are considered fraudulent and measures are taken to detect these practices.

Typical syrups include glucose syrup from wheat, barley or rice; inuline syrup from chicory and medium invert sugar syrup from beets. Adulteration with C_3 syrups may be more prevalent than C_4 syrups as the detection of C_3 syrups in the honey matrix is more difficult (Section 1.3).

Carbohydrate profiles have been shown to be useful in the detection of honey adulteration. One of the earlier methods was the use of paper chromatography to detect high levels of oligosaccharides. Paper chromatography is still occasionally used as a method of detecting adulteration despite no longer being recommended by the industry due to the prevalence of false positive results in honeys with naturally high oligosaccharide content.

Various carbohydrate ratios have been proposed to indicate authenticity of a honey. The carbohydrate composition of multifloral, acacia, linden, sunflower, rape and pine honey has been determined by GC-MS analysis of the TMS oxime derivatives.[89] It was proposed that the maltose/turanose, sucrose/turanose and maltotriose/raffinose + erlose + melezitose ratios could be used a proof of authenticity.

A combination of HPAE-PAD and GC-FID has been used to detect adulteration of honey with sugar syrups.[90] Three different adulterating syrups were used; two C_3 sugar syrups and one a mixture of C_3 and C_4 and were added to either acacia, chestnut or lavender honey. It is traditionally very difficult to detect the adulteration of honey with C_3 syrups which follow the same Calvin cycle of photosynthesis as most honey nectar sources. Principal component analysis was used to detect adulterating syrups in honey with a detection limit between 5 and 10%.

Bee feeding experiments have been undertaken to determine how sugar syrups are changed if fed to bees as opposed to mixed with honey after harvest.[91] Three sugar syrups were used, all of C_3 botanical origin: glucose syrup from wheat, barley or rice; inulin syrup from chicory and a medium invert sugar syrup from beets. Glucose and fructose concentrations were determined both before and after harvest by HPAE-PAD. All sugar syrups showed an increase in glucose and fructose concentration after harvesting which occurred due to a decrease in moisture level and the hydrolysis of oligosaccharides to monosaccharides by bee enzymes. The levels of eight disaccharides and two trisaccharides were determined by analysis of the TMS derivatives by GC-FID both before and after harvest. Both the glucose and inulin syrups contained high levels of maltose and maltotriose before harvesting whereas the medium invert syrup contained elevated sucrose levels. All of the major and most minor oligosaccharide levels decreased after harvesting which indicates conversion to the monomeric units by bee enzymes.

The mislabelling of floral origin is the most common form of fraudulence. The carbohydrate profile has been used to determine the authenticity of commercial honeys.[91] A total of 280 French honeys of verified origin (acacia, chestnut, rape, lavender, fir, linden and sunflower) were analysed by GC-FID and classified by principal component analysis. Commercial honeys (47 samples) with a given origin

were then authenticated using the PCA parameters established with the authentic samples. This method was effective at identifying non-conforming samples but can not be used to conclusively establish fraudulence.

3.1.3 Floral Origin

Various HPLC and GC methods have been used in the quantitation of oligosaccharides in honey. The oligosaccharide profile can be used to distinguish the floral origin of honey and to detect adulteration.

The oligosaccharide content of some Western Canadian honeys and their nectar sources has been investigated to determine the origin of oligosaccharides in honey.[92] Nectar and honey samples from alfalfa, alsike, canola, red clover and trefoil were studied. Glucose, fructose and sucrose concentrations were determined by HPLC; all three sugars were found to be present in nectar samples. Trace amounts of maltose were found in alsike nectar at a level significantly lower than honey, no other oligosaccharides were found.

The oligosaccharide profile of alfalfa, alsike, canola and trefoil honey samples has been determined by HPAE-PAD.[79] Small differences were observed in the relative proportion of each oligosaccharide between floral sources however the overall "fingerprint" was very similar.

A variation of the above method has been used to determine the oligosaccharide content of New Zealand manuka, heather (*Calluna vulgarius*), clover and beech honeydew honeys.[93] The disaccharide portion of manuka honey was found to be dominated by maltose. Clover honey was found to have a very similar profile to manuka honey while heather honey contained a higher ratio of isomaltose to maltose and significantly less erlose. The oligosaccharide portion of beech honeydew honey was characterised by the abundance of isomaltose in the disaccharide region and the trisaccharides melezitose, panose and maltotriose. It was suggested that the prominence of melezitiose could be used as a floral marker for beech honeydew honey. As classification was achieved

solely on relative retention time with several oligosaccharides coeluting, this study should be regarded as indicative only.

The oligosaccharide profile of New Zealand beech honeydew honey has been determined using a combination of LC and GC.[82] Isolated oligosaccharides were subsequently characterised by NMR. Maltose, turanose, palatinose and nigerose were found to be the most abundant disaccharides with erlose the predominant trisaccharides. In contrast to a previous study on beech honeydew honey by Weston, melezitose was found to be a minor constituent and therefore can not be regarded as a floral marker for beech honeydew honey. This finding must have more credibility as the identification of oligosaccharides was achieved by isolation and characterised by NMR.

The oligosaccharide profiles of unifloral honeys produced in the Province of Soria (Spain) have been analysed by HPLC-PAD.[85] A combination of principal component analysis and Canonical Discriminant Analysis was used to examine the relationship between carbohydrate profile and botanical origin. Samples of ling (*Calluna vulgaris*), spike lavender (*Lavandula latifolia*), French lavender (*Lavandula stoechas*) thyme (*Thymus* sp.) oak forest (predominantly *Quercus* sp.) and multifloral honey were analysed. Some oligosaccharides, particularly trehalose, erlose, nigerose, melezitose, isomaltose and panose, showed significant differences in the mean concentration between different floral sources. Cross validation produced a 100% correct classification for French lavender and 93% correct classification for ling and oak forest honeys. Spike lavender and thyme achieved 87% and 80% correct classification respectively.

A similar study by HPAE-PAD has been conducted on 91 authentic UK honeys from either bramble, ling heather, oil seed rape, white clover, hawthorn or willow-herb.[94] Canonical Discriminant Analysis successfully classified 100% of ling heather samples while 62% and 70% of seed rape and bramble samples were classified correctly. No clover samples were correctly classified.

Principal component analysis and stepwise discriminant analysis has been used to classify the floral origin of Moroccan honeys.[95] The TMS oxime derivatives of 98 honeys; 59 multifloral and 39 from eucalyptus, citrus, *Lythrum* sp., members of

apiaceae and honeydew were analysed by GC-MS. All honeydew honeys were correctly classified however only moderate (70 - 75%) correct classification rates were achieved for citrus, *Lythrum* and eucalyptus honeys. *Apiaceae* honeys achieved a low classification rate of 43%. It can therefore be concluded that oligosaccharide profile can effectively be used to discriminate nectar honeys from honeydew honeys. This method may not be suited to the classification of all nectar honeys.

As well as providing an insight to botanical origin, the carbohydrate profile may also be linked to the geographical origin of a honey.[87] The levels of maltose, nigerose, turanose and maltotriose have been linked to the geographical origin of honeys from various states in Brazil.

3.1.4 Oligosaccharide Profile of Manuka Honey

Two separate studies have been undertaken on the oligosaccharide profile of manuka honey. An initial method utilising HPAE-PAD stated that "there were no differences whatsoever between the oligosaccharide composition of antibacterial active and inactive manuka honeys."[93] Due to the small sample set (1 active and 2 inactive honeys) and variability in oligosaccharide content (20-200%) it is difficult to ascertain if any true difference in oligosaccharide composition exists.

A subsequent investigation of ling heather (*Calluna vulgaris*) honey and manuka honey by GC-FID found that manuka honey had lower levels of turanose/maltulose, palatinose and isomaltose than ling heather honey.[66] Ling heather honey was found to contain higher levels of panose than manuka honey. No significant difference was found in the oligosaccharide composition of active and inactive manuka honey.

The two main limitations of the above mentioned studies of manuka honey are the limited number of samples used for each floral source (maximum of 5) and difficulties in quantifying overlapping peaks.

3.2 Experimental

3.2.1 Preparation of Honey Samples

The carbohydrate composition of manuka honey was evaluated with respect to UMF™ activity in order to determine if an association exists between the carbohydrate composition and UMF™ activity.

The monosaccharide concentration of 38 manuka honeys was determined using an HPLC method as described in Section 2.1.6. Each honey was analysed in duplicate and quantified using external calibration curves. The percentage of glucose and fructose in ether extracted freeze-dried honey and glucose/fructose (g/f) ratio was calculated as described in Section 2.1.6.3.

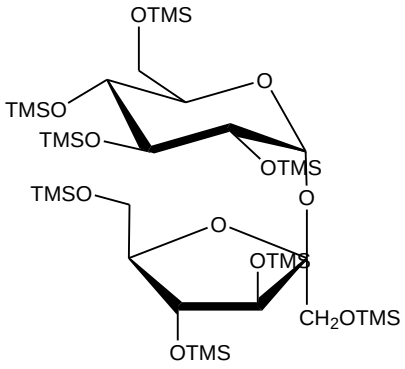
The sample preparation procedures for the analysis of the disaccharide composition of manuka honey by GC-FID are outlined in Section 2.1.7. Deuterated samples and standards were prepared using sodium borodeuteride as the reducing agent.

A total of 12 disaccharide standards were used (Table 3.2), reduced and silylated structures of which are depicted in Figure 3.1. Reducing sugars with fructose at the reducing end form two products upon reduction; both reduction products are given. Non-reducing sugars such as sucrose do not undergo reduction.

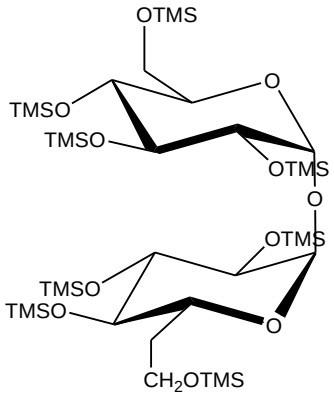
Table 3.2 Standard disaccharides examined in this investigation

Standard	Derivative	Linkage	Configuration
sucrose	-	2↔1	β,α
α,α-trehalose	-	1↔1	α,α
cellobiose	cellobiitol	1→4	α
laminaribiose	laminaribiitol	1→3	α
nigerose	nigeritol	1→3	β
turanose	nigeritol; α-D-glc*p*-(1→3)-D-man	1→3	β
maltulose	maltitol; α-D-glc*p*-(1→4)-D-man	1→4	β
maltose	maltitiol	1→4	β
kojibiose	kojibiitol	1→2	β
gentibiose	gentiobiitol	1→6	α
palatinose	isomaltitol; α-D-glc*p*-(1→6)-D-man	1→6	β
isomaltose	isomaltitol	1→6	β

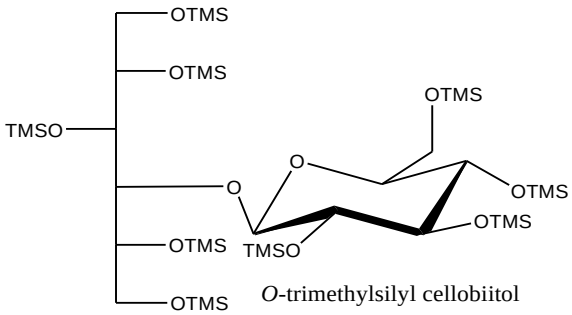
man = mannitol, glc = glucitol



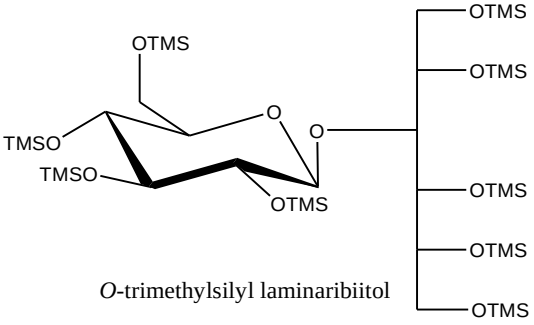
O-trimethylsilyl sucrose



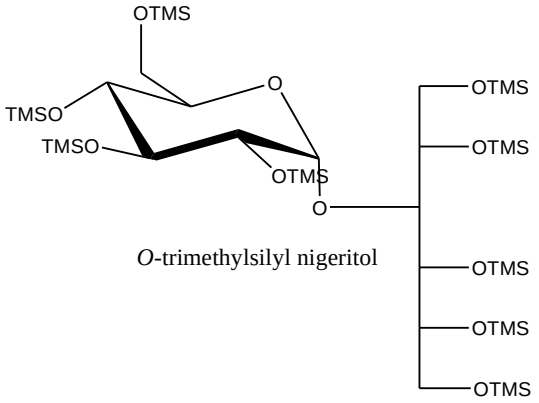
O-trimethylsilyl α,α–trehalose



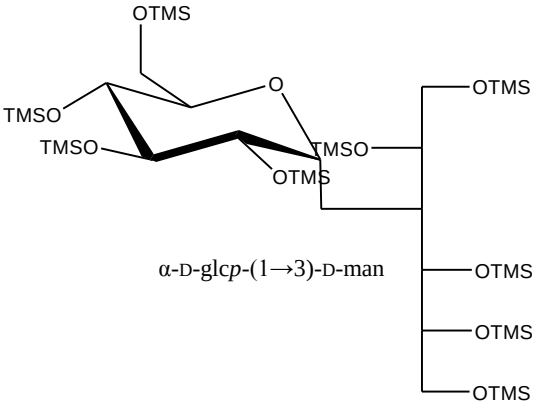
O-trimethylsilyl cellobiitol



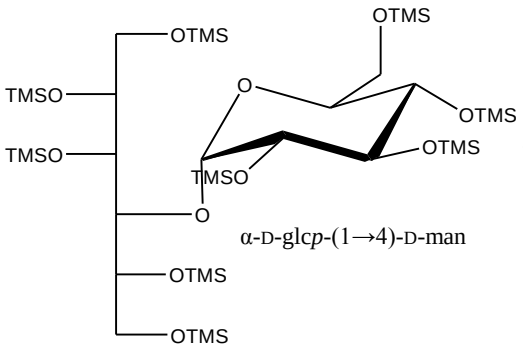
O-trimethylsilyl laminaribiitol



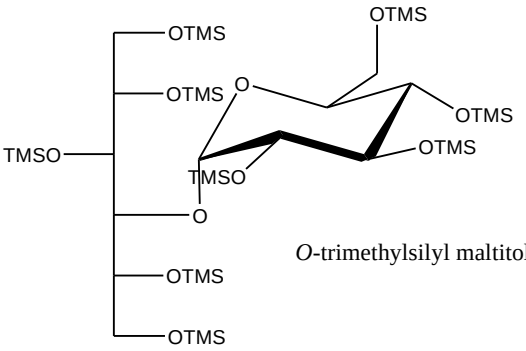
O-trimethylsilyl nigeritol



α-D-glc*p*-(1→3)-D-man



α-D-glc*p*-(1→4)-D-man



O-trimethylsilyl maltitol

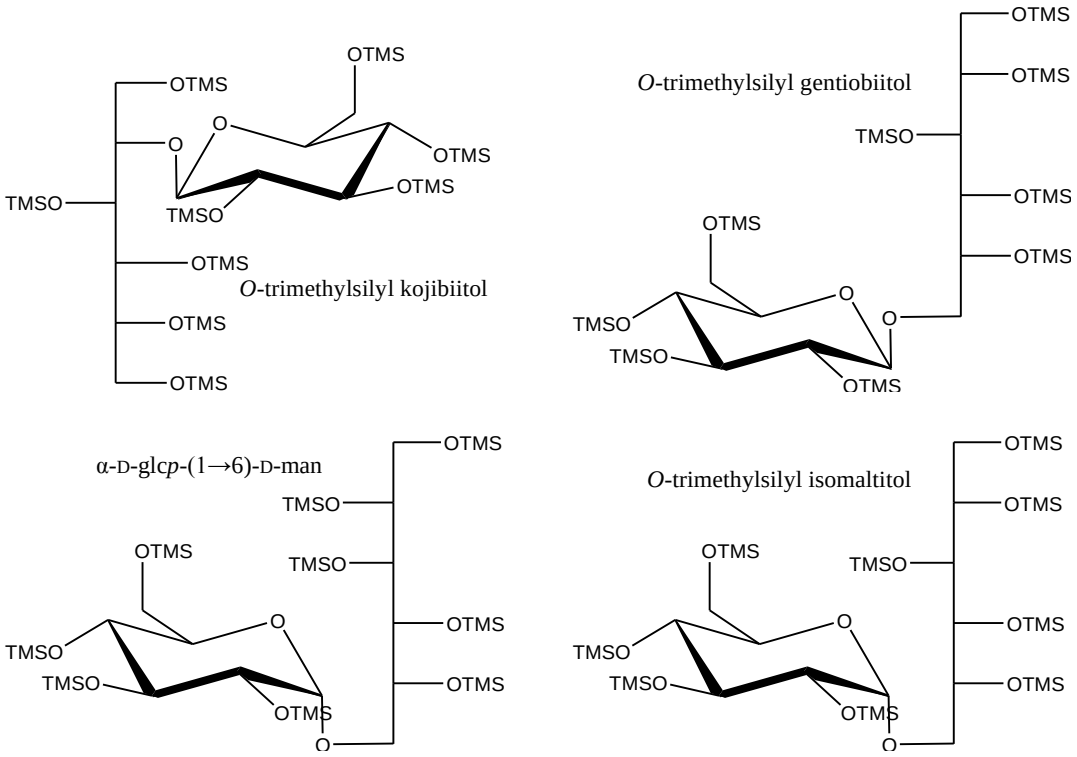


Figure 3.1 Structure of borohydride reduced *O*-trimethylsilyl standards

The non-peroxide antibacterial activity (UMF™) of each manuka honey was assessed according to the procedure reported in Section 2.1.11.

3.3 Results and Discussion

3.3.1 Monosaccharides in Honey

The glucose and fructose concentration of 38 manuka honeys was determined as described in Section 2.1.6. Each honey was analysed in duplicate and quantified using external calibration curves.

The percentage of glucose and fructose in ether extracted freeze-dried honey and glucose/fructose (g/f) ratio was calculated as described in Section 2.1.6.3. The averaged result from each honey is reported in Table 3.3 with full results in Appendix A1.1.

The non-peroxide antibacterial activity (UMF™) of each manuka honey was assessed according to the procedure reported in Section 2.1.11.

Table 3.3 Average % glucose and fructose in ether extracted freeze-dried honey

Honey	UMF™	% Dry weight		σ		g/f ratio
		glucose	fructose	glucose	fructose	
1	25.4	35.16	50.32	0.02	0.05	0.699
2	28.0	35.25	51.24	0.16	0.19	0.688
3	24.3	35.49	49.11	0.51	0.76	0.723
4	22.9	35.61	47.75	0.30	0.41	0.746
5	20.9	35.81	47.96	0.10	0.17	0.747
6	29.8	36.56	49.48	0.08	0.07	0.739
7	17.0	37.05	44.41	0.04	0.08	0.834
8	17.8	36.48	44.42	0.00	0.24	0.821
9	19.4	35.79	47.82	0.27	0.40	0.748
10	16.8	37.15	48.59	0.35	0.44	0.765
11	17.7	37.07	46.89	0.21	0.26	0.791
12	17.7	38.85	48.66	0.13	0.15	0.798
13	15.7	37.25	43.74	0.15	0.15	0.851
14	16.0	41.24	46.63	0.09	0.04	0.884
15	17.9	39.05	45.27	0.01	0.02	0.863
16	18.2	36.64	47.38	0.02	0.01	0.773
17	15.9	39.60	46.04	0.28	0.30	0.860
18	15.6	38.88	46.21	0.30	0.37	0.841
19	14.8	37.64	45.90	0.38	0.46	0.820
20	13.0	38.56	44.31	0.12	0.19	0.870
21	10.0	38.78	47.17	0.55	0.66	0.822
22	10.4	38.81	46.75	0.01	0.01	0.830
23	10.6	39.06	46.63	0.34	0.43	0.838
24	9.2	37.50	45.67	0.39	0.44	0.821
25	16.1	36.14	45.99	0.57	0.71	0.786
26	9.0	38.19	43.54	0.16	0.19	0.877
27	8.6	37.13	46.33	0.24	0.31	0.801
28	7.6	38.24	47.72	1.09	1.40	0.801
29	8.2	38.54	46.48	0.12	0.18	0.829
30	11.9	38.12	46.74	0.25	0.31	0.816
31	8.5	39.79	46.24	0.04	0.02	0.860
32	8.0	39.01	44.95	0.14	0.16	0.868
33	7.6	40.99	47.11	0.69	0.79	0.870
34	10.2	41.93	46.72	0.01	0.00	0.897
35	9.8	39.73	45.70	0.55	0.60	0.869
36	8.0	42.24	46.97	0.65	0.72	0.899
37	9.9	38.37	48.62	0.26	0.33	0.789
38	8.2	36.55	46.54	0.42	0.55	0.785

σ = standard deviation

The % glucose and fructose in honey was plotted against UMF™ values in order to ascertain if any relationship exists (Figure 3.2). These results show a weak correlation (R^2 of 0.41 for glucose and 0.29 for fructose) between the concentration of these monosaccharides and UMF™, the general trend being for glucose concentration to fall and fructose to rise with increasing UMF™.

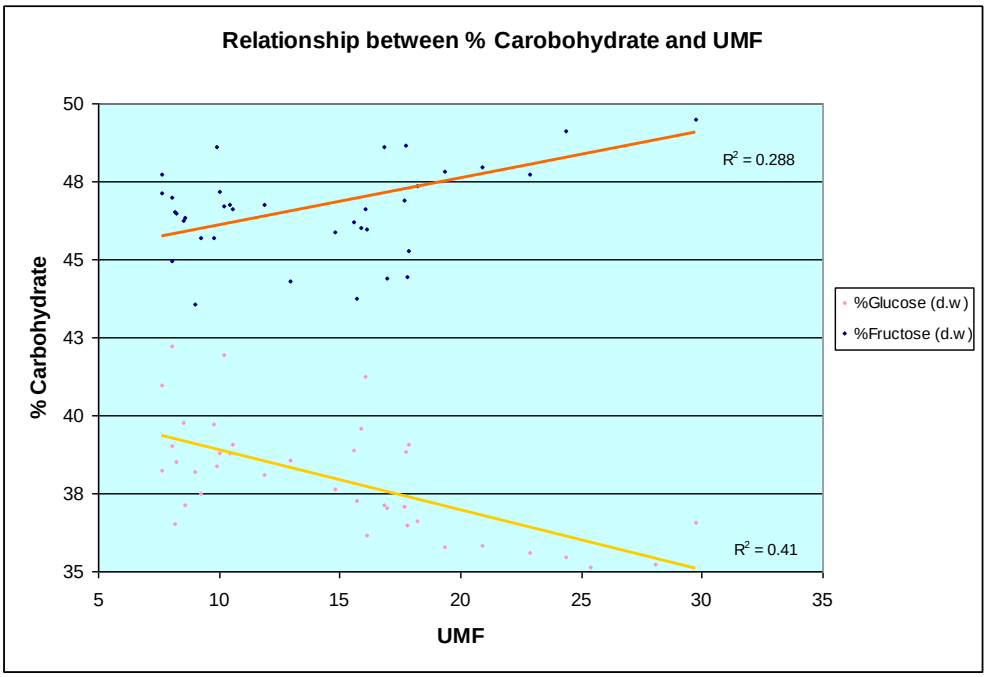


Figure 3.2 Plot of % Carbohydrate vs UMF™

Subtle differences in glucose and fructose concentration were magnified by plotting the g/f ratio against UMF™ (Figure 3.3). This produces a moderate correlation with an R^2 of 0.52.

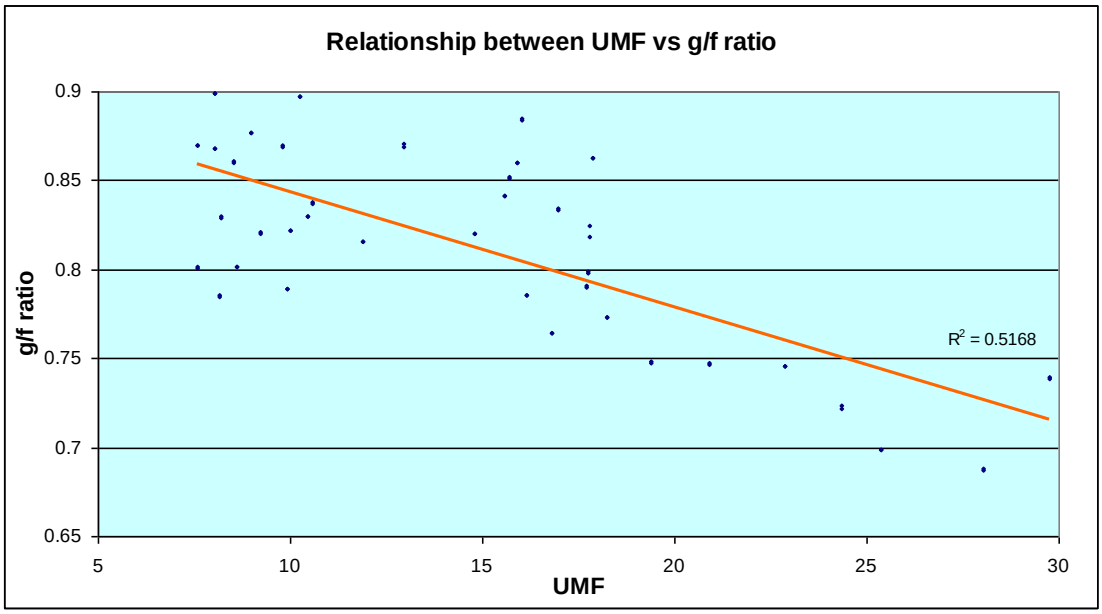


Figure 3.3 Plot of UMF™ vs g/f ratio

The accurate UMF™ measurement of some honeys is complicated by partial activity. Partial activity is a cloudy zone where some but not all bacteria have been inhibited around the well. The edges of partial activity zones are often hard to define and thus

measuring an accurate diameter is difficult. In the set of 38 manuka honeys used in this study, 13 displayed partial activity. The comparison of the % glucose and fructose to UMF™ was repeated with the partial activity honeys removed (Figure 3.4). This produced a slightly stronger correlation between % carbohydrate and UMF™ (R^2 of 0.47 for glucose and 0.41 for fructose).

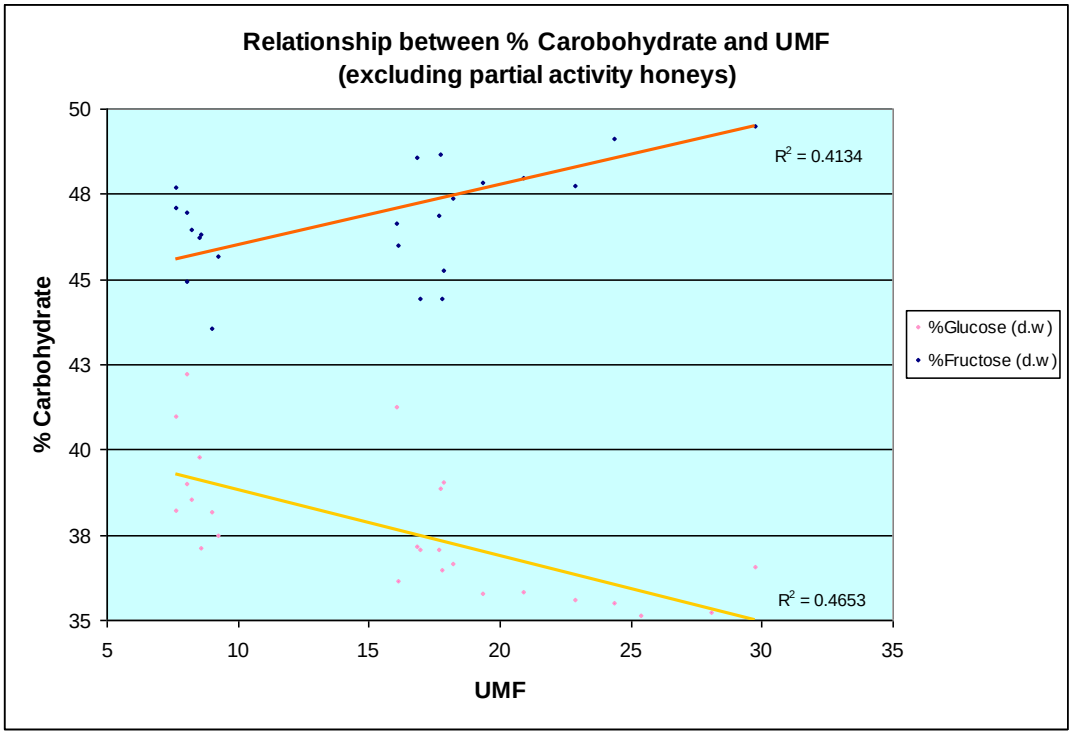


Figure 3.4 Plot of % carbohydrate vs UMF™ (excluding partial activity honeys)

The exclusion of partial activity results from the g/f ratio plot also increased the R^2 from 0.52 to 0.63 (Figure 3.5).

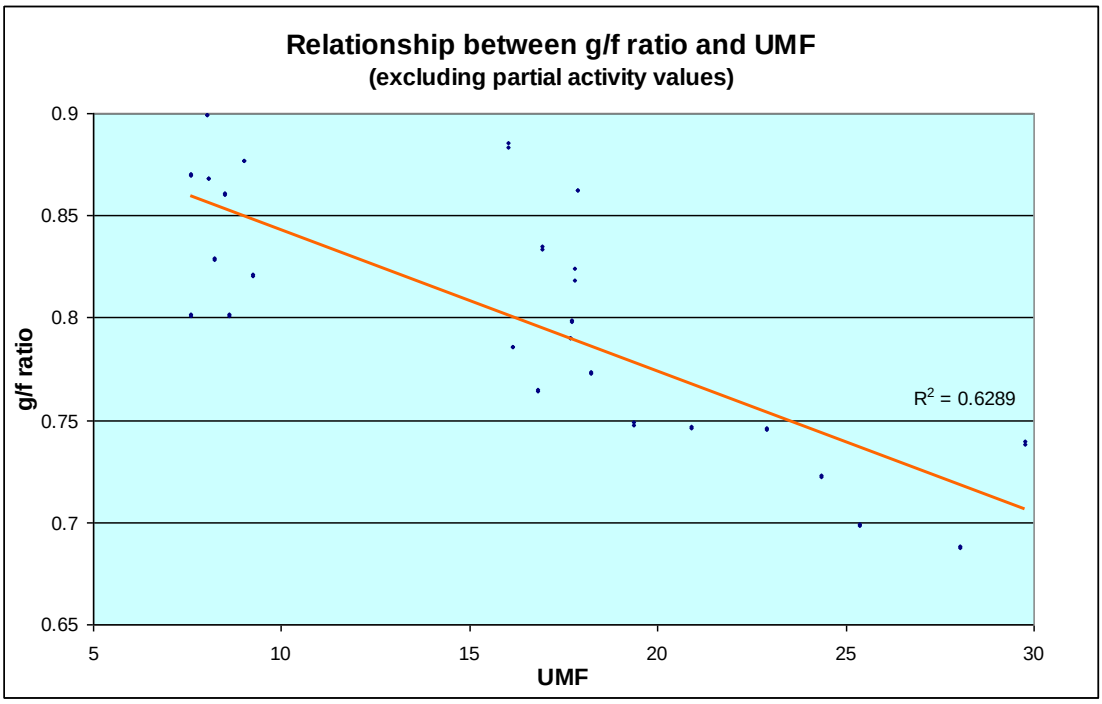


Figure 3.5 Plot of g/f ratio vs UMF™ (excluding partial activity results)

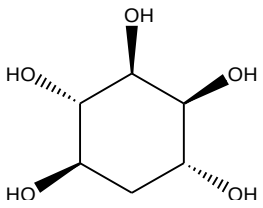
It is now known that the non-peroxide activity of manuka honey is due to the formation of methyl gloxal (MGO).[43, 44] The mechanism by which MGO is formed in honey is unknown, however MGO is known to be a carbohydrate degradation product, most likely from the degradation of glucose.[58] As the UMF™ activity (and thus MGO concentration) increases in a honey, a lower glucose level is detected (as evidenced by the decreasing g/f ratio with increasing activity in Figure 3.5. This is in keeping with the theory that MGO production in honey is linked to the degradation of glucose.[58] As to why this occurs at a significantly higher rate in manuka honey compared to any other floral source is unknown. Manuka honey has a lower than average moisture content and is therefore more viscous which can make harvesting difficult. Manuka honey combs must be pricked and centrifuged with heating in order to extract the honey from the comb. This intensive extraction process may also promote the degradation of sugars to MGO.

It has been shown that monosacharides are converted enzymatically to disaccharides with prolonged storage with the free glucose concentration decreasing more rapidly than fructose resulting in a lower g/f ratio.[96] The 38 manuka honeys used in this investigation were harvested in the 2003 season and once extracted stored under the

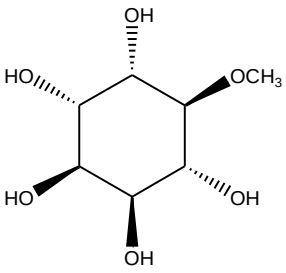
same conditions. This indicates that these honeys must contain an enzyme/s which increases the rate of conversion to higher saccharides independent of storage conditions.

3.3.1.1 Identification of Inositol in Honey by GC-MS

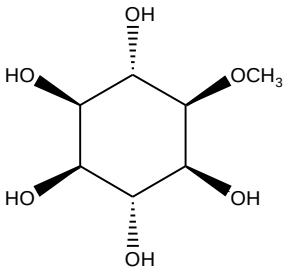
During the course of this investigation, an inositol like peak was observed in honey. The inositols quercitol **(7)**, pinitol **(8)**, methyl-muco-inositol **(9)**, muco-inositol **(10)** and myo-inositol **(11)** have previously been identified in honey.[97, 98]



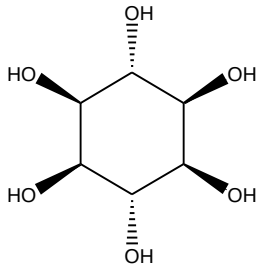
(7)



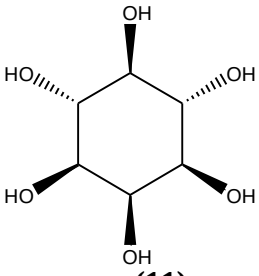
(8)



(9)



(10)



(11)

The inositol in manuka honey was identified as myo-inositol **(11)** by comparison of retention time and MS fragmentation pattern of *O*-trimethylsilyl myo-inositol (Figure 3.6) and the corresponding honey peak (Figure 3.7). All 38 manuka honey samples were found to contain myo-inositol with an average concentration of 0.041 ± 0.03 (% ether extracted freeze-dried honey), full results are given in Appendix A1.2.

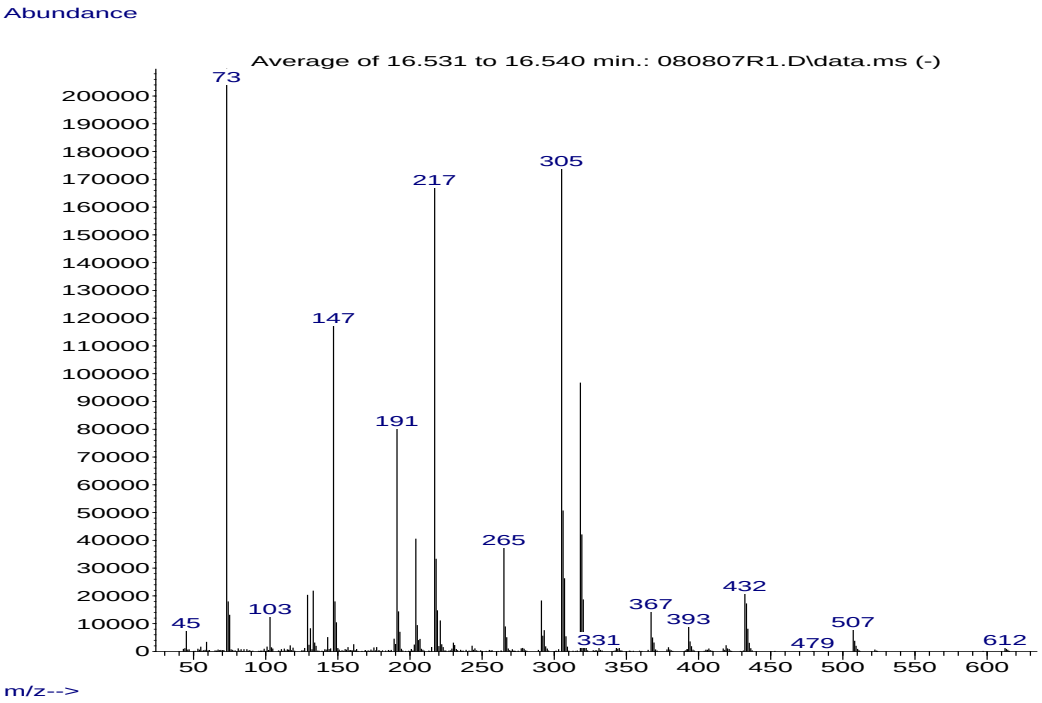


Figure 3.6 Mass spectra of *O*-trimethylsilyl myo-inositol

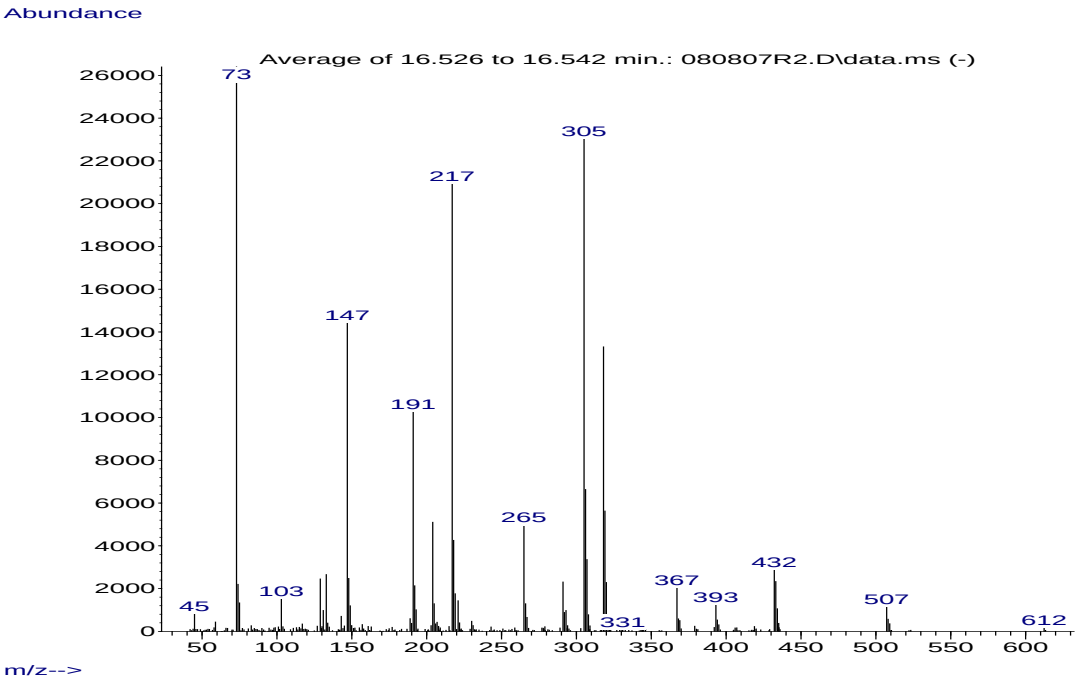


Figure 3.7 Mass spectra of the corresponding *O*-trimethylsilyl myo-inositol peak in manuka honey

3.3.2 Disaccharides in Honey

3.3.2.1 Quantitation of Nigerose,Turanose, Maltulose and Maltose

The analysis of complex mixtures of disaccharides by traditional methods is complicated by the coelution of some components. The reduction of a ketohexose forms two different products due to epimerisation at C2 of the alditol. Mixtures of nigerose, turanose, maltulose and maltose reduced with borohydride produce three products (Figure 3.8- Figure 3.9) and hence only three peaks (A, B and C) in the GC-FID chromatogram.

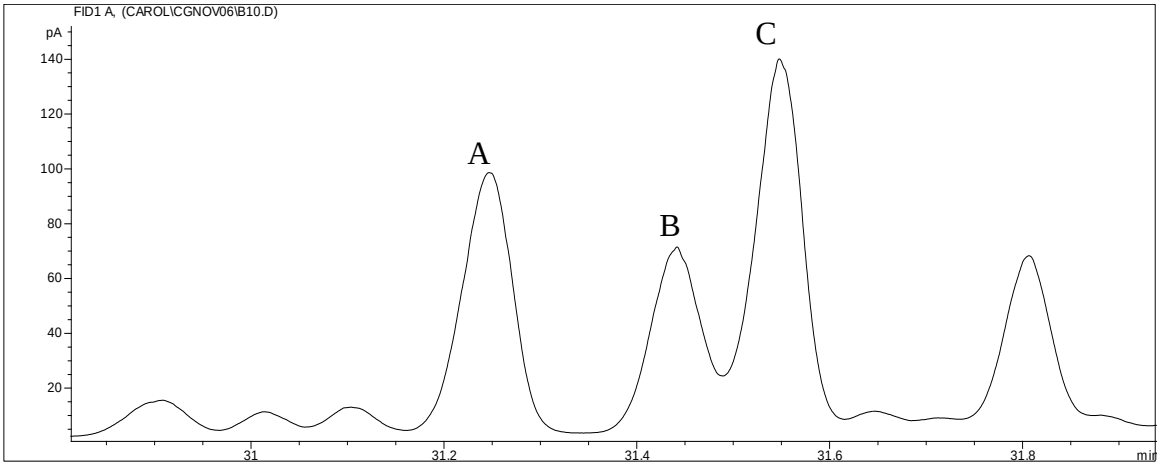
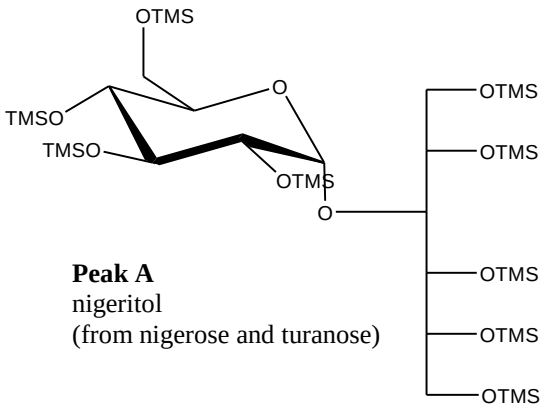
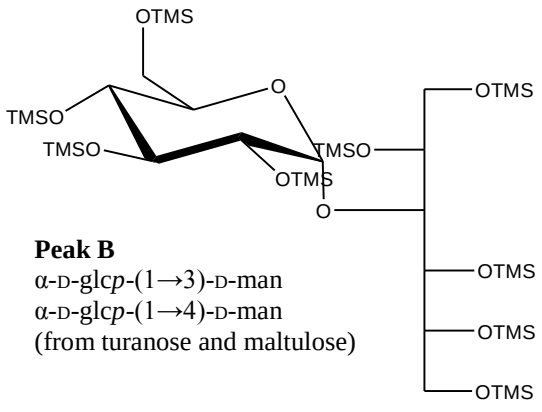


Figure 3.8 Chromatogram of reduced and silylated honey. A = nigeritol; B = α-D-glc*p*-(1→3)-D-man + α-D-glc*p*-(1→4)-D-man, C = maltitol



Peak A
nigeritol
(from nigerose and turanose)



Peak B
α-D-glc*p*-(1→3)-D-man
α-D-glc*p*-(1→4)-D-man
(from turanose and maltulose)

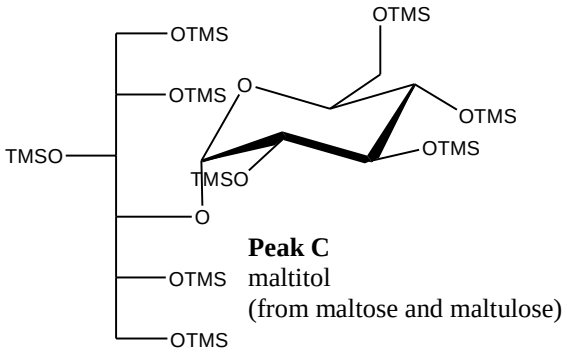


Figure 3.9 Reduction products of nigerose, turanose, maltulose and maltose

A previous study by Wu demonstrated that each pair of products could not be separated which results in the elution of three peaks. It was however suggested that the relative proportion of each parent disaccharide could be calculated by measuring the relative intensity of the *m/z* 307 and 308 ions by GC-MS-SIM after reduction with borodeuteride (Figure 3.10).[66]

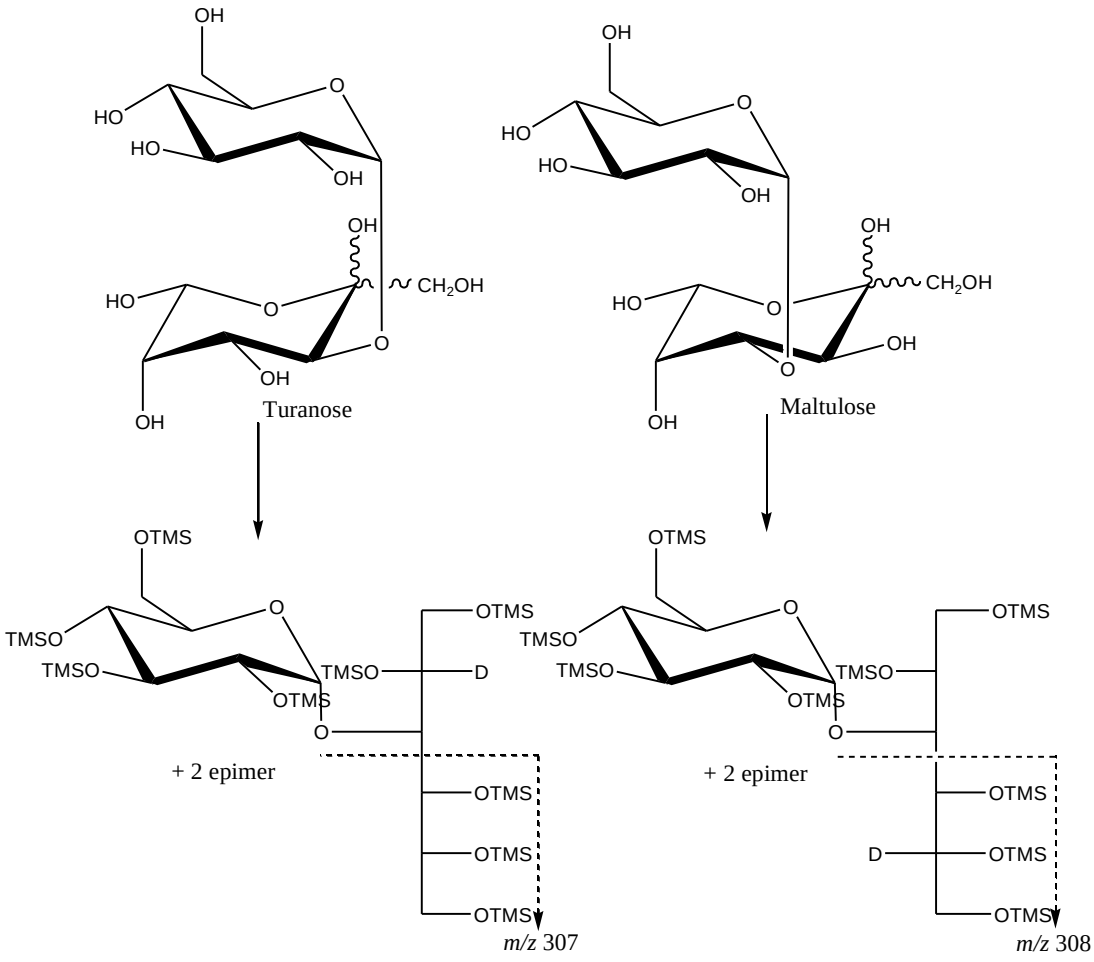


Figure 3.10 Borodeuteride reduction and silylation of turanose and maltulose

A series of mixed borodeuteride reduced nigerose and turanose standards were prepared and analysed by GC-MS-SIM. The ratio of the *m/z* 307 and 308 ion in the turanose/maltulose peak was measured by plotting the ion current arising from each ion and integrating the peak area.

Standards of known composition were used to establish the relationship between the ratio of turanose by weight and the abundance of the *m/z* 307 ion relative to *m/z* 308 ion (Table 3.4, Figure 3.11). This relationship was then used to calculate the ratio of turanose in honey.

Table 3.4 Contribution of turanose to *m/z* 307 ion

Weight (mg)		Area		% Turanose (weight)	% *m/z* 307 ion
Turanose	Maltulose	*m/z* 307 ion	*m/z* 308 ion		
1.20	2.30	45715	67641	34.3	40.3
1.97	1.28	171887	152596	60.6	53.0
1.80	1.38	91436	93852	56.6	49.3

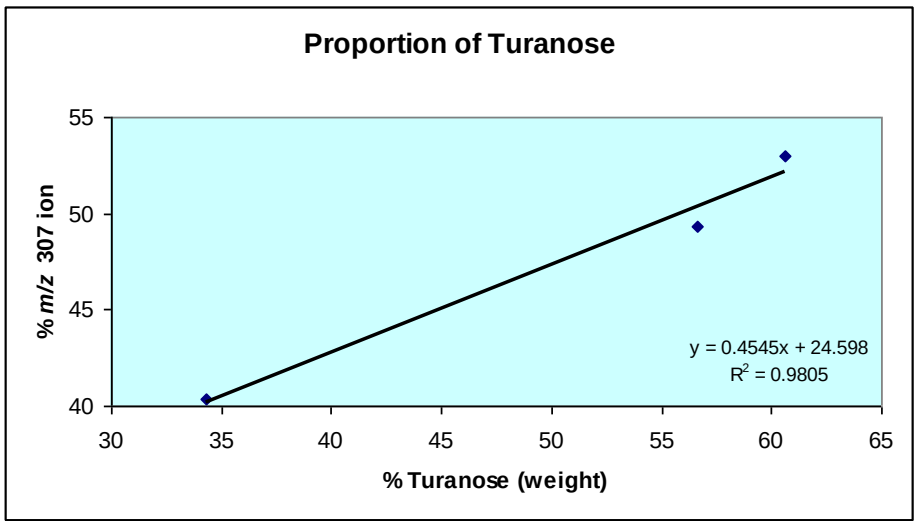


Figure 3.11 Relationship between the proportion of turanose and *m/z* 307 ion

The relative proportion of turanose in the manuka honey set is reported in Table 3.5. These results were used to calculate the proportion of nigerose, maltulose and maltose using Peak A/Peak B and Peak B/Peak C ratios (Section 2.1.9.3).

Table 3.5 Proportion of turanose in Peak B

Honey	Area		% *m/z* 307 ion	% Turanose
	m/z 307 ion	*m/z* 308 ion		
1	52215	47839	52.2	60.7
2	47048	46497	50.3	56.5
3	73652	69457	51.5	59.1
4	89197	79539	52.9	62.2
5	70432	67325	51.1	58.4
6	50491	43434	53.8	64.2
7	66919	54886	54.9	66.8
8	65689	52931	55.4	67.7
9	88877	81312	52.2	60.8
10	13719	12409	52.5	61.4
11	23213	20713	52.8	62.2
12	26037	22551	53.6	63.8
13	51838	41546	55.5	68.0
14	20994	18736	52.8	62.1
15	16105	15132	51.6	59.3
16	12271	10800	53.2	62.9
17	427028	363449	54.0	64.7
18	16441	13463	55.0	66.8
19	88910	73780	54.6	66.1
20	26994	24360	52.6	61.5
21	907063	851438	51.6	59.4
22	345384	340745	50.3	56.6
23	730388	774189	48.5	52.7
24	145918	116221	55.7	68.4
25	506006	480029	51.3	58.8
26	68645	65296	51.3	58.6
27	267563	244237	52.3	60.9
28	105025	100977	51.0	58.1
29	100364	82938	54.8	66.3
30	60788	41757	59.3	76.3
31	74656	63453	54.1	64.8
32	152148	131441	53.7	63.9
33	67697	59989	53.0	62.5
34	41803	32297	56.4	70.0
35	9327	9612	49.2	54.2
36	41360	37179	52.7	61.7
37	28435	26686	51.6	59.4
38	17334	15177	53.3	63.2

The proportion of each reduction product formed from a ketohexose is affected by stereochemical interactions. Often one product will be slightly more stereochemically favoured than the other and thus be present in greater quantity. The proportion of formation of each reduction product was calculated by measuring the Peak A and Peak B area of turanose (Figure 3.12) and Peak B and Peak C area of maltulose standards (Figure 3.13). Due to the restricted amount of material available, only 4

points for turanose, and three for maltulose were prepared. Each data point represents a separate preparation of the relevant sugar reduced with borodeuteride.

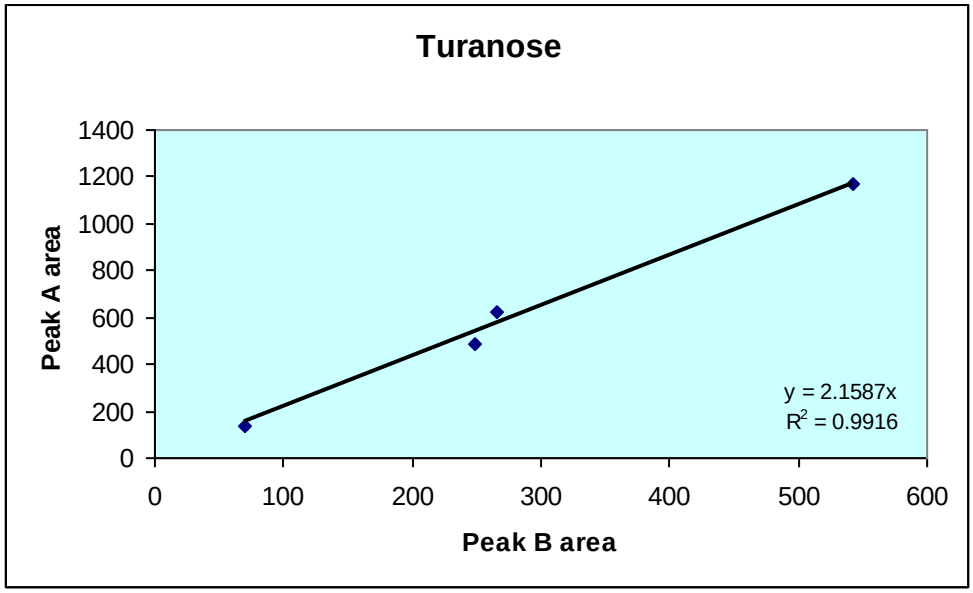


Figure 3.12 Relationship between Peak A and Peak B products of reduced and silylated turanose

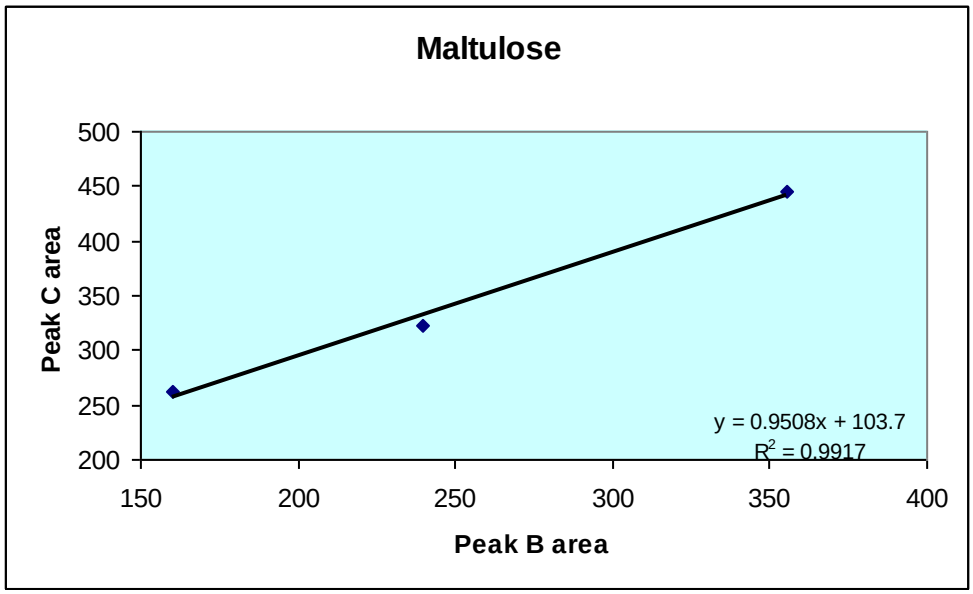


Figure 3.13 Relationship between Peak B and Peak C products of reduced and silylated maltulose

3.3.2.2 Standard Disaccharide and Honey Co-injections

The co-injection of standards with honey is commonly used to identify disaccharides in honey. Earlier work on disaccharides in manuka honey used co-injections as the principal identification technique.[66] Due to an improvement in chromatographic resolution, the peak assignment of three disaccharides differ from that of the previous investigation.

The co-injection of cellobiose with honey (Figure 3.14) appears to straddle two small peaks. Cellobiose was assigned to the first of these two peaks on the bases of ion ratio results (Section 4.3.2).

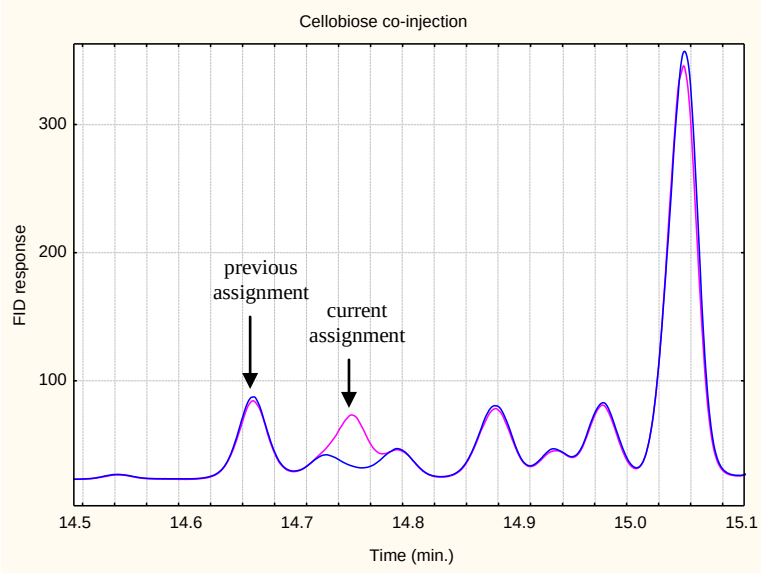


Figure 3.14 Co-injection of cellobiose with honey

Due to the improvement in chromatographic resolution, laminaribiose is now fully resolved from the two following peaks (Figure 3.15).

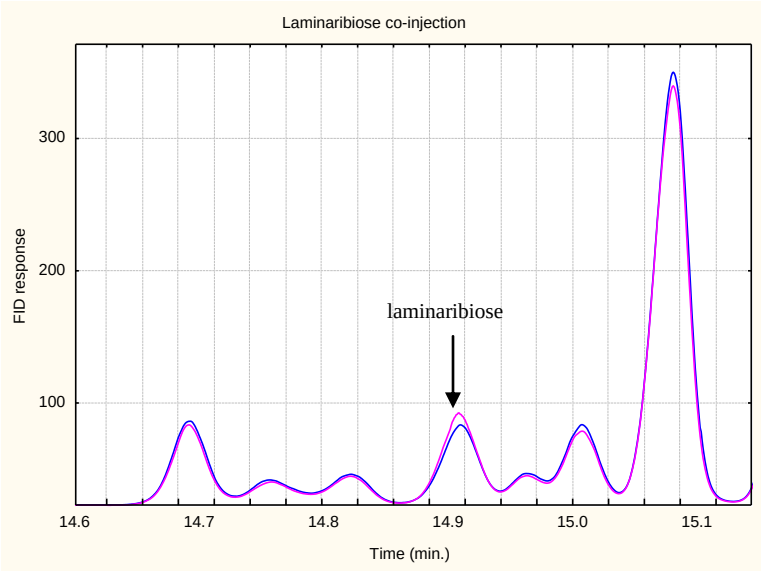


Figure 3.15 Co-injection of laminaribiose with honey

After the co-injection of gentibiose with honey it became clear that gentibiose elutes as a shoulder on a later eluting peak which has previously been identified as gentibiose (Figure 3.16). This shoulder is only apparent when a new column is used and was not detected by GC-MS. The identification of disaccharides by GC-MS-SIM produced ratio results which were consistent with a peak identification of gentibiose (Section 4.3.2). This suggests that the main component of this peak is also a β1-6 linked disaccharide. Due to the poor resolution of gentibiose and the subsequent disaccharide, both are reported as a single peak and assigned as gentibiose.

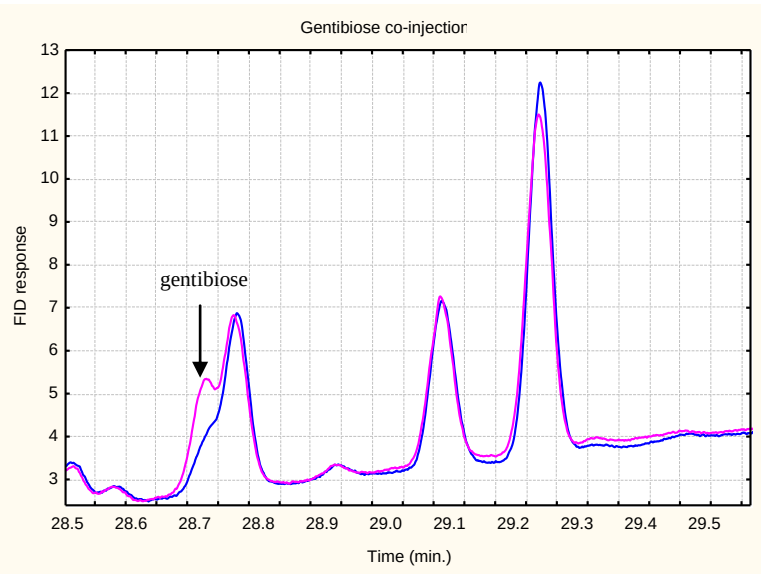


Figure 3.16 Co-injection of gentibiose with honey

There were no further changes in the assignment of the remaining disaccharides based on the co-injection results (Appendix A1.4). Using a combination of co-injection and MS fragment ratio data (Section 4.3.2), the quantification of disaccharides in honey was carried out using the following peak identification (Figure 3.17, Table 3.6). The linkage and configuration of unknowns is discussed in Section 4.3.2.

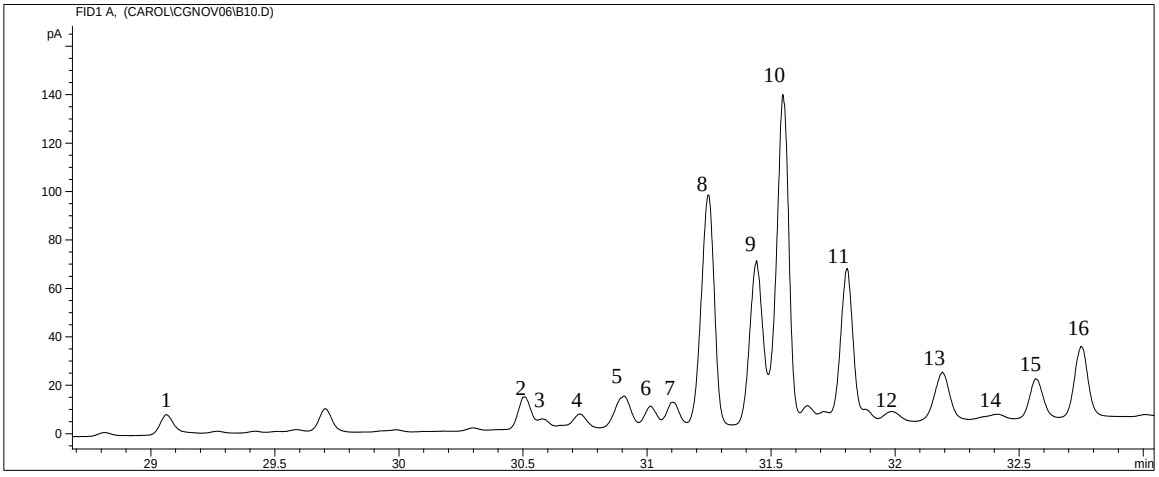


Figure 3.17 Identification of disaccharides in honey

Table 3.6 Peak identification of disaccharides

Peak	Disaccharide
1	sucrose
2	unknown 1
3	cellobiose
4	unknown 2
5	laminaribiose
6	unknown 3
7	unknown 4
8	nigerose/turanose
9	turanose/maltulose
10	maltulose/maltose
11	kojibiose
12	unknown 5
13	gentibiose*
14	unknown 6
15	unknown 7
16	isomaltose

*component of peak

3.3.2.3 Disaccharide Content of Manuka Honey

The disaccharide content of 38 manuka honeys was calculated as described in section 2.1.9. The average disaccharide content of manuka honey is given in Figure 3.18, full results are reported in Appendix A1.5.

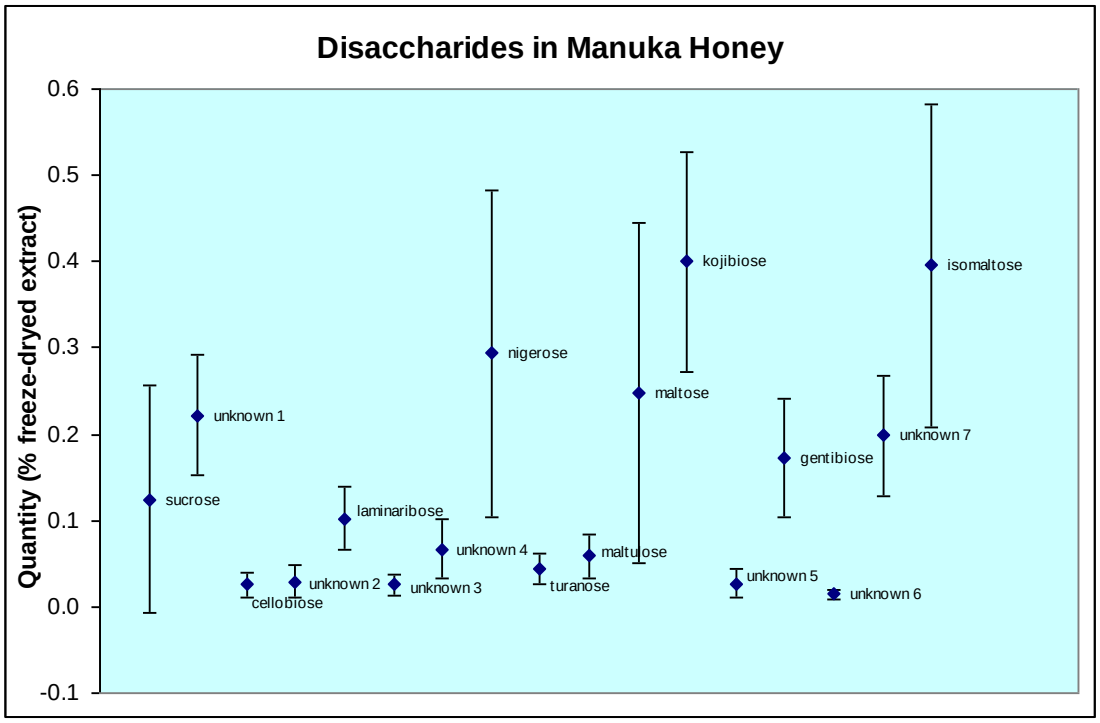


Figure 3.18 Average content and standard deviation of disaccharides in manuka honey

The relative contribution of each disaccharide to total disaccharides is reasonably consistent throughout all 38 manuka honeys. This "fingerprint" may not be unique to manuka honey as a previous investigation of manuka and ling heather honeys found no significant difference in the proportion of ling heather and manuka honey disaccharides.[66] This investigation by Wu was however limited to a small number of samples.

In this study, some variation is seen in the abundance of the more predominant disaccharides; in particular sucrose, nigerose, maltose and isomaltose. This variability appears to be present not only between different honeys but within replicates of the same honey and is not inconsistent with previous investigations.[66]

3.3.3 Trisaccharide Content of Manuka Honey

The trisaccharide composition of manuka honey was determined by GC-FID using the same preparation and analysis methods as for disaccharides (Sections 2.1.7 - 2.1.9). A typical manuka honey trisaccharide trace is given in Figure 3.19. A total of fourteen trisaccharides were detected, several of which were identified by co-injection with standards (Appendix A1.6). Peak assignments of identified trisaccharides are listed in Table 3.7.

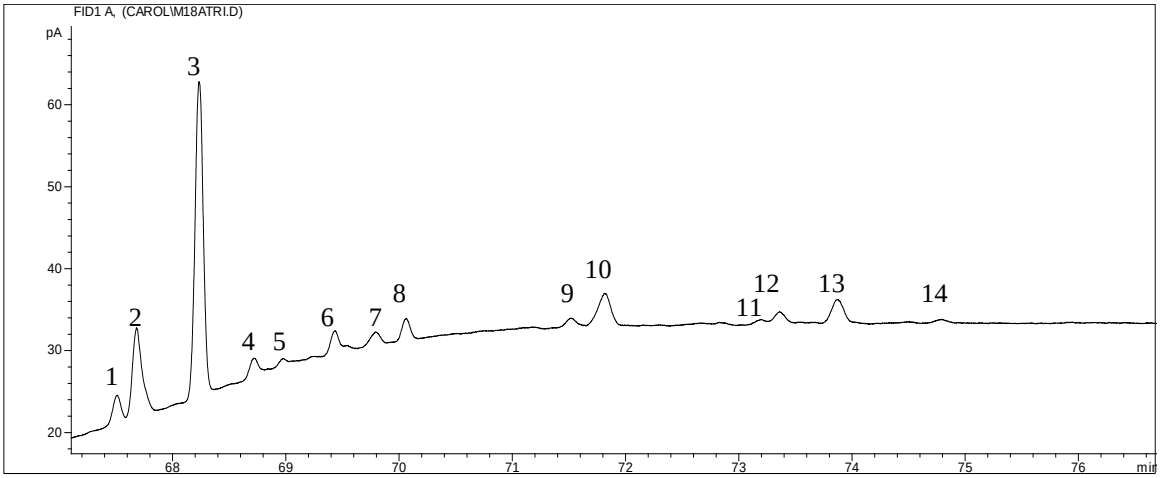


Figure 3.19 Trisaccharides in manuka honey

Table 3.7 Trisaccharides identified in manuka honey

Peak	Trisaccharide
2	1-kestose
3	erlose
4	melezitose
10	maltotriose
13	panose
14	isomaltotriose

One of the advantages of using a GC method for the analysis of trisaccharides is the very low detection limits obtainable. Due to the vast inter-sample variation achieved using this method, a decision was made to report results as qualitative only. A more appropriate method for the quantitative analysis of trisaccharides in honey would be HPAE-PAD providing minor constituents were not required or samples were concentrated prior to analysis due to lower sensitivity. It was deemed outside the requirements of this thesis to pursue this area further.

3.4 Statistical Analysis

The carbohydrate profile of manuka honey was evaluated using a variety of different statistical analyses to determine if a relationship exists between the carbohydrate composition and antibacterial activity (UMF™). A data set was constructed containing the concentration of the mono and disaccharides determined in each honey as described in Section 2.1.12.

3.4.1 Data Pre-Processing

In data sets comprising of a wide range of values, a relationship is often seen between the mean and variance of the data, otherwise known as heteroscedasticity. This is undesirable as it will bias results. In order to correct for this, data must first be transformed in a suitable manner to remove this relationship.

Commonly used transformations for this purpose include: reciprocal, reciprocal square root, log and square root.[99] In situations when the standard deviation is proportional to the mean, a data transformation of Y = log y will stabilise variance. This is often the case with analytical data.

In order to establish if any transformable inhomogeneity exists in the raw data, a scatter graph was constructed plotting the mean of the individual disaccharide concentration (mean of 38 honeys) against the individual disaccharide standard deviation (average of 38 honeys). Glucose and fructose results were excluded from this graph (Figure 3.20) in order to improve visualisation as these compounds were present in far higher concentrations than the disaccharides. Each point in Figure 3.20 represents an individual disaccharide.

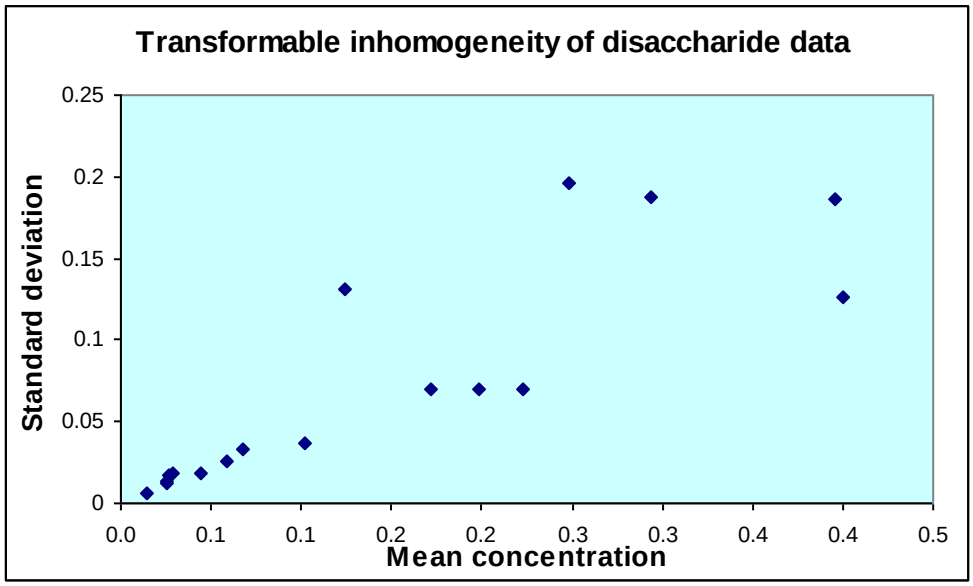


Figure 3.20 Plot of the standard deviations for each individual disaccharide vs mean of concentration of each individual disaccharide in 38 manuka honeys

A general linear trend, which is undesirable, can be seen in the graph of transformable inhomogeneity which indicates that a log transformation is required. After the log transformation was undertaken, no relationship was seen between the mean and standard deviation. All subsequent statistical analyses were therefore performed on the log transformed carbohydrate data set.

3.4.2 Exploratory Statistical Techniques

3.4.2.1 Cluster Analysis

Cluster analysis is used to identify whether any underlying patterns or structure exist in data. Hierarchical clustering is explained in more detail in Section 2.4.1. A range of different distance measures were used in the hierarchical cluster analysis of the log carbohydrate data. In order to aid visualisation, only the first out of three triplicate results were included in the analysis. The dendrogram of the resulting analysis using Ward's method as the distance measure is depicted in Figure 3.21.

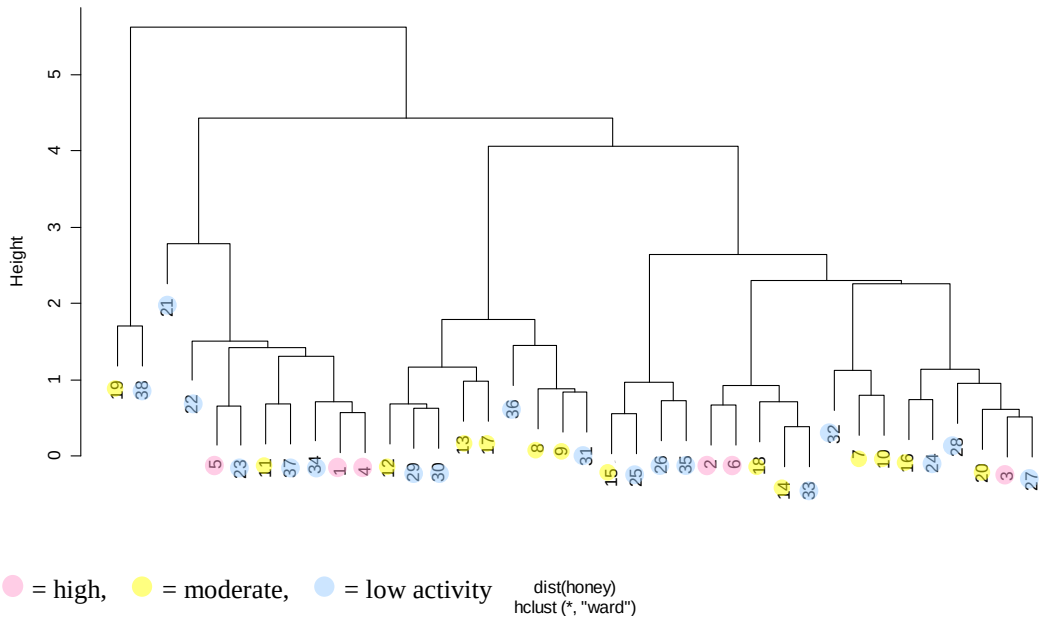


Figure 3.21 Dendrogram of the log carbohydrate matrix as a function of UMF™ activity using Ward's method

Very little clustering between activity levels was apparent in any of the dendrograms using various distance measure therefore it can be concluded that a more sophisticated level of modelling is required.

3.4.2.2 Principal Components Analysis

Principal components analysis (PCA) is used to reduce the dimensionality of the data while retaining useful information. PCA was undertaken on the log carbohydrate data set using the statistical package R as described in Section 2.4.1. No standardisation or centering of the data was performed prior to PCA. The variance was found to be spread over a large number of components with the first 10 components accounting for 93% of the variance (Figure 3.22). A score plot of the first two principal components is shown in Figure 3.23.

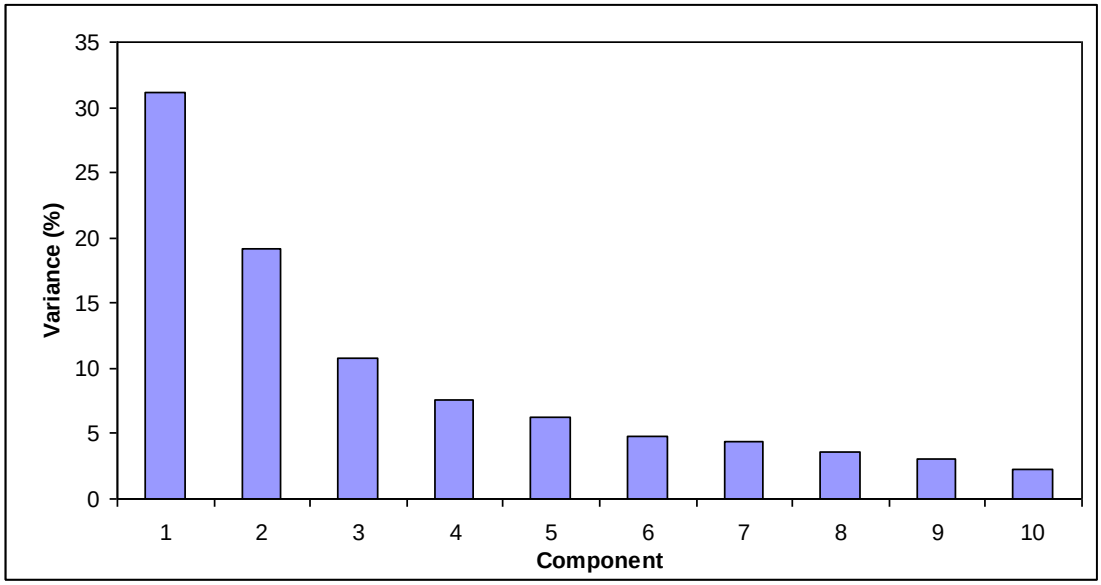
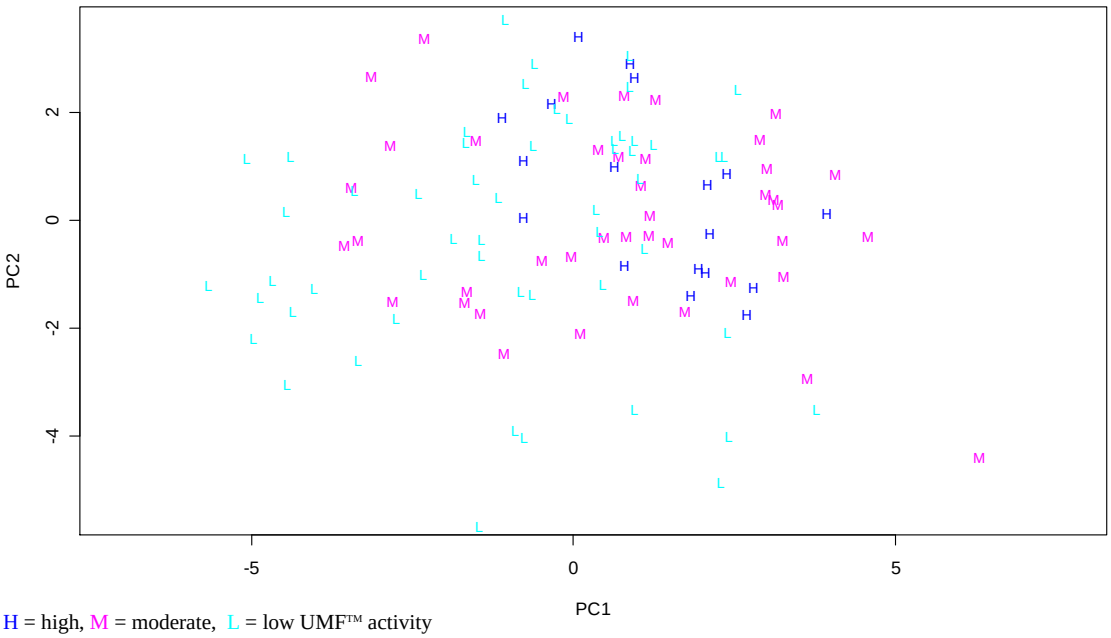


Figure 3.22 Scree plot from PCA of log carbohydrate matrix



H = high, M = moderate, L = low UMF™ activity

Figure 3.23 Score plot of PC2 vs PC1 for the log carbohydrate data matrix

While no true separation is seen between activity levels in the first two principal components, high activity honeys are centered towards the top right of the score plot compared to the low activity honeys which are distributed more to the left.

Hierarchical analysis was applied to the PCA scores using the same distance measures described above. Ward's method once again produced the best separation with the high activity samples being grouped in the central two clusters (Figure 3.24). The hierarchical cluster analysis on the PCA scores obtained slightly better separation compared to the full log carbohydrate matrix (Figure 3.21). High activity honeys were centered in the central clusters in the dendrogram. The moderate and low activity honeys were spread relatively evenly between all clusters.

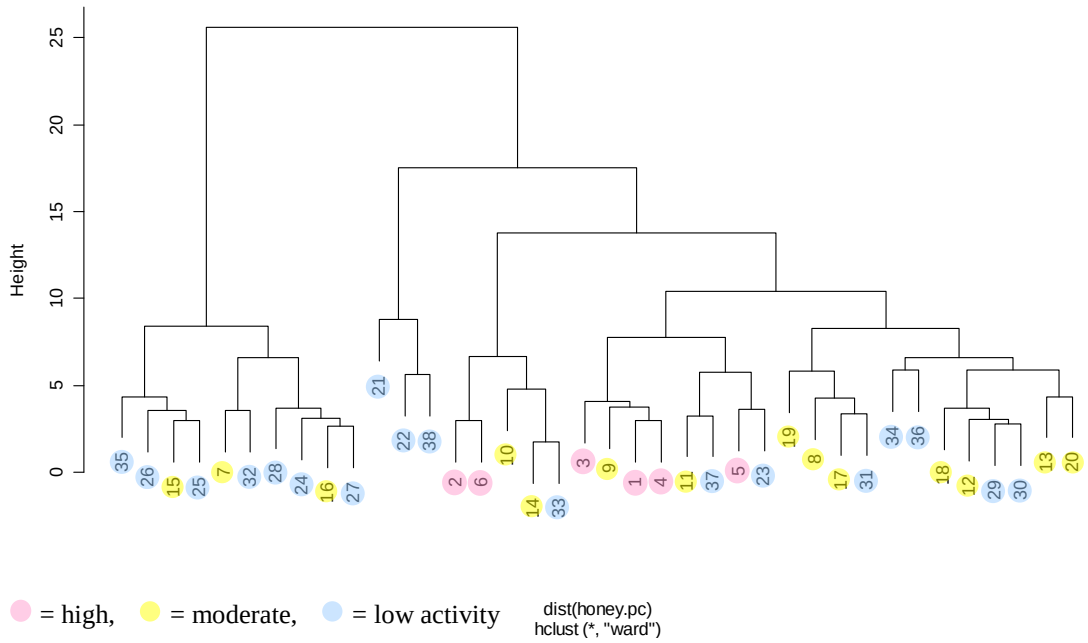
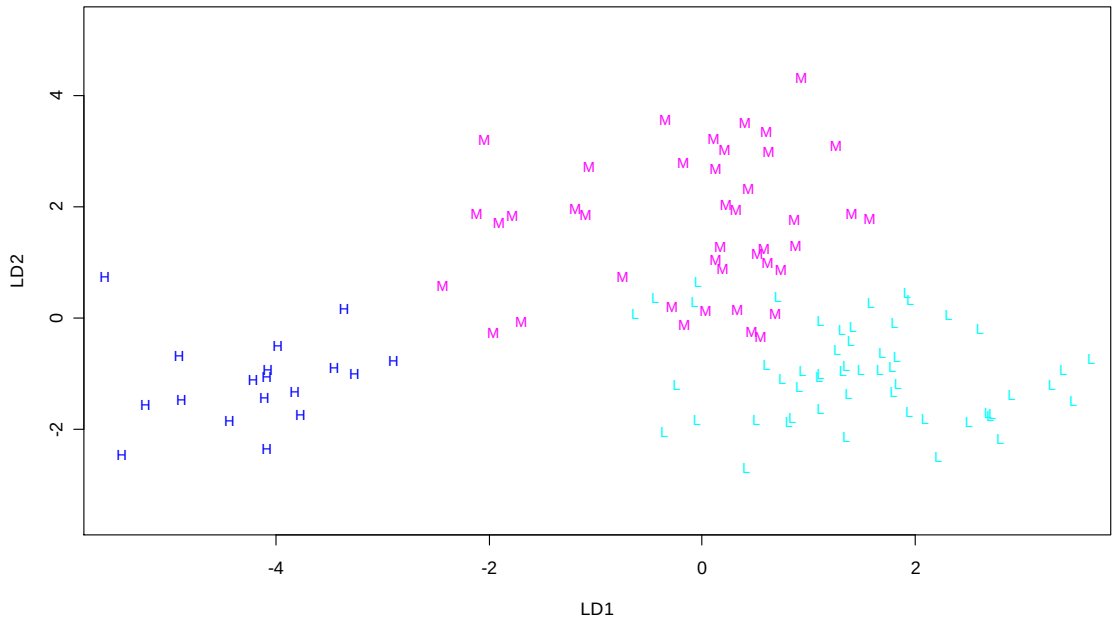


Figure 3.24 Dendrogram of PCA scores a function of UMF™ activity using Ward's method

Due to the poor degree of separation between activity levels, unsupervised methods were deemed insufficient for classifying the activity level based on the carbohydrate profile.

3.4.2.3 Linear Discriminant Analysis

Linear Discriminant Analysis (LDA) was conducted on the log carbohydrate data set as described in Section 2.4.1. The score plot of the first two Linear Discriminants is given in Figure 3.25.



H = high, M = moderate, L = low UMF™ activity

Figure 3.25 Score plot of LD2 vs LD1 conducted on the log carbohydrate data set

A degree of separation between the activity levels is apparent with minimal overlap between low and moderate activity honeys. The first Linear Discriminant accounts for a majority of the separation between the three groups. The separation between moderate and low activity honeys is improved with the inclusion of the second discriminant. Some overlap between moderate and low activity honeys may be as a consequence of how these honeys were classified as the splitting between activity levels was arbitrary.

The coefficients of Linear Discriminants produced from LDA indicate which compounds can be attributed to the differentiation between activity levels. The coefficients of Linear Discriminants obtained from LDA on the log carbohydrate data set are given in Table 3.8. The magnitude of each coefficient indicates the importance of each component in the discriminant model.

Table 3.8 Coefficients of Linear Discriminants

Compound	LD1	LD2
glucose	79.93	-12.00
fructose	3.63	-49.81
sucrose	1.24	2.21
unknown 1	1.54	-0.50
cellobiose	0.06	-5.29
unknown 2	-0.28	0.15
laminaribiose	-10.71	4.41
unknown 3	-0.58	2.86
unknown 4	5.18	-2.38
nigerose	-0.04	-1.48
turanose	5.69	-1.80
maltulose	-5.87	1.65
maltose	-0.43	-0.08
kojibiose	-0.19	4.59
unknown 5	2.09	-0.43
gentibiose*	1.08	-6.91
unknown 6	0.71	0.15
unknown 7	-0.80	0.62
isomaltose	3.53	-0.29

From an examination of the coefficients of Linear Discriminants, it can be seen that glucose is by far the most important compound in the first discriminant followed by laminaribiose and maltulose. Fructose is the most important compound in the second discriminant followed by glucose and gentibiose. Unknown 1, unknown 2, nigerose, maltose, unknown 6 and unknown 7 are of little importance for either discriminant.

Given that a linear relationship was seen between UMF™ and glucose/fructose ratio (Section 3.3.1), it is not surprising that both glucose and fructose are important components in the Linear Discriminant model.

3.5 Conclusions and Recommendations

The quantitative analysis of the carbohydrate profile of manuka honey was achieved using a combination of HPLC, GC-FID and GC-MS.

The analysis of carbohydrates in honey can serve several purposes, but in this case only two are relevant.

- Provide some evidence to assist in establishing the origin of UMF™ activity and to act as a potential marker for UMF™ activity.

- To provide a library of disaccharide profiles for comparison purposes when detecting adulteration.

A relationship between monosaccharide composition and UMF™ activity was observed, where the glucose/fructose ratio was found to decrease with increasing UMF™ activity. This may be linked to methyl glyoxal content of the honey (a publication which appeared subsequent to submission of this thesis indicates that methyl glyoxal in manuka honey originates from dihydroxyacetone in the nectar of the flower[1]; this taken together with the evidence of the glucose/fructose ratio may indicate some abmormality of glycolysis). No relationship between the disaccharide profile and UMF™ activity was immediately obvious due to the large variance observed between honeys; however, when the results were analysed by more sophisticated methods, more information was obtained.

The saccharide profile was successfully used to distinguish between high, moderate and low UMF™ activity honeys by Linear Discriminant Analysis. An examination of the Linear Discriminants indicates that glucose concentration was the single most important compound in the first discriminant which accounts for the majority of the separation in the model. Fructose was the most important compound in the second Linear Discriminant which improves the separation between moderate and low activity honeys. The disaccharides laminaribiose, unknown 4, turanose and maltulose also contributed to the first discriminant despite the large degree of variability seen in disaccharide concentrations determined using the GC method.

Despite the small data set, a strong relationship can be seen between the carbohydrate composition (in particular glucose) and the activity level.

[1] Adams, C.J.; Manley-Harris, M.; Molan, P. The origin of methylglyoxal in New Zealand manuka (*Leptospermum scoparium*) honey. *Carbohydrate Research*. **2009**, 344, (8), 1050-1053.

The data obtained here can be used as a benchmark for detection of adulteration of manuka honey. Given the high price of manuka honey, it is a tempting target for adulteration by sugar syrups or bee feeding. No similar disaccharide profiles have been prepared for any other New Zealand unifloral honeys other than beech honeydew honey.[66, 82] As some of the other unifloral honeys also command high prices, it would be appropriate to prepare a more comprehesive library. While the above-mentioned GC-FID/MS method was acceptable for disaccharides; it is time consuming, tedious and prone to giving inconsistent results. The proportion of nigerose, turanose, maltose and maltulose was calculated in honey using the ratio of *m/z* 307 to *m/z* 307 ion responses as determined by GC-MS-SIM. The quantitation of these four disaccharides should be viewed as an approximation only as very few points were used to construct the calibration graphs (due to the limited supply of disaccharide standards) and cumulative errors in the calculation process. The development of an HPAE-PAD or similar method would enable the rapid determination of all main di and trisaccharides in honey.

By using multivariate statistical methods, the prevalence of overlapping peaks and multiple unknown compounds does not negatively impact on the performance of the model. It would be of interest to ascertain the extent to which the carbohydrate profile can be used to predict UMF™ activity, floral origin and adulteration using an analysis method such as HPAE-PAD which is more suited to the rapid analysis of large numbers of samples.

Chapter 4

Identification of Disaccharides in Honey by GC-MS-SIM

4.1 Introduction

The intensity of specific ions in the GC-MS of reduced and silylated disaccharides have been found to provide useful structural information. To date sixteen disaccharides have been found in honey (Table 3.1), twelve of which were used in this investigation (Table 3.2).

The identification of disaccharides in honey is generally achieved by comparison of relative retention time to prepared standards. Disaccharides in honey are commonly derivatised to the trimethlysilyl ether form either as the oxime or reduced version. Mixtures of disaccharides derivatised to form oximes or reduced to form alditols can produce multiple products which are unable to be separated by gas chromatography. This can lead to misidentification of parent disaccharides if retention time is the only form of identification.

The introduction of other classification variables such as linkage position will significantly increase the likelihood of correct identification.

4.2 Review

O-trimethylsilyl disaccharide aldosyls and alditols produce very similar fragmentation patterns by GC-MS, an example of a typical spectra is depicted in Figure 4.1. Several studies have been conducted using the relative intensity of specific ions to determine structural characteristics of disaccharides. The linkage position of (1↔1), (1→2), (1→3), (1→4), (1→5), (1→6) *O*-trimethylsilyl aldosyl oligosaccharides can be determined by the relative peak intensities of selected ions relative to the (*m/z*) 361 ion.[100]

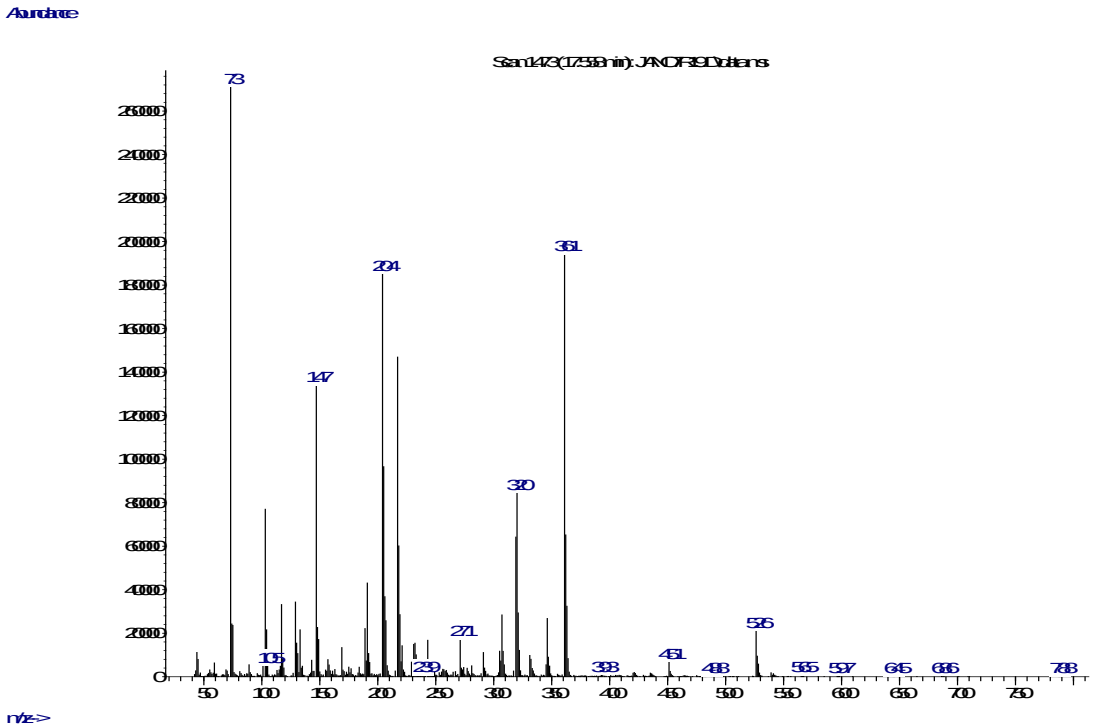


Figure 4.1 GC-MS of *O*-trimethylsilyl kojibiitol

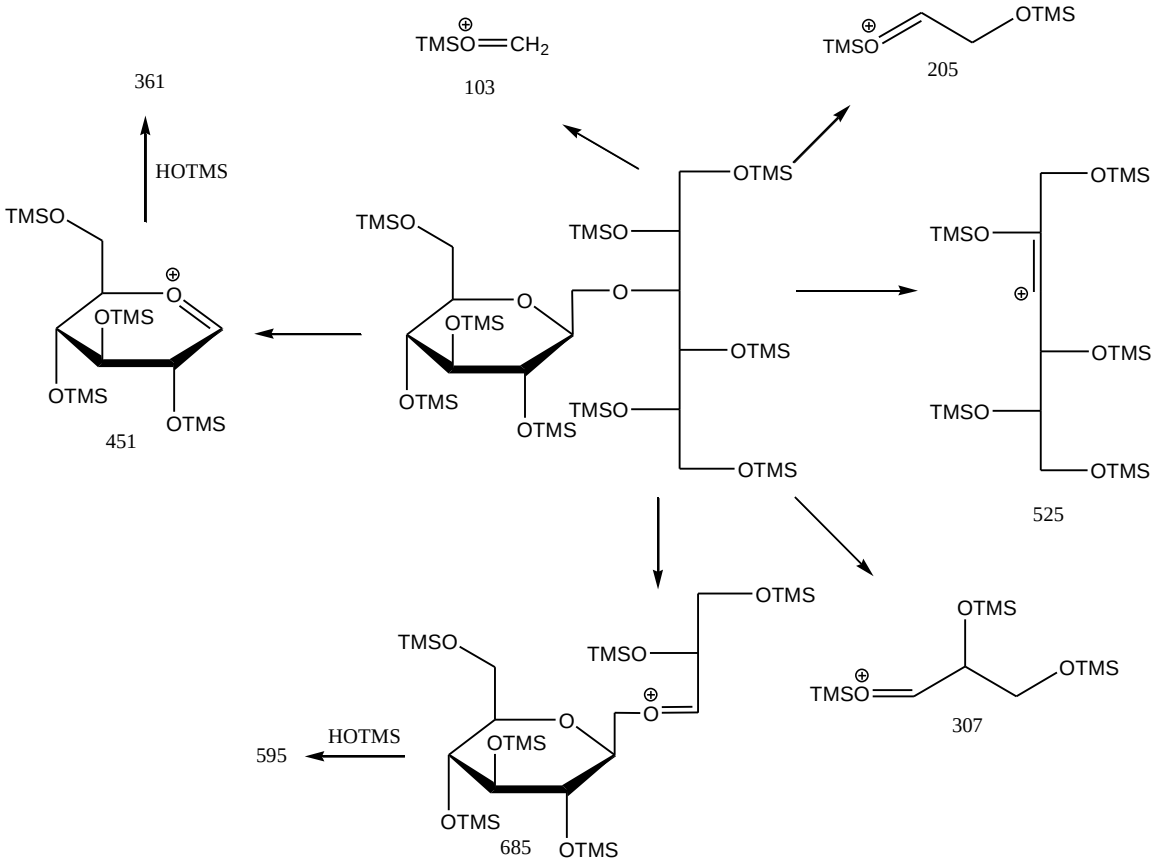
The linkage position of (1→3), (1→4) and (1→6) linked *O*-trimethylsilyl disaccharide alditols reduced with sodium borodeuteride, can be distinguished by the relative intensity of selected ions relative to the (*m/z*) 217 ion (Table 4.1).[101] The same ions were seen in almost all *O*-trimethylsilyl disaccharide alditols with only the relative intensity of some ions being affected by linkage position (Scheme 4.1).

After reduction with borodeuteride, a proportion of the *m/z* 307 ion is converted to the deuterated *m/z* 308 ion. The extent of this conversion is dependent on linkage position. A greater conversion of the *m/z* 307 ion to the deuterated *m/z* 308 ion occurs with (1→4) and (1→6) linked disaccharides compared to (1→3) linked disaccharides. The analogous conversion of the *m/z* 685 and 595 ions to the corresponding deuterated *m/z* 686 and 596 ions, respectively is prevalent in (1→3) linked structures but does not occur with (1→4) and (1→6) linked disaccharides.

Table 4.1 Relative conversion levels of selected ions observed in the mass spectra of silylated borodeuteride reduced disaccharides[101]

	Linkage position		
Conversion	**(1→3)**	**(1→4)**	**(1→6)**
m/z 205 → *m/z* 206	low	low	high
m/z 307 → *m/z* 308	low	high	high
m/z 595 → *m/z* 596	high	high	low
m/z 685 → *m/z* 686	high	low	low

Scheme 4.1 Fragmentation of *O*-trimethylsilyl cellobiitol (*m/z*)[102, 103]



To date all studies have been conducted using pure standards, the potential of the above mentioned methods to distinguish linkage position in complex mixtures has not been reported.

A number of ions which are present in the mass spectrum of reduced and silylated disaccharides were examined to determine if a relationship could be found between ion intensity and linkage position. Initially, pure standards were used for this investigation to establish intensity ranges for each linkage position. Data obtained from the

investigation of standards were used to evaluate the possibility of using this technique for determining linkage position in complex mixtures such as honey.

4.3 Results and Discussion

4.3.1 Differentiation of Linkage Position in Pure *O*-Trimethylsilyl Disaccharide Alditols

An examination of the relative intensity of ions suggested by Kärkkäinen and a range of other ions identified in the mass spectrum suggested that the intensity of selected ions could be used to differentiate linkage position.[101] It was proposed that relative ion intensities should be expressed as a percentage of the *m/z* 217 ion intensity as this ion displays the least variability.[101] The relative intensity of ions has also been calculated relative to the predominant *m/z* 361 ion.[100] The *m/z* 73 ion, a predominant ion in all spectra was also included in this investigation.

The reduced and silylated analogues of standard disaccharides (Table 3.2) were examined by GC-MS-SIM. A total of fourteen ion intensities were measured; seven of these ions are shown Scheme 4.1, however some of the ions are deuterated analogues where the deuterated atom may be present at C_1 or C_2 (Figure 4.2). These ions were measured relative to the *m/z* 217, 73 and 361 ion intensities observed for the respective compounds (Figure 4.3, Table 4.1 - 4.3). The fourteen ions comprised of eight ions identified by Kärkkäinen along with six other ions (Figure 4.2) visually identified from the mass spectra as possibly correlating with linkage position. However, these latter ions are not expected to be relevant in determining structure or configuration of non-reducing disaccharides.

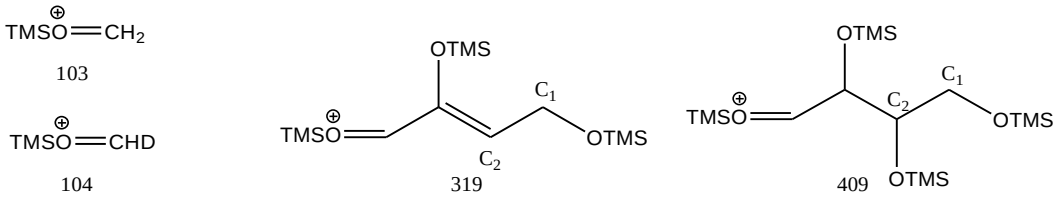


Figure 4.2 Structure of fragmentation ions chosen for analysis where either C_1 or C_2 of *m/*z 319 and 409 ion may be deuterated

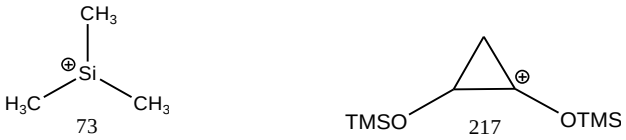


Figure 4.3 Structure of *m/z* 73 and 217 fragmentation ions[102, 103]

Reduction of ketohexoses produces two products differing in configuration at C2 of the alditol. The mass spectra of both products were examined separately to produce fifteen products from twelve standards.

The relative intensity of selected ions were plotted in order to visually establish correlations between linkage position and/or configuration of each standard (Figure 4.4 - Figure 4.9). The relative intensity of these ions are presented in Appendix A2.1.

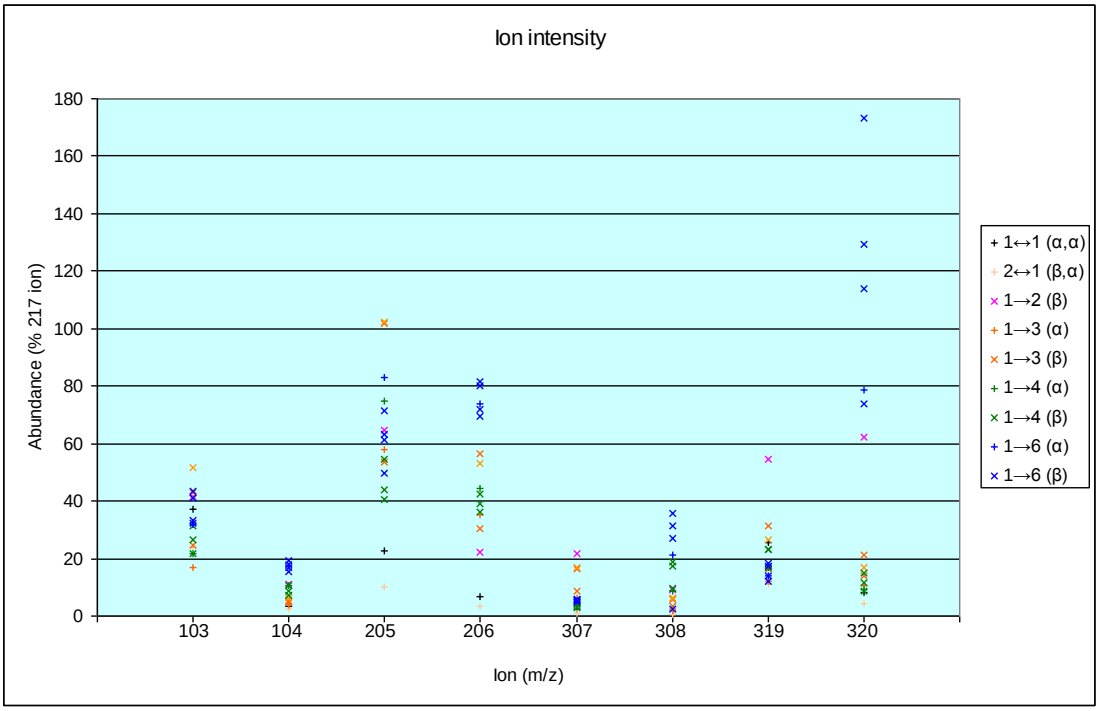


Figure 4.4 Plot of selected ion intensities as a % of the *m/z* 217 ion intensity

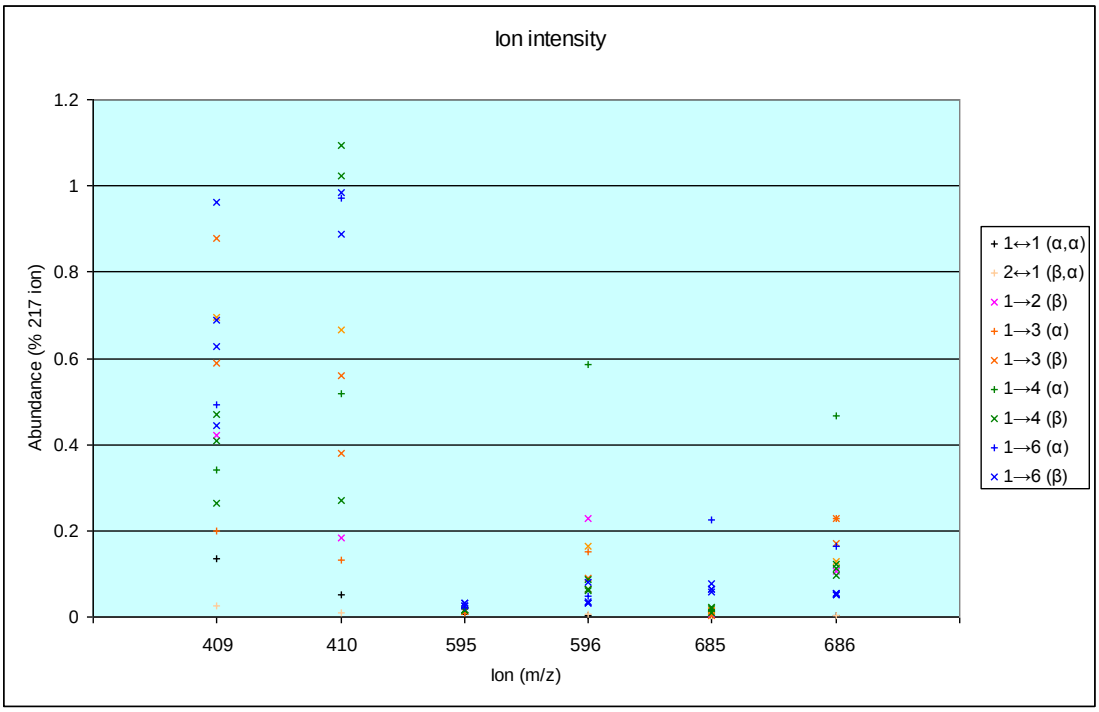


Figure 4.5 Plot of selected ion intensities as a % of the *m/z* 217 ion intensity

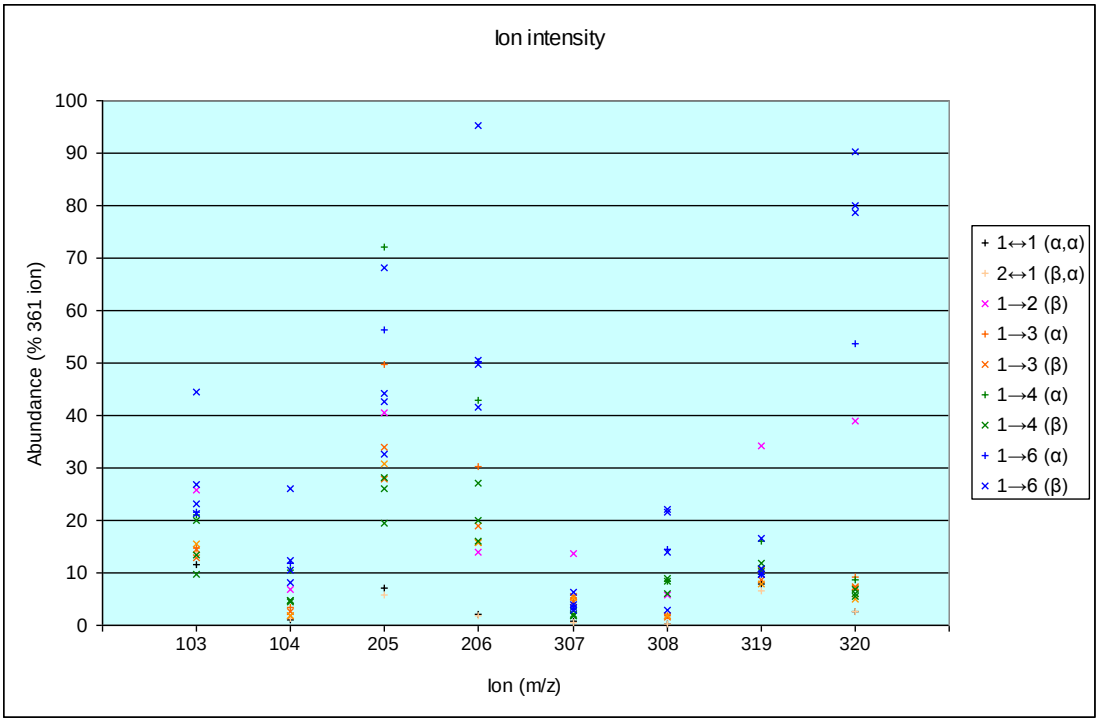


Figure 4.6 Plot of selected ion intensities as a % of the *m/z* 361 ion intensity

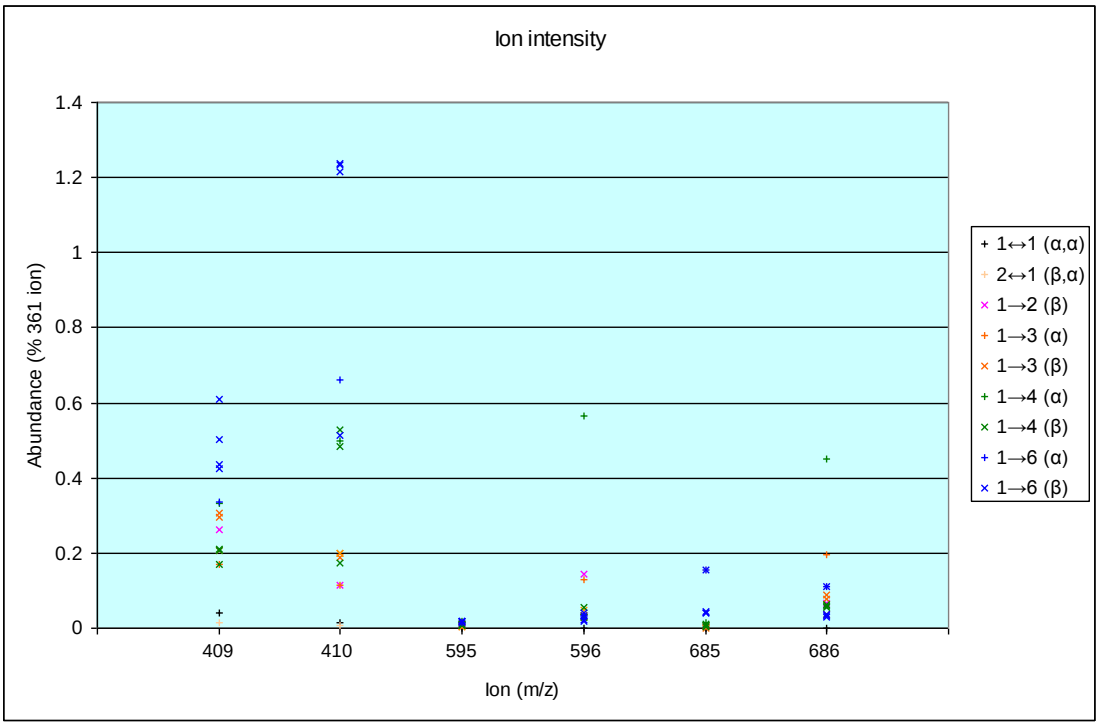


Figure 4.7 Plot of selected ion intensities as a % of the *m/z* 361 ion intensity

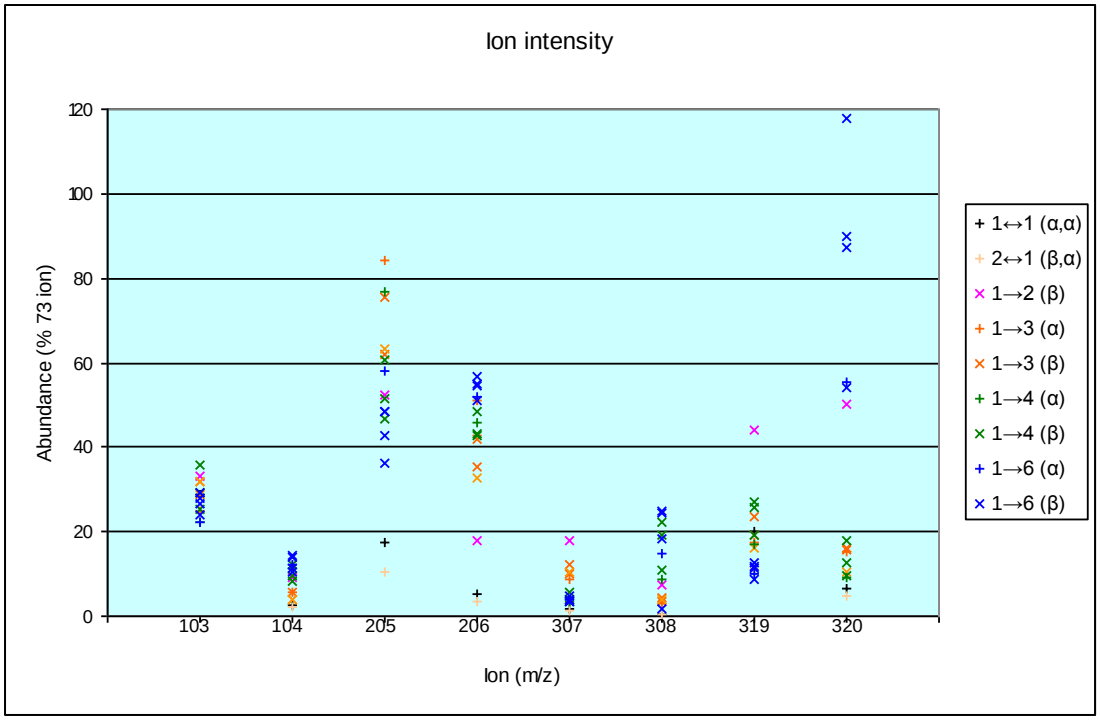


Figure 4.8 Plot of selected ion intensities as a % of the *m/z* 73 ion intensity

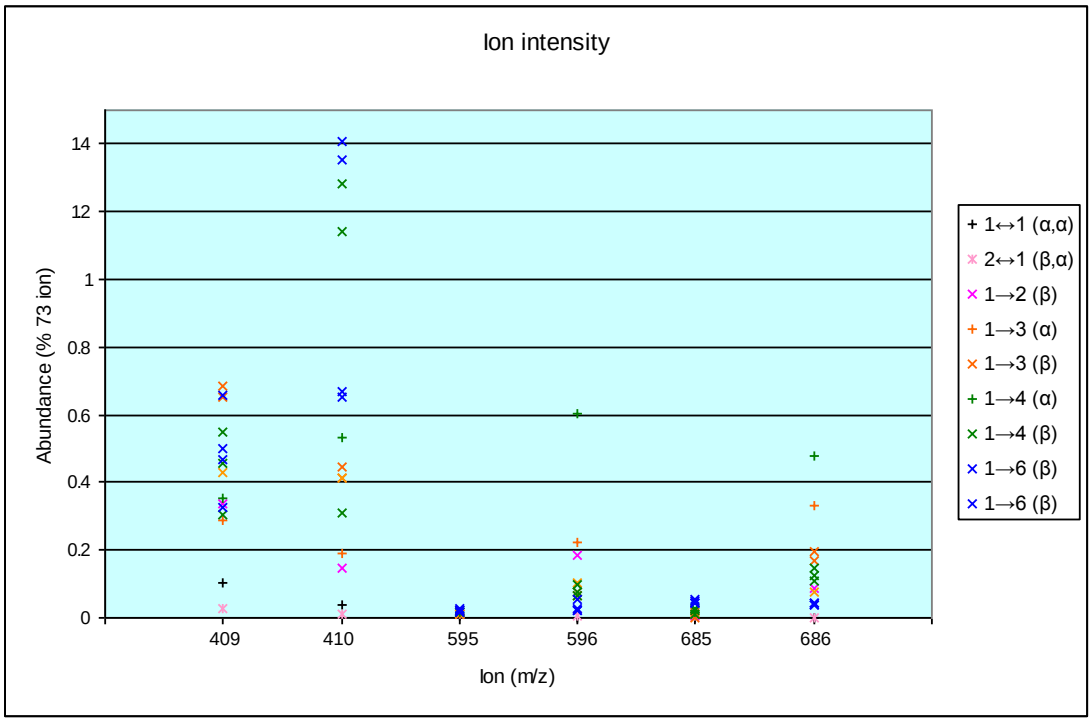


Figure 4.9 Plot of selected ion intensities as a % of the *m/z* 73 ion intensity

On the basis on the differentiation of linkage position by selected ions, six ions presented as ion intensity ratios were chosen for further examination. These chosen ions were those which displayed a difference in intensity between linkage positions in a visual examination of ion intensity ratios differing by one mass unit. These ions were examined as pairs: *m/z* 307 vs *m/z* 308 ion intensities (relative to *m/z* 73 ion), *m/z* 319 vs *m/z* 320 ion intensities (relative to *m/z* 73 ion) and the *m/z* 205 vs *m/z* 206 ion intensities (relative to *m/z* 361 ion). The normalisation ion is important because it introduces a unique scaling factor. This can be demonstrated by examining two plots of identical ion intensities relative to different normalisation ions (Figure 4.10).

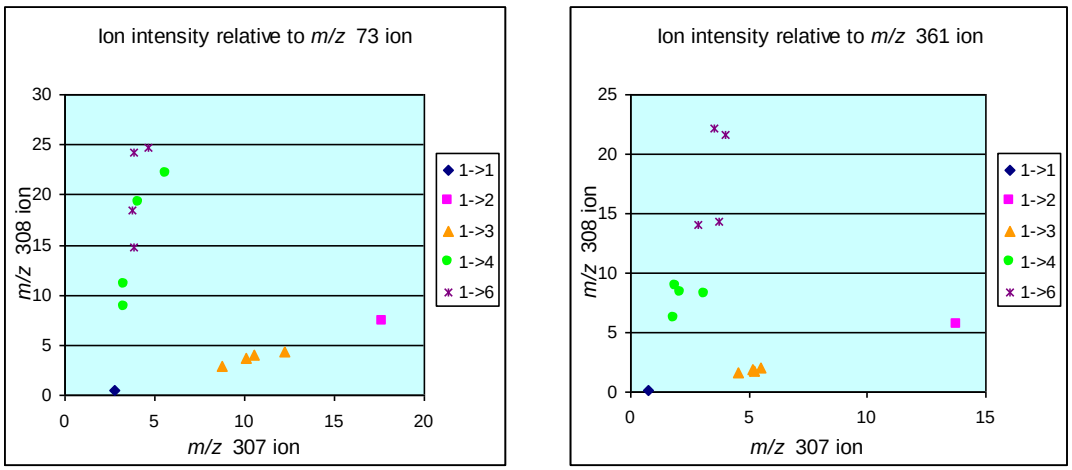


Figure 4.10 Plot of *m/z* 308 vs *m/z* 307 ion intensities normalised against the *m/z* 73 or *m/z* 361 ion

While some ions are relatively unaffected by the normalisation ion, the relative intensity of others ions such as with 1→4 and 1→6 linked *O*-trimethylsilyl disaccharide alditols seen above can have a significant effect on the clustering.

As the relative abundance of the *m/z* 205, 206, 307 and 308 ions is relatively low, a separate analysis was conducted scanning for only the necessary ions in order to maximise response.

To investigate the reproducibility of the relative proportion of selected ions of *O*-trimethylsilyl disaccharide alditols, five consecutive injections of *O*-trimethylsilyl cellobiitol were performed by GC-MS-SIM (Appendix A2.2). This produced an average % coefficient of variation of 7.8%. The intra-run variability is conceivably higher than 7.8% if run over a long time period so to minimise this variability all results were generated within a twelve hour period.

The *m/z* 307 and *m/z* 308 ion intensity ratios were found to differentiate 1→3 from 1→4 linked disaccharides. The 1→3 linkage produced *m/z* 307 and *m/z* 308 ion intensity ratios with a range of 5.5 - 7.8 and 1.8 - 2.9 respectively. The 1→4 linkage produced lower *m/z* 307 ion intensity ratios of 2.3 - 4.0 and considerably higher *m/z* 308 ion intensity ratios of 8.8 - 14.8. The *m/z* 307 and *m/z* 308 ion intensity ratios also readily differentiated 1→3 and 1→4 linked disaccharides from 1↔1, 2↔1 and 1→2 linked analogues (Table 4.2).

Table 4.2 Relative intensity of selected ions in *O*-trimethylsilyl disaccharide alditols

Disaccharide	Linkage	Conform.	Intensity (%) relative to *m/z* 361 ion		Intensity (%) relative to *m/z* 73 ion			
			205	206	307	308	319	320
sucrose	2↔1	β,α	5.9	1.9	0.9	0.3	9.5	3.8
trehalose	1↔1	α,α	6.1	1.9	2.0	0.6	21.1	6.8
kojibiose	1→2	α	40.0	13.5	16.1	6.6	39.9	46.5
laminaribiose	1→3	β	51.8	26.5	5.5	1.8	17.9	10.2
nigerose	1→3	α	30.6	16.7	7.2	2.6	13.4	10.9
turanose	1→3	α	30.5 36.4	16.3 18.6	7.8 7.8	2.8 2.9	15.1 11.5	10.4 7.0
cellobiose	1→4	β	62.3	40.5	3.3	8.8	17.7	9.6
maltulose	1→4	α	28.1 24.6	26.6 20.5	2.3 2.6	7.2 12.3	12.4 15.9	6.3 7.8
maltose	1→4	α	21.3	18.7	4.0	14.8	17.6	11.1
gentibiose	1→6	β	62.6	57.8	3.1	12.1	7.6	43.6
palatinose	1→6	α	35.1 40.2	46.8 51.0	2.9 3.2	13.9 20.0	9.0 9.8	85.9 75.0
isomaltose	1→6	α	38.1	48.0	3.7	20.4	9.7	77.2

Conform. = conformation

By plotting two ion intensity ratios against each other, one dimensional data becomes two dimensional which can maximise differences between correlated and uncorrelated data. The following plots pair up ion intensity ratios which may be correlated in order to maximise differences visually (Figure 4.11 - Figure 4.13). The ellipses on the plots are to aid in visualising clusters and do not have any statistical significance.

The *m/z* 307 vs *m/z* 308 ion intensity ratio effectively separates 1→3 from 1→4 and 1→6 linkage positions (Figure 4.11). While some separation occurs between 1→4 and 1→6 linkages, a degree of overlap is apparent.

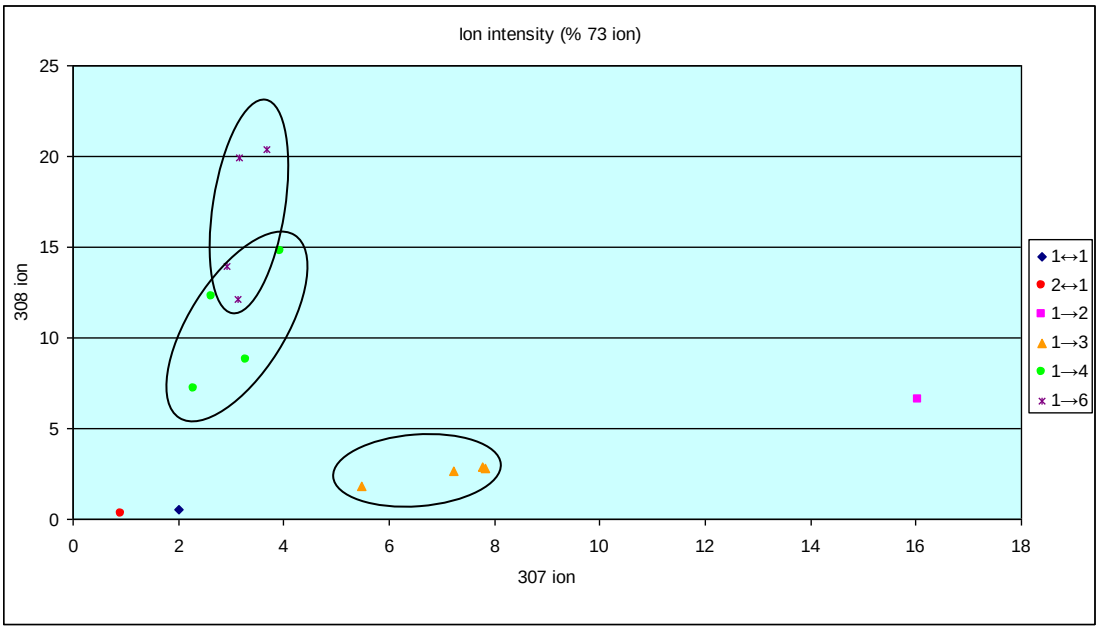


Figure 4.11 Plot of *m/z* 308 vs *m/z* 307 ion intensity ratios of *O*-trimethylsilyl disaccharide alditol standards relative to *m/z* 73 ion

In contrast the *m/z* 319 vs *m/z* 320 ion intensity ratio plot was very effective at distinguishing 1→6 linkages from all other linkage types (Figure 4.12). The *m/z* 307 and *m/z* 308 ion intensity ratios for 1→6 linkages are in the range of 2.9 - 3.7 and 43.6 - 85.9 which is far removed from 1→3 and 1→4 linkages which cluster between 11.5 - 17.9 and 6.3 - 11.1. The 1→2 linkage position is also well separated from all other linkage types with a value of 39.9 and 46.5.

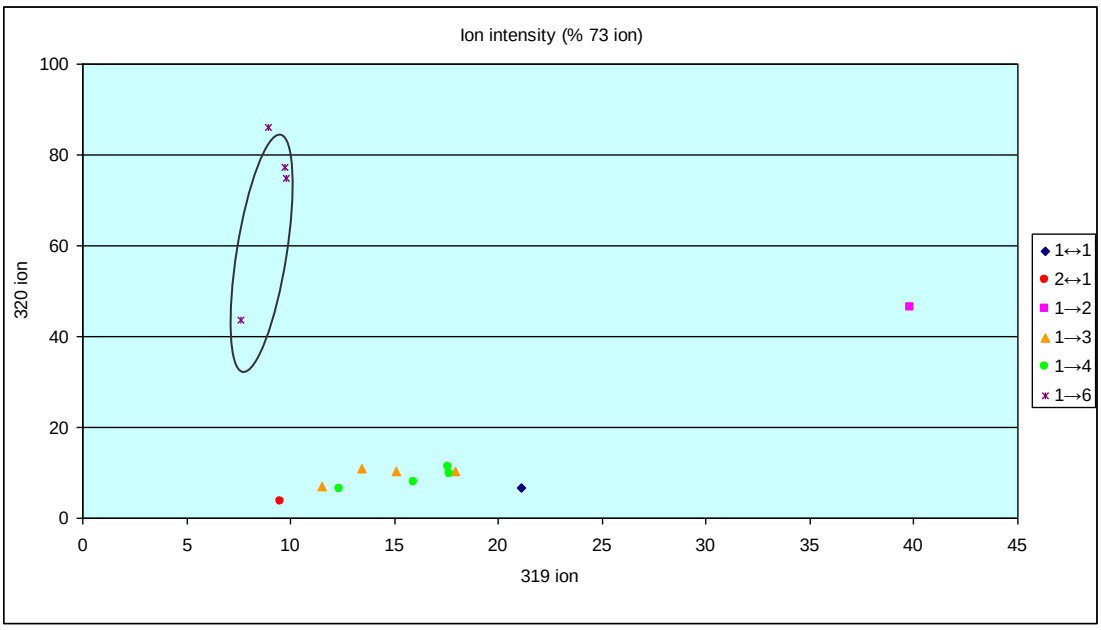


Figure 4.12 Plot of *m/z* 320 vs *m/z* 319 ion intensity ratios of *O*-trimethylsilyl disaccharide alditol standards relative to *m/z* 73 ion

The *m/z* 205 vs *m/z* 206 ion intensity ratio plot (Figure 4.13) can be used to distinguish disaccharides based on linkage position as well as configuration. In all three linkage groups where disaccharides of both α and β configuration were available, the α configuration produced significantly lower ion ratios than the related β configuration. With the exception of 1↔1 and 1↔2 linkages, all linkage positions were clearly separated.

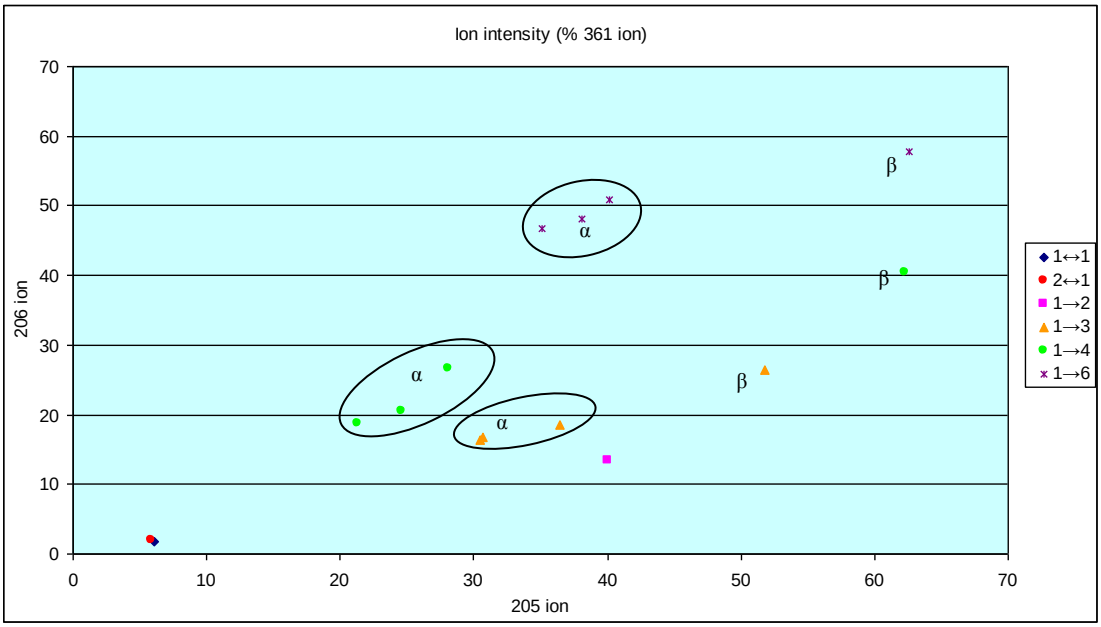


Figure 4.13 Plot of *m/z* 206 vs *m/z* 205 ion intensity ratios of *O*-trimethylsilyl disaccharide alditol standards relative to *m/z* 361 ion

4.3.2 Identification of Linkage Position of O-Trimethylsilyl Disaccharide Alditols in Honey

A number of disaccharides are present in honey, a typical chromatogram of manuka honey is shown in Figure 4.14. The linkage position and configuration of disaccharide alditols in honey was predicted by comparison of results obtained from the analysis of standards (Table 4.3). These results were generated in the same run as the standards in order to minimise ion ratio variations.

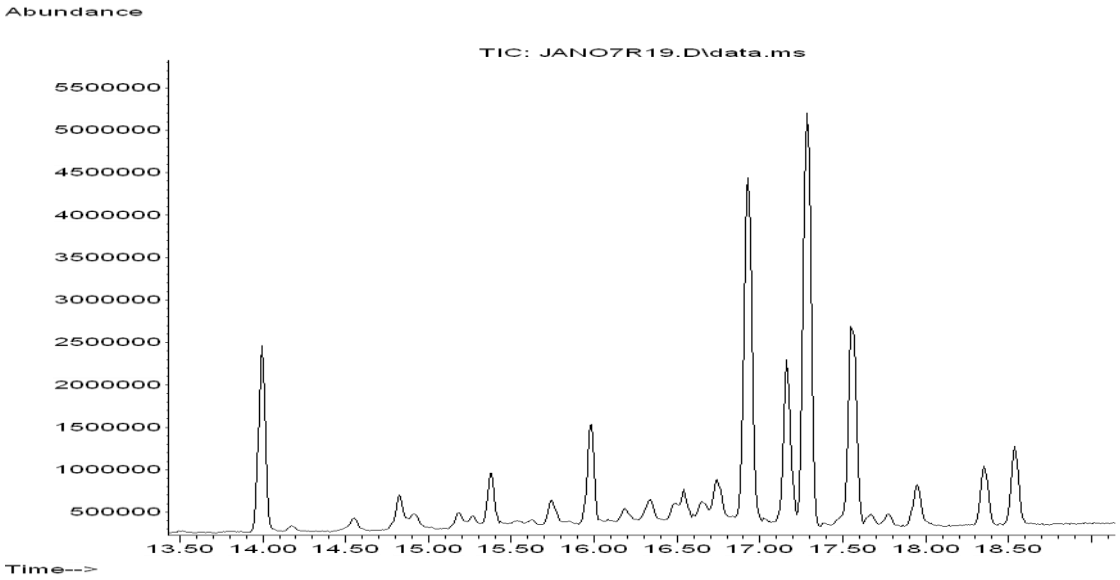


Figure 4.14 Chromatogram of disaccharides in honey

The α and β configuration standards have *m/z* 205 and *m/z* 206 ion intensity ratios in the range of 21.3 - 40.0 and 51.8 - 62.6. All honey disaccharides with a *m/z* 205 ion intensity ratio less than 45 were predicted as having an α configuration. A β configuration was assigned to *m/z* 205 ion intensity ratios greater than 45.

The *m/z* 205 ion is formed either through the cleavage between C_2 and C_3 or C_4 and C_5 of the alditols and was formed more readily in β linked disaccharides.

Table 4.3 Relative ion intensity of *O*-trimethylsilyl disaccharide alditols in honey with linkage and configuration predicted by comparison to standard ratio plots

RT (min)	Intensity (%) relative to *m/z* 361 ion		Intensity (%) relative to *m/z* 73 ion				Predicted linkage	Predicted Configuration
	205	206	307	308	319	320		
13.78	39.9	32.8	4.1	5.4	23.7	20.2	1→3	α
14.01	5.2	1.9	1.0	0.	9.8	4.2	2↔1	β,α
14.2	76.6	52.9	1.3	3.8	2.1	1.5	1↔1	
15.22	39.7	40.5	1.9	1.3	6.7	2.4	1↔1	
15.46	56.2	32.4	3.2	1.3	9.6	5.5	1↔1	
15.64	7.7	2.4	12.7	0.4	12.1	4.0	1↔1	α,α
15.86	36.8	37.5	1.1	3.5	11.0	7.4	1↔1	
16.02	8.1	2.6	1.2	0.3	11.2	3.5	1↔1	
16.22	61.0	40.0	5.6	6.7	14.9	9.8	1→4	β
16.37	41.0	47.2	3.0	8.3	6.0	15.1	1→4	α
16.58	63.8	41.2	5.5	4.2	15.0	11.1	1→3	β
16.69	85.5	26.1	6.2	5.4	26.3	10.9	1→3	β
16.79	35.8	63.4	2.4	9.0	3.7	18.6	1→4	α
16.96	30.1	16.2	7.8	2.8	15.0	11.1	1→3	α
17.19	33.4	23.1	5.2	4.8	11.7	7.1	1→3	α

RT (min)	Intensity (%) relative to *m/z* 361 ion		Intensity (%) relative to *m/z* 73 ion				Predicted linkage	Predicted Configuration
	205	206	307	308	319	320		
17.32	23.6	21.5	2.9	11.8	14.8	8.4	1→4	α
17.58	43.0	15.4	14.8	6.4	35.2	43.4	1→2	α
17.68	24.6	32.5	4.0	19.7	19.5	33.5	1→6	α
17.79	63.4	69.4	3.2	11.7	11.8	42.0	1→6	β
17.96	57.1	19.3	13.1	5.0	68.5	21.6	1→2	β
18.37	61.5	73.5	12.3	6.2	68.2	35.4	1→2	β
18.56	38.9	52.1	3.4	18.4	9.1	68.2	1→6	α
18.79	73.9	59.1	3.4	13.0	15.8	47.7	1→6	β

A total of 22 disaccharides were detected in this manuka honey sample, many of which were minor peaks. The relative ion intensity of *O*-trimethylsilyl disaccharide alditols in honey was found to be more variable than pure standards. This is not entirely unexpected as the presence of impurities can affect the relative proportion of each ion especially if the disaccharide is present in very small quantities.

The linkage type of each disaccharide in honey was predicted by comparison of the relative ion ratios for the pure standards. Each predicted linkage position was plotted with the standard results for the three main ion pairs and colour coded to aid visualisation.

Two main peaks with a retention time of 16.96 and 17.20 min in the honey samples are known to correspond with three standards, two with a 1→3 linkage and the third a 1→4 linkage. It was therefore expected that these two peaks may have both 1→3 and 1→4 linkage character. In the *m/z* 307 vs *m/z* 308 ion ratio plot (Figure 4.15), the predicted 1→3 linkages in the honey sample had a higher *m/z* 308 ion ratio than the standards which suggests it may have some 1→4 linkage character. A single honey disaccharide with a *m/z* 307 ion ratio of 5.6 and *m/z* 308 ion ratio of 6.7 was classified as 1→4 linked despite being within the normal range for the 1→3 linkage position. This was done as the *m/z* 319 and *m/z* 320 ion ratios for the same peak matched with a standard with a similar retention time. The predicted 1→2 linkages had a lower *m/z* 307 ion ratio than the standard but as only one standard with a 1→2 linkage was studied, these ratios may still be within the normal range.

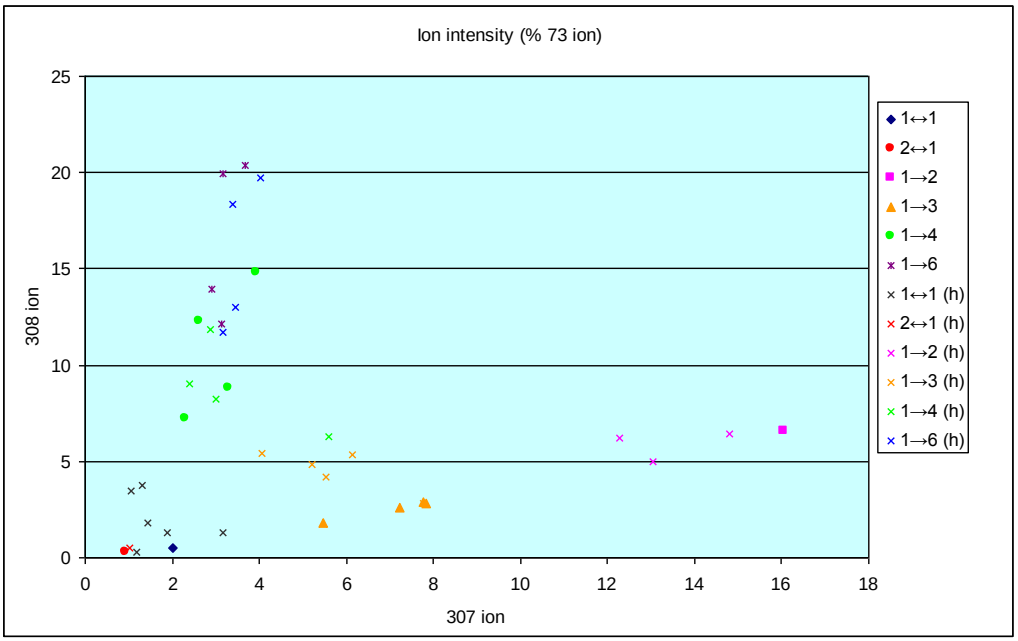


Figure 4.15 Plot of *m/z* 308 vs *m/z* 307 ion intensity ratios of *O*-trimethylsilyl disaccharide alditols of standards and honey disaccharides (h) relative to *m/z* 73 ion

The *m/z* 319 vs *m/z* 320 ion ratio plot (Figure 4.16) confirms the 1→6 linkage prediction. As with the above example the 1→2 linkage position for honey has two points which display different ion ratios to the standard with a higher *m/z* 319 ion ratio compared to the standard example.

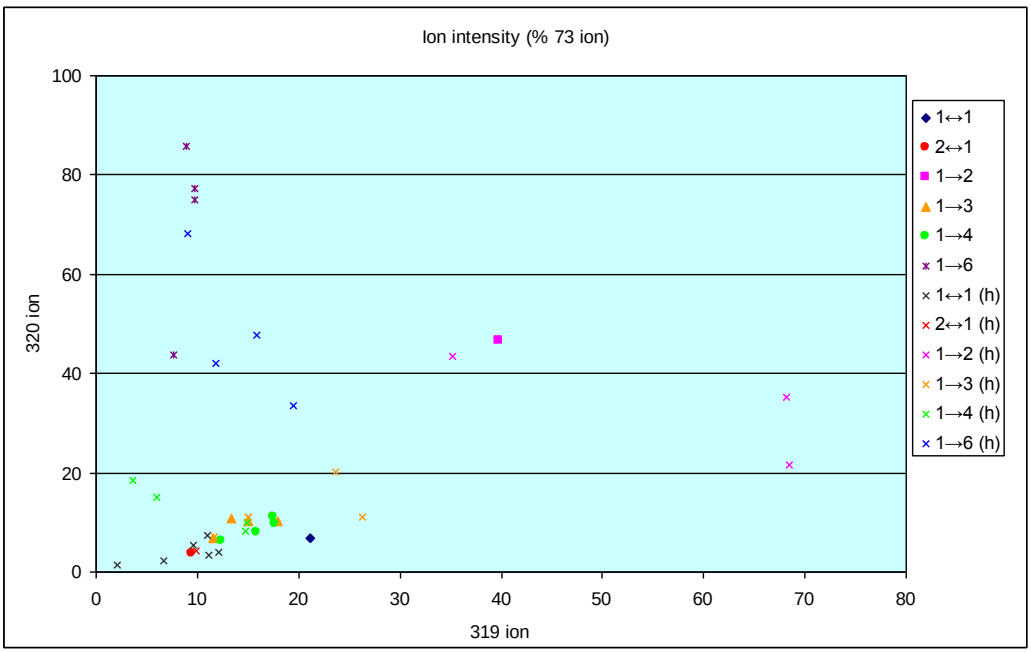


Figure 4.16 Plot of *m/z* 320 vs *m/z* 319 ion intensity ratios of *O*-trimethylsilyl disaccharide alditols of standards and honey disaccharides (h) relative to *m/z* 73 ion

The *m/z* 205 vs *m/z* 206 ion ratio plot (Figure 4.17) does not separate linkage position and configuration as clearly with the addition of the honey data. The exceptions are 2↔1 linked and a single 1↔1 linked honey disaccharides which are well matched to the standard data however all other 1↔1 linked predictions are spread throughout the plot. Due to the limited number of disaccharides available without a free hemiacetal group, all other honey disaccharides with ratios similar to the 1↔1 and 2↔1 linked standards in the *m/z* 307 vs *m/z* 308 and *m/z* 319 vs *m/z* 320 plots were classified as 1↔1 linked.

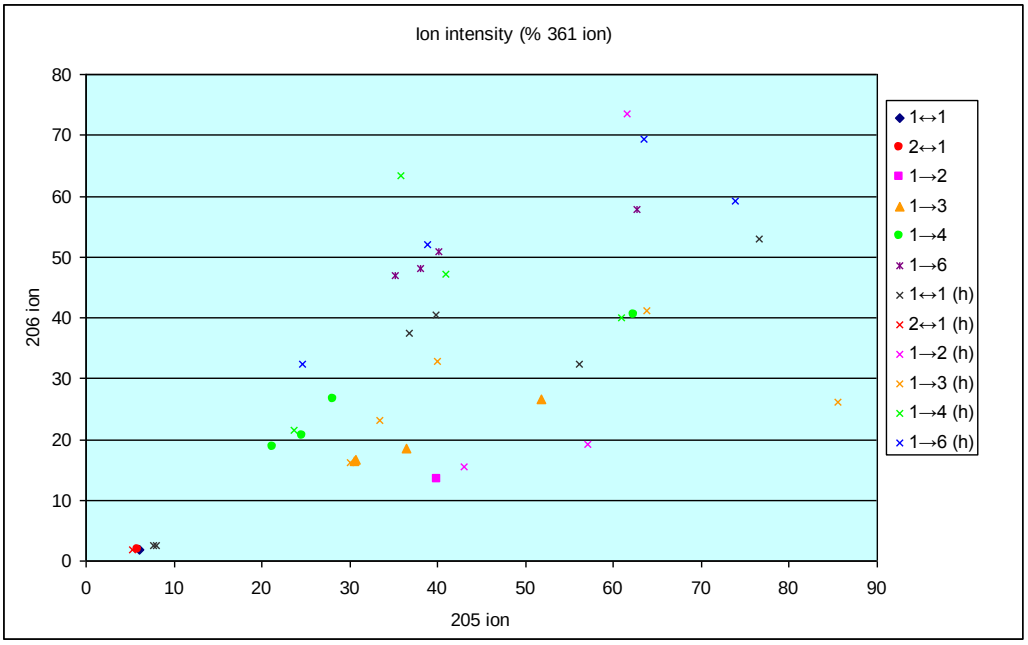


Figure 4.17 Plot of *m/z* 206 vs *m/z* 205 ion intensity ratios of *O*-trimethylsilyl disaccharide alditols of standards and honey disaccharides (h) relative to *m/z* 361 ion

The predicted linkage, configuration and retention time were used to determine the identity of disaccharide alditols in honey (Table 4.4).

Table 4.4 Predicted compared to actual linkage and configuration of reduced *O*-trimethylsilyl disaccharides in honey

Predicted Identity	RT (min)		Linkage		Configuration	
	Standard	Honey	Standard	Honey	Standard	Honey
		13.78		1→3		α
sucrose	14.03	14.01	2↔1	2↔1	β,α	β,α
		14.2		1↔1		
		15.22		1↔1		
		15.46		1↔1		
α,α-trehalose	15.71	15.64	1↔1	1↔1	α,α	α,α
		15.86		1↔1		

Predicted Identity	RT (min)		Linkage		Configuration	
	Standard	Honey	Standard	Honey	Standard	Honey
		16.02		1↔1		
cellobiose*	16.33	16.22	1→4	1→4	β	β
		16.37		1→4		α
laminaribiose	16.54	16.58	1→3	1→3	β	β
		16.69		1→3		β
		16.79		1→4		α
nigerose[a]	16.96	16.96	1→3	1→3	α	α
turanose/maltulose	17.20	17.19	1→3,1→4	1→3	α	α
maltose[b]	17.32	17.32	1→4	1→4	α	α
kojibiose	17.60	17.58	1→2	1→2	α	α
		17.68		1→6		α
gentibiose	17.82	17.79	1→6	1→6	β	β
		17.96		1→2		β
palatinose	18.39	18.37	1→6	1→2	α	β
palatinose/isomaltose	18.58	18.56	1→6	1→6	α	α
		18.79		1→6		β

*Tentative assignment
[a] Minor turanose contribution (calculated in Section 3.3.2.3)
[b] Minor maltulose contribution (calculated in Section 3.3.2.3)

The reduction of nigerose, turanose, maltulose and maltose with borodeuteride produces multiple identical products (Section 3.3.2.1). These are separated by GC into three peaks (two products in each). The analysis of specific ions suggest that the first of these peaks is mostly 1→3 linked in character (nigerose) and the third peak predominantly 1→4 linked (maltose). These results correlate with the calculated contribution (Section 3.3.2.3). The calculated contribution for the second peak (turanose and maltulose) indicates both disaccharides are present in similar concentrations. The relative intensity of the *m/z* 307 vs *m/z* 308 ion ratio suggest that this peak is 1→3 linked.

Palatinose appears to have been previously misidentified. Although the retention times of the standard and honey peaks are identical (18.37 and 18.39 min), the intensity of specific ions indicate the disaccharide is α-1→2 linked as opposed to β-1→6 linked. The fragmentation pattern of the first eluting palatinose peak and corresponding honey peak are shown in Figure 4.18 -Figure 4.19. The predominance of the *m/z* 320 ion in the palatinose spectra in contrast to the dominant *m/z* 319 ion in the honey spectra is the most characteristic difference between the two spectra. Palatinose contains relatively higher *m/z* 204 and 361 ions and lower *m/z* 73 ions. Based on these differences it is very unlikely that the honey peak with an identical retention time to palatinose originates from this disaccharide. The identity of this peak could be determined through

isolation and characterisation, this however was deemed outside the scope of this project.

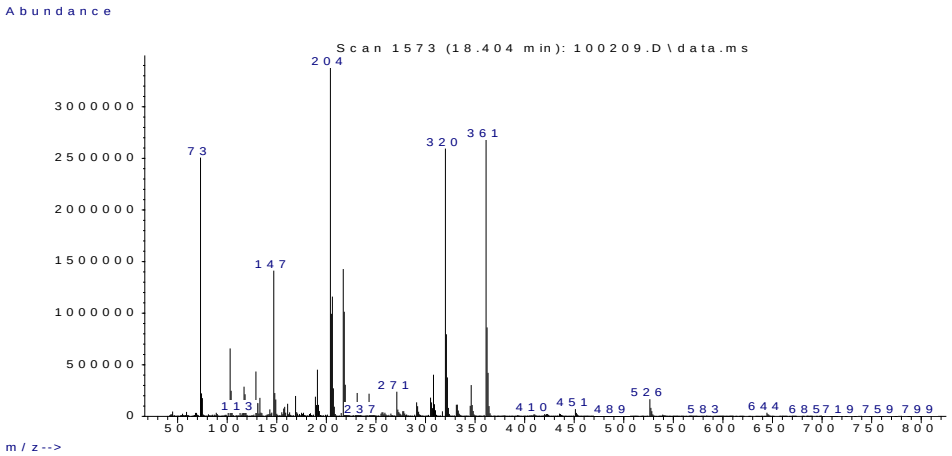


Figure 4.18 Fragmentation pattern of the first eluting palatinose deuterated reduction product

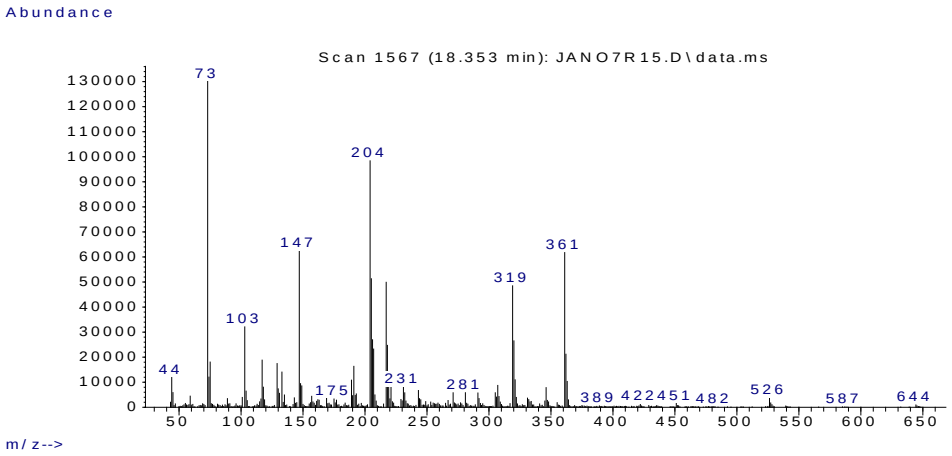


Figure 4.19 Fragmentation pattern of the corresponding honey deuterated reduction product

4.4 Conclusions

A method has been developed which successfully uses ion intensity ratios to determine linkage position of disaccharides in honey. This is the first reported use of this technique in complex disaccharide mixtures.

Determination of ion ratio data for other disaccharides not found in honey would improve the robustness of this technique. This may also enable the identification of some of the thus far unidentified disaccharides in honey.

The analysis of the likely linkage position of disaccharides found in honey using mass spectral ion ratio data has highlighted the importance of using multiple techniques in the identification process. While identification using a combination of retention time and ion ratio data provides a high degree of certainty, the only unequivocal method of identification remains isolation and structure characterisation using one and two-dimensional NMR methods (solution state) or X-ray crystallographic analysis (solid state) if a suitable crystal can be obtained.

Chapter 5

Extractable Organic Substances from New Zealand Honeys

5.1 Introduction

A series of papers reporting the extractives of New Zealand honeys have been published (Table 5.1). It has been suggested that some of these extractives could serve as floral markers.

Table 5.1 Proposed floral marker compounds in extracts of some New Zealand unifloral honeys

Honey	Characteristic and/or floral marker(s)	Range (mg/kg)	Ref.
Clover (*Trifolium repens*)	Low extractable organic substances	≈50	10
Ling heather (*Calluna vulgaris*)	High in degraded carotenoids including: 3,5,5-trimethylcyclohex-2-en-1-one 3,5,5-trimethylcyclohex-2-ene-1,4-dione 2-methoxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione 4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one 4-(3-oxobut-1-enylidene)-3,5,5-trimethylcyclohex-2-en-1-one 4-(3-hydroxybut-1-enyl)-3,5,5-trimethylcyclohex-2-en-1-one 4-hydroxy-4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one 4-hydroxy-4-(3-hydroxy-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one (isomer 1) 4-hydroxy-4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one (isomer 2) 4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one	 2.1 - 5.0 1.2 - 1.6 1.1-1.4 27 - 36 1.4 - 1.6 0.3 - 4.4 30 - 60 107 - 185	11
Kamahi (*Weinmannia racemosa*)	Kamahines A-C Meliracemoic acid		104, 105 106
Manuka (*Leptospermum scoparium*)	High extractable organic substances phenyllactic acid + 4-methoxyphenyllactic acid	 >700	10, 13

Honey	Characteristic and/or floral marker(s)	Range (mg/kg)	Ref.
	syringic acid + 3,4,5-trimethoxybenzoic acid	>35	
	acetophenone + 2-methoxyacetophenone	>20	
Nodding thistle (*Carduus nutans*)	High in linalool derivatives including: (*E*)-2,6-dimethyl-6-hydroxy-2,7-octadienoic acid (*E*)-2,6-dimethyl-3,7-octadiene-2,6-diol (*Z*)-2,6-dimethyl-2,7-octadiene-1,6-diol (*Z*)-2,6-dimethyl-6-hydroxy-2,7-octadienal α,5-dimethyl-5-ethenyl-2-tetrahydrofuranacetaldehydes β,5-dimethyl-5-ethenyl-2-tetrahydrofuranacetaldehydes	15 - 87	14
Rewarewa (*Knightea excelsa*)	High in aliphatic diacids	64 - 111	15
	2-methyoxybutanedioic acid	2.3 - 3.3	
	4-hydroxy-3-methyl-*trans*-2-pentenedioic acid	0.2 - 3.9	
Thyme (*Thymus* sp.)	1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethylcyclohexane-*trans,cis*-1,2,3-triol	>40	12
	methyl-3-hexenoate	3.5 - 8.1	
	3-aminoacetophenone	0.7-5.1	
Viper's bugloss (*Echium vulgare*)	Low levels of extractable organic substances	≈50	16
	1,4-hydroquinone	>15	
Willow (*Salix* sp.)	*cis,trans*-abscisic acid	106	12
	trans,trans-abscisic acid	42	

A number of small surveys have been conducted on other New Zealand unifloral honeys (including studies by Tan and other Waikato University graduate students[68, 107-111]) however due to the difficulty in obtaining multiple samples of certified unifloral honey of sufficient quality these results have not been published in the literature in refereed journal articles. A subsequent study by Senanayake[68] on certified unifloral beech honeydew honey and kamahi honey was not published due to the presence of contaminants such as phthalates, solvent stabilizers and anti-oxidants unintentionally introduced during the extraction process.

The aim of this survey is to reliably characterise the organic extractable substances in beech honeydew honey, kamahi, pohutukawa, rata and tawari honey to publishable standard and identify possible floral marker compounds. The identification of these marker compounds will be aided by undertaking a series of statistical analyses to classify honey type based on the extractives data.

5.2 Experimental

5.2.1 Sample preparation and analysis

Ten samples from each floral source (beech honeydew, kamahi, pohutukawa, rata and tawari) were extracted in diethyl ether and methylated as described in Section 2.2.

A total of 67 compounds were detected in the methylated ether extracts by GC-MS. Identification of compounds was achieved with the aid of NIST98 mass spectral library and by comparison of retention time to standards where available. Generally a match of >80% was considered acceptable however all spectra were visually inspected to determine the legitimacy. The concentration of identified compounds was calculated using a relative response factor assigned to the class of compound. The assigned class compounds are given in Table 5.2 along with the equation of the calibration graph. This equation was used to calculate the concentration of the extractives as described in Section 2.2.5.2. The calibration graphs of each class standard are shown in Appendix A3.1. Compounds of a given class were assumed to have a similar relative response factor as the chosen response factor compound. Unidentified compounds or compounds which did not fall into any of the compound classes were assigned a unit response relative to heptadecanoic acid ethyl ester.

Table 5.2 Assigned response compounds and calibration graph equations

Class	Assigned response compound	Equation (calibration graph)	R^2
aliphatic mono-acids	palmitic acid	1.833x - 0.698	0.997
aliphatic di-acids	pimelic acid	1.127x - 0.265	0.999
aromatic acids	benzoic acid	1.150x - 0.252	0.999
aromatic carboxylic acids	phenyllactic acid	1.391x - 0.708	0.994
aromatic ketones	acetophenone	0.942x - 0.216	0.999
unidentified/unclassified	heptadecanoic acid ethyl ester	1	NA

The concentration of identified compounds was calculated relative to either palmitic acid, pimelic acid, benzoic acid, phenyllactic acid or acetophenone where appropriate using experimentally determined response factors relative to heptadecanoic acid ethyl ester. All other compounds were measured relative to heptadecanoic acid ethyl ester (unit response factor of 1). The retention time of standards, quantified peaks and response factors assigned to peaks are given in Table 5.3.

Table 5.3 Retention time, identity and relative response compounds. Acids are detected as the corresponding methyl ester

RT_{honey} (min)	$RT_{standard}$ (min)	Compound	Assigned response compound
3.69		unknown (*m/z* 45,118)	
4.41		valeric acid	palmitic acid
5.29	5.31	succinic acid	pimelic acid
5.59		3-methyl-2-furanone	
5.83		methylbutanedioic acid	pimelic acid
6.20		2,2-dimethylbutanedioic acid	pimelic acid
6.42	6.4	benzoic acid	benzoic acid
7.01		glutaric acid	pimelic acid
7.20	7.19	3,5,5-trimethyl-2-cyclohexene-1,4-dione	acetophenone
7.36		unknown (*m/z* 59, 71, 129, 141)	
7.52		2-methyleneglutaric acid	pimelic acid
7.64		2,2,6-trimethylcyclohexane-1,4-dione	acetophenone
7.80	7.8	phenylacetic acid	phenyllactic acid
8.03		2,6-dimethylocta-3,7-diene-2,6-diol	pimelic acid
8.04		unknown (*m/z* 55, 114, 128, 158)	
8.19	8.17	salicylic acid	benzoic acid
8.19		nonanoic acid	palmitic acid
8.54		unknown (*m/z* 70, 107, 125, 140)	
8.71		2-coumaranone	
8.89		unknown (*m/z* 54, 82, 110, 151)	
8.90		2-methoxyacetophenone	acetophenone
9.86		unknown (*m/z* 139)	
10.05		unknown (*m/z* 67, 82, 110, 123, 138)	
10.21		unknown (*m/z* 67, 82, 110, 123, 138)	
10.23		unknown (*m/z* 70, 95, 125, 140, 168)	
10.33		unknown (*m/z* 70, 95, 140, 154, 168)	
10.42		unknown (*m/z* 69, 97, 101, 129, 156)	
10.59		unknown (*m/z* 69, 97, 101, 129, 156	
10.63	10.65	2-methoxybenzoic acid	benzoic acid
10.63		unknown (*m/z* 55, 71, 79, 91)	
10.76		unknown (*m/z* 115, 143)	
11.02		2-hydroxyphenylacetic acid	
11.13		unknown (*m/z* 55, 67, 83, 91)	
11.30	11.33	4-methoxybenzoic acid	benzoic acid
11.35	11.36	phenyllactic acid	phenyllactic acid
11.41		4-methoxyphenylacetic acid	phenyllactic acid
12.37	12.37	octanedioic acid	pimelic acid
12.57		4-ethoxybenzoic acid	
12.82	12.77	4-hydroxybenzoic acid	benzoic acid
13.23		4-hydroxyphenylacetic acid	phenyllactic acid
13.64	13.67	lauric acid	palmitic acid
14.03		nonanedioic acid	pimelic acid
14.37		4-methoxymandelic acid	phenyllactic acid
14.64	14.61	3,5-dimethoxybenzoic acid	benzoic acid
14.82		3,4-dimethoxybenzoic acid	benzoic acid
15.51	15.54	4-methoxyphenyllactic acid	pheyllactic acid
15.58	15.58	decanedioic acid	pimelic acid
15.80		2,4,6-trimethylacetophenone	

RT_{honey} (min)	$RT_{standard}$ (min)	Compound	Assigned response compound
16.36		decene-2-dioic acid*	pimelic acid
16.75	16.76	methyl syringate	benzoic acid
17.34		unknown (*m/z* 82, 95, 150, 210)	
17.56		*cis*-3,4-methoxycinnamic acid	phenyllactic acid
17.59		4-hydroxy-3,5-dimethoxyhydrazidebenzoic acid	
18.34	18.30	indole-3-acetic acid	
18.83		unknown (*m/z* 59, 121, 160, 210)	
19.02	19.01	*trans*-3,4-methoxycinnamic acid	phenyllactic acid
19.48	19.46	palmitic acid	palmitic acid
19.73		meliracemoic acid	
20.06		unknown (*m/z* 76, 104, 149)	
20.43		kamahines A-C	
21.08		unknown (*m/z* 137, 181, 251, 266)	
21.29		unknown (*m/z* 83, 127, 155, 179)	
21.76		9-octadecenoic acid	palmitic acid
21.85	21.81	*cis,trans*-abscisic acid	
22.03	22.04	stearic acid	palmitic acid
22.76		*trans,trans*-abscisic acid	
25.46		pinostrobin chalcone	

* = identified by comparison of mass spectrum reported by Tan[108] (Appendix A3.2)

Fatty acids and aliphatic compounds eluting after stearic acid (detected as the corresponding methyl esters) were not reported as they originate from the wax component in honey. A list of substances attributed to the wax component from New Zealand honeys has been reported by Tan.[10] Several recent surveys have reported a variety of compounds in beeswax.[112-115]

5.3 Results and Discussion

5.3.1 Beech (*Nothofagus* spp.) Honeydew Honey

The organic constituents of New Zealand beech honeydew honey have been investigated in two separate surveys.[68, 110] As beech honeydew honey originates from the excretions of two species of sap sucking scale insect (*Ultracoelostoma assimile*, *Ultracoelostoma brittini*) as opposed to nectar from a flower, pollen analysis is conducted merely to discount any other large contributing nectar source. The physiochemical properties of honeydew honey tend to be very different from floral honey due to the origin of the nectar source. The physiochemical results (determined by Airborne Honey Ltd.) for the 10 beech honeydew honeys used in this investigation are listed in Table 5.4. All samples have a low pollen count and are dark in colour with high conductivity which is indicative of good quality beech honeydew honey. Nectar honeys have a total pollen count > 100,000 except those which have an extremely low frequency of pollen in the nectar. Nectar honeys which are overrepresented in pollen can have a total pollen count as high as 950,000.

Table 5.4 Physiochemical properties of beech honeydew honey

Sample ID	Colour (mm)	Moisture (%)	Total Pollen (/10g honey)	Conductivity (ohms/cm x 10^{-4})
22028	80	16.1	34900	12.06
22308	85	15.9	71600	11.81
20166	89	16.1	51600	11.89
20686	93	17.4	ND	10.3
21583	82	15.8	39950	11.55
21919	87	15.1	93250	12.71
22230	87	16	37500	9.7
22555	80	15.8	ND	11.43
22591	90	16.4	91600	10.98
22803	87	16.4	ND	11.32

ND = not determined

The methylated ether extracted honeys were analysed by GC-MS as described in Section 2.2. A representative beech honeydew honey chromatogram, peak identification and concentrations are given Figure 5.1 and Table 5.5.

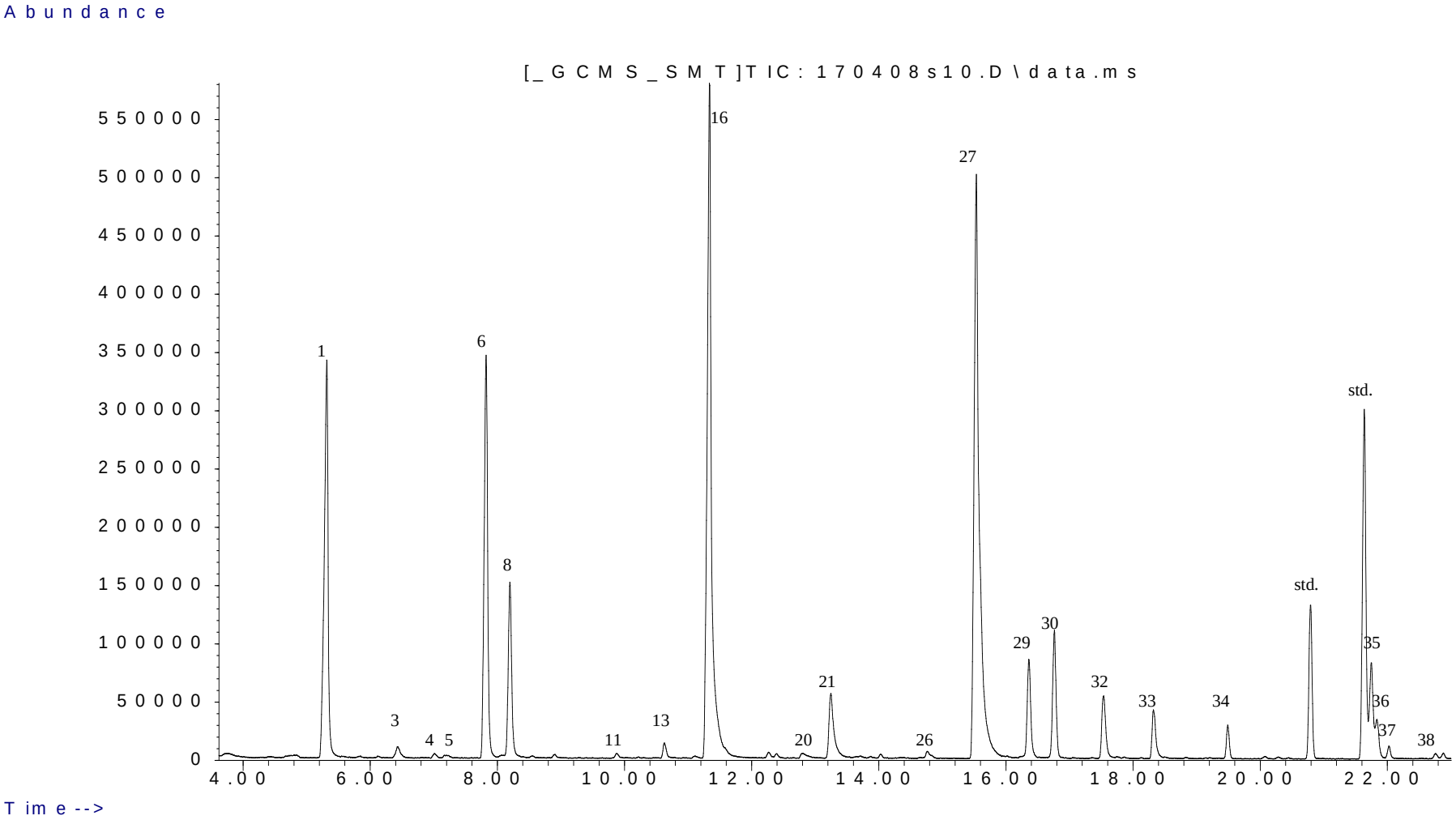


Figure 5.1 GC-MS profile of a representative methylated beech honeydew honey extract. Peak identifications are listed in Table 5.5.

Table 5.5 Concentration of compounds detected in diethyl ether extraction of beech honeydew honey (mg/kg). Acids are quantified as the corresponding methyl ester

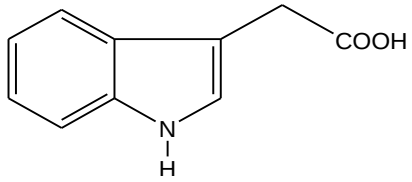
			Sample									
Peak	**RT**	**Compound**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	5.287	succinic acid	2.5	2.5	19.9	33.2	23.2	6.3	20.0	48.1	35.0	45.8
2	5.834	methylbutanedioic acid	0.6	0.6	0.5	-	-	-	-	-	-	-
3	6.419	benzoic acid	1.0	1.2	1.4	1.0	0.9	0.5	0.6	1.3	0.6	1.6
4	7.009	glutaric acid	-	-	0.2	0.4	0.3	0.1	-	0.4	0.4	0.5
5	7.229	3,5,5-trimethyl-2-cyclohexene-1,4-dione	-	0.6	0.4	3.1	1.6	1.9	-	0.8	-	0.6
6	7.827	phenylacetic acid	22.4	36.3	15.5	13.9	22.8	19.6	24.9	29.0	28.8	33.9
7	8.035	unknown (*m/z* 55, 114, 128, 158)	0.8	0.5	0.3	-	-	-	-	-	-	-
8	8.189	salicylic acid	7.1	12.8	11.2	9.2	12.4	6.2	9.0	11.1	11.9	17.7
9	8.535	nonanoic acid	-	-	0.2	-	-	0.1	0.1	0.2	-	-
10	8.892	2-coumaranone	-	-	0.4	0.4	0.2	0.1	0.6	0.6	0.5	0.4
11	9.872	2-methoxyacetophenone	-	3.1	-	2.0	0.7	0.8	0.2	3.1	0.2	0.7
12	10.223	unknown (*m/z* 67, 82, 110, 123)	-	0.7	-	1.2	-	-	-	-	0.2	-
13	10.631	2-methoxybenzoic acid	-	2.4	0.5	3.6	0.8	0.8	0.6	1.8	0.7	1.6
14	11.126	2-hydroxyphenylacetic acid	-	-	0.3	-	0.2	-	0.5	-	0.3	0.3
15	11.309	4-methoxybenzoic acid	3.3	*	*	*	*	*	*	*	*	*
16	11.353	phenyllactic acid	-	63.8	68.4	225.5	43.9	30.8	29.8	96.1	58.7	86.7
17	12.258	4-methoxyphenylacetic acid	1.8	3.3	1.2	1.8	0.3	0.4	0.3	1.1	0.4	0.5
18	12.386	octanedioic acid	-	-	0.6	1.0	0.2	0.3	0.4	0.8	0.5	0.4
19	12.574	4-ethoxybenzoic acid	0.5	0.4	0.3	-	-	-	-	-	-	-
20	12.821	4-hydroxybenzoic acid	-	0.2	1.3	2.3	1.2	1.5	0.8	2.0	1.0	1.0
21	13.234	4-hydroxyphenylacetic acid	4.1	6.5	5.7	7.8	6.7	8.3	4.2	9.0	6.0	7.8
22	13.658	lauric acid	4.6	4.1	3.5	0.1	0.2	-	0.2	-	0.3	-
23	14.034	nonanedioic acid	0.2	0.2	1.2	0.5	0.2	0.3	0.3	1.0	0.3	0.3
24	14.367	4-methoxymandelic acid	-	-	1.6	1.3	-	-	-	-	-	-
25	14.639	3,5-dimethoxybenzoic acid	-	0.6	1.3	0.6	-	0.2	-	0.4	-	-
26	14.821	3,4-dimethoxybenzoic acid	0.5	0.9	1.9	0.7	-	0.4	0.3	0.4	-	1.1
27	15.54	4-methoxyphenyllactic acid	-	79.2	132.2	217.3	45.2	38.7	27.2	170.1	76.8	103.8

			Sample									
Peak	**RT**	**Compound**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
28	15.589	decanedioic acid	2.4	*	*	*	*	*	*	*	*	*
29	16.367	decene-2-dioic acid	-	-	-	7.8	4.4	5.4	3.4	8.6	7.1	11.9
30	16.757	methyl syringate	0.7	26.2	24.0	27.3	3.3	4.4	5.1	13.7	2.4	12.5
31	17.338	unknown (*m/z* 82, 95, 150, 210)	0.6	2.7	2.8	2.1	1.2	1.9	1.5	2.4	1.7	-
32	17.594	4-hydroxy-3,5-dimethoxyhydrazidebenzoic acid	0.4	0.6	0.4	16.5	5.4	8.0	1.4	4.4	7.5	8.4
33	18.339	indole-3-acetic acid	14.7	16.8	1.1	3.8	7.7	16.8	2.3	5.9	4.8	6.2
34	19.495	palmitic acid	1.4	1.4	1.2	1.3	0.8	1.2	0.8	1.1	1.0	1.7
35	21.774	9-octadecanoic acid	4.3	3.4	3.7	4.6	3.2	3.3	-	3.2	-	6.1
36	21.897	*cis,trans*-abscisic acid	-	-	-	-	-	7.1	-	-	-	4.9
37	22.033	stearic acid	0.6	0.6	0.6	0.5	0.3	0.5	0.3	0.2	0.3	0.7
38	22.763	*trans,trans*-abscisic acid	-	-	-	-	-	1.4	-	-	-	0.6
Total organics			75	272	304	591	187	167	135	417	247	358

- = not detected, * = overlapping peak

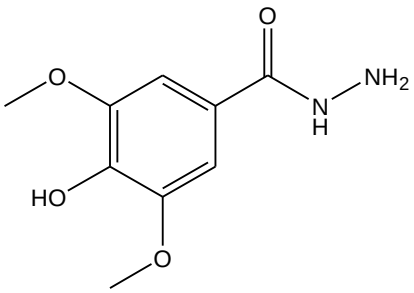
An array of phenolics characterises the beech honeydew honey extracts. By far the most dominant compounds are phenyllactic acid (peak 16, 29.8 - 225.5 mg/kg) and 1-methoxy-4-propylbenzene (peak 27, 27.2 - 217.3 mg/kg).

Previous surveys of New Zealand beech honeydew honey have determined indole-3-acetic acid **(12)** (peak 33) as the sole marker compound for beech honeydew honey.[110] In the present survey indole-3-acetic acid was found to be present in all samples ranging from 1.1 - 16.8 mg/kg. This is consistent with a previous study by Senanayake on beech honeydew from the 2002 season which determined levels ranging from 0.9 - 9.1 mg/kg.[68] Hyink also determined a range from trace - 14 mg/kg.[110]



(12)

An unusual phenolic compound, namely 4-hydroxy-3,5-dimethoxyhydrazidebenzoic acid **(13)** (peak 32) was detected in all samples ranging from 0.4 - 16.5 mg/kg. This identification was based on a library mass spectra search which exhibited an extremely strong likeness between the two spectra, particularly in the weak ions. Unlike methyl syringate, the 4-hydroxy group on the aromatic ring remains un-methylated upon the addition of excess diazomethane. Incomplete methylation occurs as the methylation of three adjacent hydroxyl groups on the aromatic ring produces a sterically unfavourable environment for the central group which has a marked effect on reactivity. The mass spectrum of both the honey-derived and library compound is given in Figure 5.2. Hydrazides are rather unusual in natural products, and have not been reported in any other New Zealand honeys. It therefore appears that this compound is enzymatically or floral source dependant. The only report of this compound from a natural source is also in honey (cotton honey) originating from Greece where it was detected in a range of 0 - 0.38 mg/kg.[116] This assignment is tentative only as partially methylated syringic acid has the same molecular weight as the azide, a comparison with an authentic standard would be required to unequivocally identify this compound.



(13)

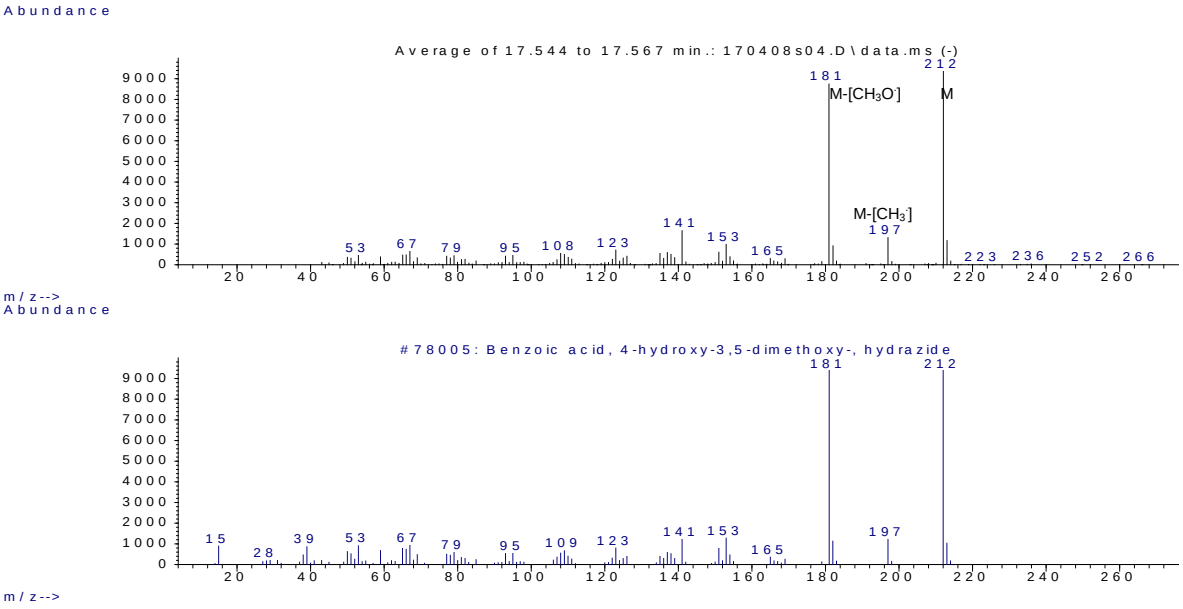
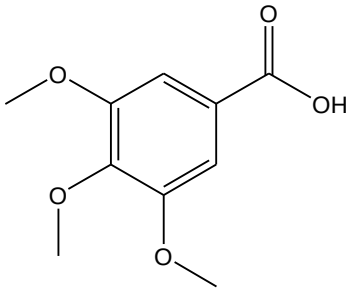


Figure 5.2 Mass spectrum of 4-hydroxy-3,5-dimethoxyhydrazidebenzoic acid found in methylated beech honeydew honey extracts (above) and the corresponding NIST.98 library spectra (below)

The related compound methyl syringate **(14)** (peak 30), a proposed floral marker for manuka honey[13] was present in all samples ranging from 0.7 - 27.3 mg/kg. Due to the exposure of honey extracts to an excess of diazomethane for a prolonged period which causes the progressive methylation of phenolic hydroxyl groups, methyl syringate is detected as the methyl ester of 3,4,5-trimethoxybenzoic acid. In an earlier study by Tan[108] in which the exposure to diazomethane was brief, the 4-hydroxy analogue was detected. The ethylation of beech honeydew honey extracts confirmed this result.[68] Methyl syringate is generally found in much higher quantities in manuka honey (26 - 470 mg/kg).[10]

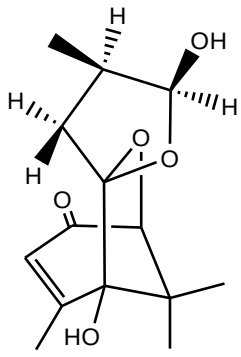


(14)

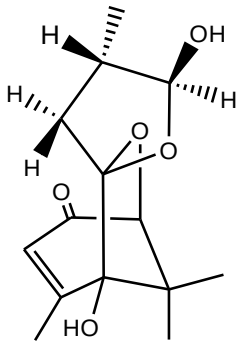
In general, aliphatic acids and diacids are a minor constituent of the organic composition of beech honeydew honey (average 11%). Succinic and palmitic acid, common fatty acids in many honeys, were found in all samples. Glutaric acid was detected in low levels in seven beech honeydew honey samples but was absent in all other honey types in this survey. Glutaric acid has previously been reported in the aliphatic acid rich rewarewa honey.[15]

5.3.2 Kamahi (*Weinmannia racemosa*) Honey

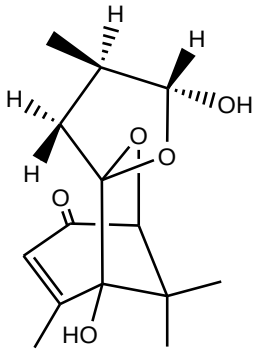
Several previous studies have been undertaken on the extractable organic substances in kamahi honey. An investigation of 11 kamahi honeys by Tan[108] and a subsequent survey by Senanayake[68] revealed the presence of a vast array of compounds, many of which were not able to be identified. Kamahi honey has been found to contain the novel *nor*-sesquiterpenoids: kamahines A-C **(15-17)** and meliracemoic acid **(18)**.[105, 106]



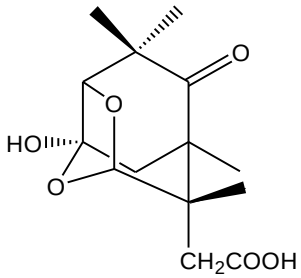
(15)



(16)



(17)



(18)

These novel *nor*-sesquiterpenoids were isolated from kamahi honey and subsequently characterised using a combination of multidimensional ^{1}H and ^{13}C NMR experiments and single crystal x-ray crystallography.[104, 105]

While the results of the earlier investigations are accessible, as yet no comprehensive survey on the organic constituents in kamahi honey has been published, in part because pollen data was not available for the majority of samples investigated in the earlier studies and their floral integrity could not therefore be unequivocally established.

Ten unifloral kamahi honeys supplied by Airborne Honey Ltd. were chosen for this survey. Pollen analysis results (provided by Airborne Honey Ltd.) indicated that while kamahi was the dominant floral source, a variety of secondary floral sources were present (Table 5.6).

Table 5.6 Kamahi honey pollen analysis data

Sample ID	1st Pollen type	1st Pollen (%)	2nd Pollen type	2nd Pollen (%)	3rd Pollen type	3rd Pollen (%)	Total Pollen (/10 g honey)
20256	Kamahi	85.1	Clover	6.5	OT	5.5	218250
19376	Kamahi	57.1	OT	15.8	Lotus	11.2	171600
19702	Kamahi	66.2	Rata	30.2	Lotus	2.2	174950
19774	Kamahi	83.3	OT	7.2	Clover	5	199900
19775	Kamahi	78.3	Willow	9.2	OT	6.6	168300
21902	Kamahi	76	Quintinea	18.8	OT	3.1	139900
20233	Kamahi	74	Rata	13.5	Clover	4	235000
20249	Kamahi	73.8	Rata	7.2	Clover	7.2	208300
21900	Kamahi	71.7	Manuka	13.2	Quintinea	5.7	189950
22462	Kamahi	74.6	Rata	14.4	Quintinea	2.5	184900

OT = other type

A typical kamahi honey GC-MS spectra is given in Figure 5.3, peak identifications and concentrations are listed in Table 5.7.

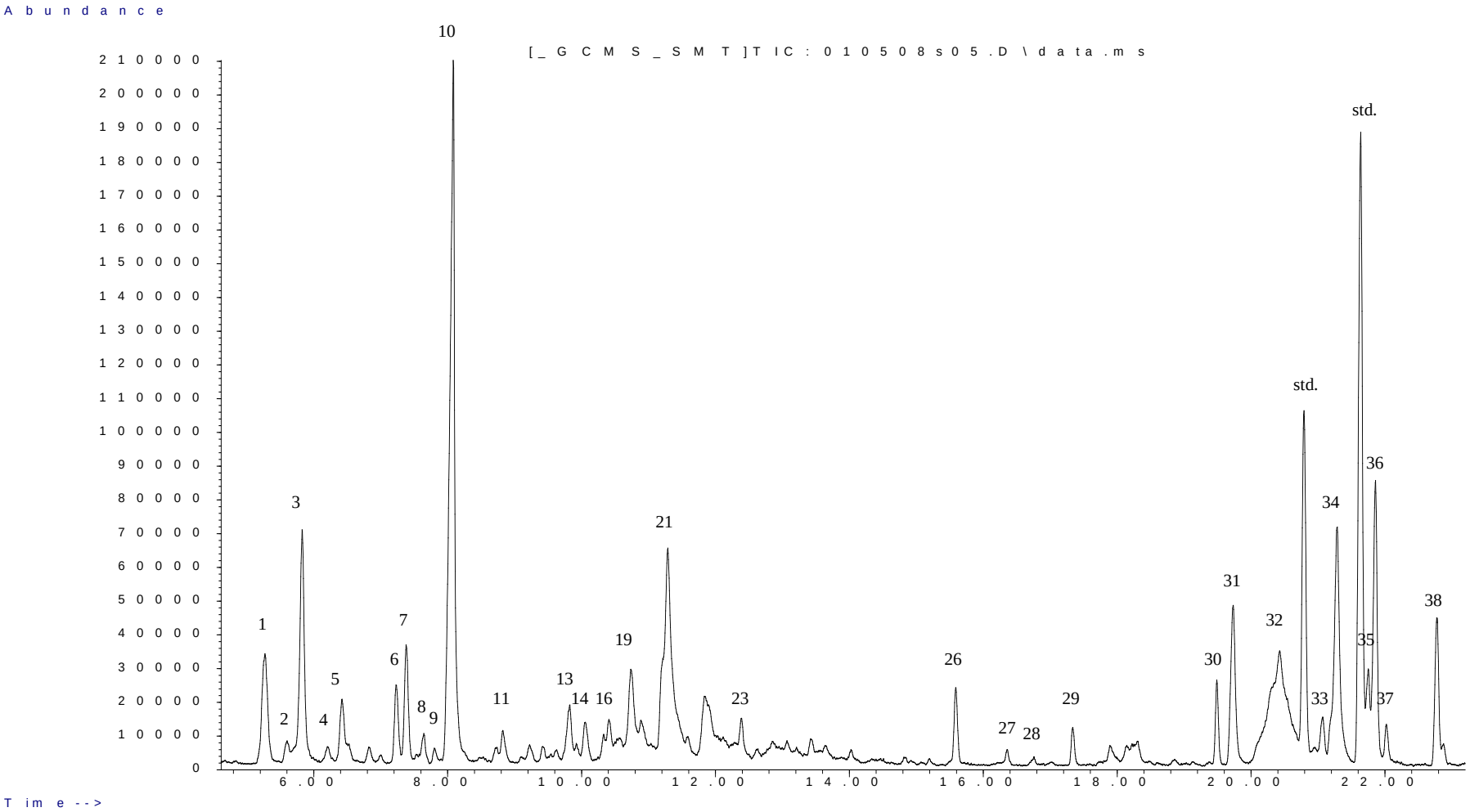


Figure 5.3 GC-MS profile of a representative methylated kamahi extract. Peak identifications are listed in Table 5.7.

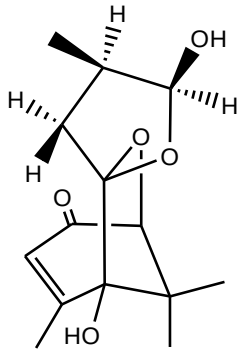
Table 5.7 Concentration of compounds detected in diethyl ether extraction of kamahi honey (mg/kg). Acids are quantified as the corresponding methyl ester

			Sample									
Peak	**RT**	**Compound**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	5.292	succinic acid	-	9.9	4.3	4.1	6.7	5.3	5.1	4.1	4.6	1.2
2	5.585	3-methyl-2-furanone	0.5	0.4	-	0.8	1.2	1.0	1.7	0.8	0.9	-
3	5.850	methylsuccinic acid	0.2	5.4	7.5	7.0	12.0	2.0	8.9	9.3	2.6	3.1
4	6.199	2,2-dimethylsuccinic acid	-	0.5	1.2	0.6	0.8	0.8	0.9	0.8	0.6	-
5	6.435	benzoic acid	-	0.8	2.0	0.8	3.7	2.4	2.6	1.6	2.8	-
6	7.220	3,5,5-trimethyl-2-cyclohexene-1,4-dione	2.3	3.5	6.2	4.6	4.0	4.0	3.6	3.9	3.9	2.4
7	7.375	unknown (*m/z* 59, 71, 129, 141)	-	1.3	2.5	3.3	5.6	6.0	5.8	3.6	5.3	0.6
8	7.644	2,2,6-trimethylcyclohexane-1,4-dione	0.2	0.5	1.8	1.0	1.3	1.4	-	1.2	0.7	-
9	7.811	phenylacetic acid	-	0.4	-	0.5	0.5	-	-	-	0.3	-
10	8.070	2,6-dimethylocta-3,7-diene-2,6-diol	27.1	7.2	20.9	35.2	41.4	48.4	34.2	23.2	36.6	9.4
11	8.705	unknown (*m/z* 70, 107, 125, 140)	0.3	-	0.3	0.7	0.6	-	-	-	0.8	-
12	8.899	unknown (*m/z* 54, 82, 110, 151, 166)	0.3	0.9	1.4	1.2	1.5	4.1	4.7	3.0	3.8	-
13	9.864	2-methoxyacetophenone	-	2.3	-	2.2	3.4	2.8	3.2	1.6	4.9	-
14	10.050	unknown (*m/z* 139)	-	2.5	1.7	2.1	2.1	-	1.8	2.5	1.0	0.8
15	10.226	unknown (*m/z* 67, 82, 110, 123, 138)	-	-	1.3	-	-	5.6	-	-	1.1	1.9
16	10.332	unknown (*m/z* 70, 95, 125, 140, 168)	-	0.5	0.5	0.6	0.8	-	-	-	-	0.3
17	10.419	unknown (*m/z* 70, 95, 140, 154, 168)	1.7	1.8	1.5	1.6	2.0	-	-	2.1	-	1.1
18	10.602	unknown (*m/z* 69, 97, 101, 129, 156)	-	10.8	1.3	1.8	1.6	-	-	-	4.6	-
19	10.763	unknown (*m/z* 55, 71, 79, 91)	1.5	-	2.1	4.3	6.0	2.8	4.0	2.8	-	1.1
20	11.22	unknown (*m/z* 55, 67, 83, 91)	-	-	-	2.9	3.8	3.7	-	-	-	-
21	11.299	4-methoxybenzoic acid	19.0	-	6.6	8.3	14.4	9.6	15.1	13.6	-	5.2
22	11.404	phenyllactic acid	-	147.0	-	-	-	-	-	-	137.2	-
23	12.378	octanedioic acid	0.4	-	-	1.2	1.4	0.8	-	-	-	-
24	13.643	lauric acid	3.8	-	-	-	-	-	-	-	-	-
25	15.511	4-methoxyphenyllactic acid	-	2.2	-	-	-	-	1.6	2.2	13.0	-
26	15.589	decanedioic acid	2.1	4.0	1.9	2.7	3.0	2.2	2.6	2.5	-	1.9
27	15.801	2,4,6-trimethylacetophenone	-	-	0.7	-	-	3.5	-	-	1.6	0.4

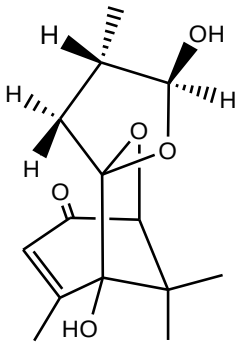
			Sample									
Peak	**RT**	**Compound**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
28	16.701	methyl syringate	0.6	15.3	-	0.3	0.4	2.0	1.3	0.6	5.6	0.4
29	17.353	unknown (*m/z* 82, 95 150, 210)	-	1.5	0.9	1.6	1.5	1.0	0.6	0.5	0.5	0.9
30	19.490	palmitic acid	3.9	0.9	0.8	1.3	1.7	1.1	0.8	0.9	0.6	1.2
31	19.742	meliracemoic acid	11.2	2.6	6.0	10.1	9.4	8.1	7.3	5.8	6.5	4.5
32	20.432	kamahines A-C	15.8	6.6	20.6	27.8	25.1	11.9	22.2	20.0	16.2	10.5
33	21.080	unknown (*m/z* 137, 181, 251, 266)	2.4	0.3	0.7	2.0	2.2	1.3	1.4	1.2	0.9	1.0
34	21.289	unknown (*m/z* 83, 127, 155, 179)	9.2	2.3	6.3	16.1	16.2	10.9	10.3	7.6	6.2	6.0
35	21.775	9-octadecenoic acid	7.6	2.9	0.6	1.8	2.2	1.5	2.1	1.1	0.7	1.0
36	21.852	*cis,trans*-abscisic acid	-	-	-	2.7	13.4	0.6	2.3	3.5	1.1	0.5
37	22.030	stearic acid	3.3	0.3	0.4	0.5	0.7	0.4	0.3	0.3	0.2	0.3
38	22.768	*trans,trans*-abscisic acid	0.9	-	0.1	1.2	6.9	0.2	0.7	1.2	0.2	-
Total organics			114	234	102	153	198	145	145	122	265	56

- = not detected

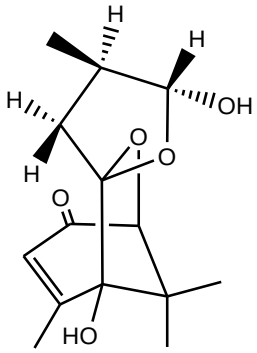
The most predominant feature of kamahi honey is a broad band (peak 32) attributed to kamahines A-C **(15-17)**. In the present survey kamahines A-C were detected in the range of 6.6 - 27.8 mg/kg. These levels are significantly lower than that found in a previous study which reported a range of 43 - 144 mg/kg.[68] A similar range was also observed by Tan (10 - 150 mg/kg) for unknown peak 208.[108]



(18)

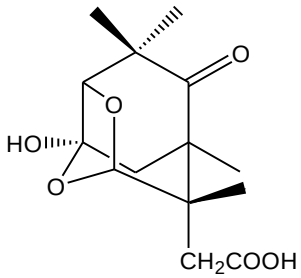


(19)



(20)

Like the kamahines A-C, meliracemoic acid **(21)** (peak 31) is a novel *nor*-sesquiterpenoid isolated from kamahi honey.[106] The methyl ester of meliracemoic acid was detected in all honeys ranging from 2.6 - 11.2 mg/kg. This is similar to previous findings from the 2002 season (9.1 - 21 mg/kg)[68] and the 1985-1987 season (1 - 10 mg/kg unknown peak 202).[108]



(21)

The mass spectrum of the methylated peak identified in this investigation as meliracemoic acid methyl ester is given in Figure 5.4 and was found to be identical to the mass spectrum represented in a previous study.[68]

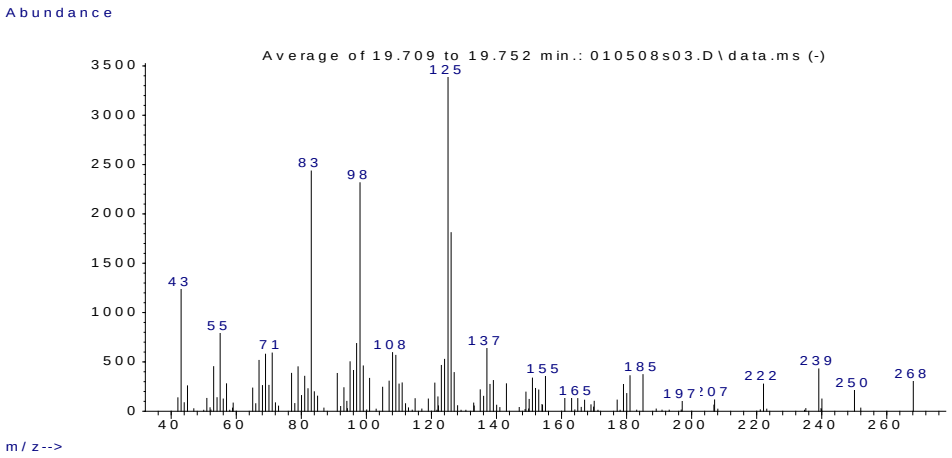
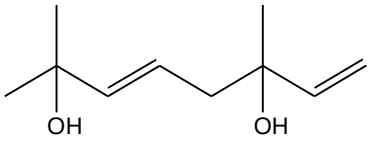


Figure 5.4 Mass spectrum of meliracemoic acid methyl ester found in methylated kamahi honey extracts

Moderate to high levels of 2,6-dimethylocta-3,7-diene-2,6-diol **(22)** (peak 10) were observed ranging from 7.2 - 48.4 mg/kg. Both hydroxyl groups remained un-methylated upon the addition of excess diazomethane. 2,6-Dimethylocta-3,7-diene-2,6-diol is not uncommon in honey and has been reported in a previous investigation of kamahi honey as well as New Zealand rewarewa honey and Australian yellow box honey.[15, 68, 117]



(22)

The presence of 2,6-dimethylocta-3,7-diene-2,6-diol in kamahi honey extracts has been confirmed by isolation and NMR structure analysis.[68] The mass spectrum of peak 10 is given in Figure 5.5, and was found to be identical to the corresponding spectra reported in the earlier study.[68]

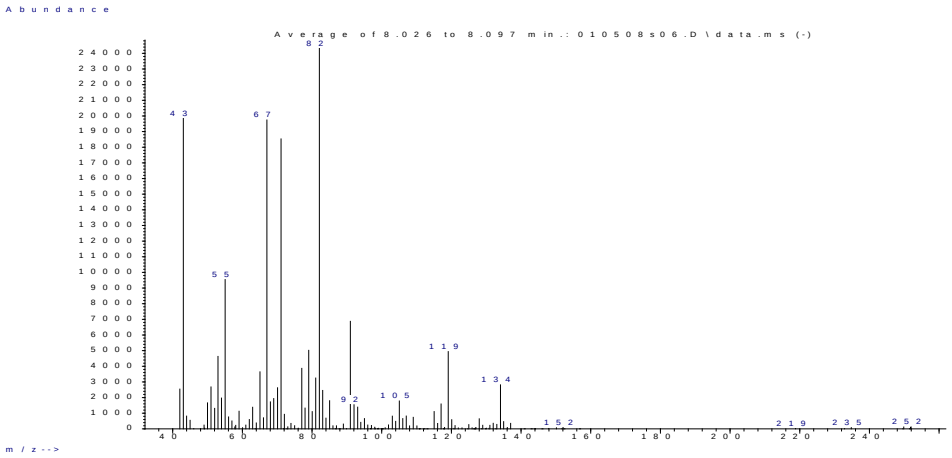
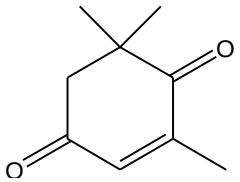
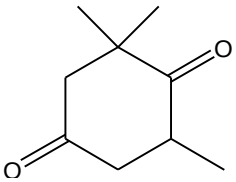


Figure 5.5 Mass spectrum of 2,6-dimethylocta-3,7-diene-2,6-diol found in methylated kamahi honey extracts

The degraded carotenoid 3,3,5-trimethylcyclohex-2-ene-1,4-dione **(23)** (peak 6) was detected in all samples ranging from 2.3 - 6.2 mg/kg. This degraded carotenoid is found in New Zealand ling heather and rewarewa honey as well as Australian yellow box honey.[11, 15, 117] The related compound 2,2,6-trimethylcyclohexane-1,4-dione **(24)** (peak 8) was detected in low levels in eight kamahi honey samples and has not been reported in any other New Zealand honeys. This compound has previously been detected in Australian yellow box honey.[117] The mass spectrum of peak 6 is given in Figure 5.6 and peak 8 in Figure 5.7.



(23)



(24)

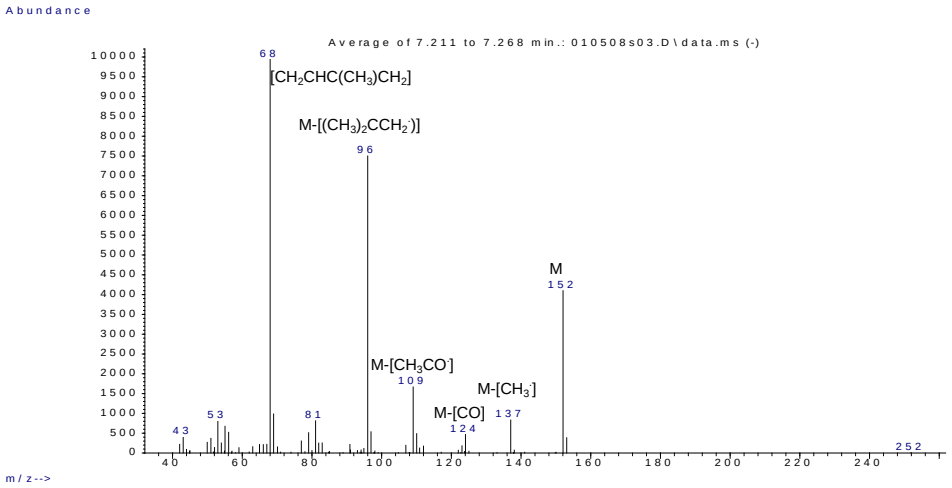


Figure 5.6 Mass spectrum of 3,3,5-trimethylcyclohex-2-ene-1,4-dione found in methylated kamahi honey extracts

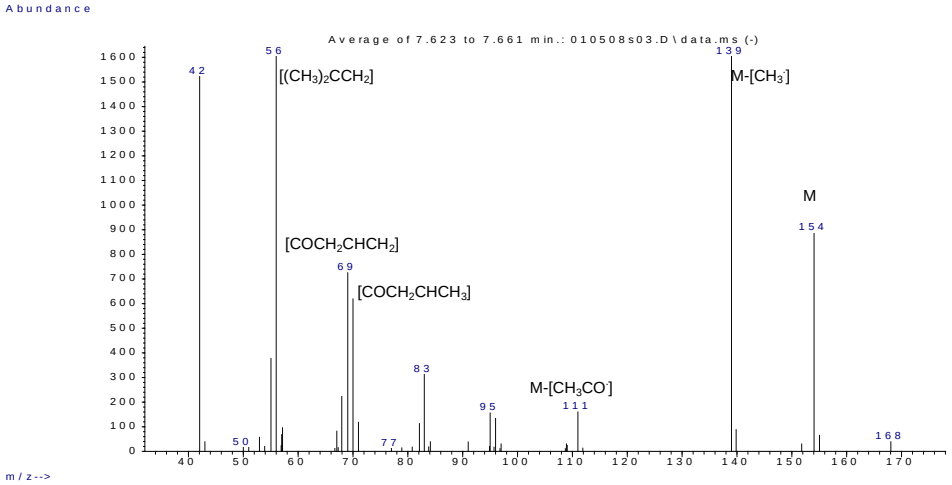
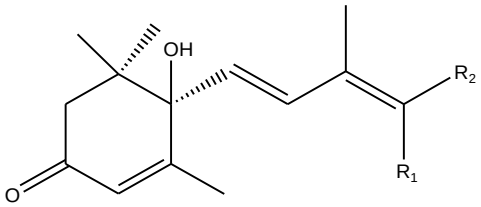


Figure 5.7 Mass spectrum of 2,2,6-trimethylcyclohexane-1,4-dione found in methylated kamahi honey extracts

Both *cis,trans*-abscisic acid **(25)** and *trans,trans*-abscisic acid **(26)** were present in low levels in the majority of kamahi honeys. Analysis of ethylated subsamples of the extractive solutions showed that (unlike that of methyl syringate in manuka honey extracts) neither of these acids were in the kamahi honey extracts as the corresponding methyl ester. One honey (sample 5) was found to contain considerably more abscisic acid than the rest. According to the pollen analysis results this sample contains a moderate proportion of willow pollen. Willow honey has been found to contain high levels of both *cis,trans*- and *trans,trans*-abscisic acid.[12] The elevated levels of abscisic acid can therefore be attributed to the contribution of willow in this sample.



(25) R_1 = H, R_2 = COOH

(26) R_1 = COOH, R_2 = H

The present extractives data, combined with pollen analysis results verifies that kamahines A-C and meliracemoic acid can be deemed to be floral markers for kamahi honey.

A single unidentified compound was present exclusively in kamahi honey, the mass spectrum of which is given in Figure 5.8. The structure of this compound (peak 33) which exhibited significant *m/z* 137, 181, 251 and 266 ions is not known however its mass spectral features and apparent molecular weight (266 Da) indicate that this may be an oxygenated degraded carotenoid-like compound analogous to kamahines A-C and meliracemoic acid which Broom and Ede have previously isolated from kamahi honey.[104-106, 111] Given the low quantity of this compound present in kamahi honey (0.3 - 2.4 mg/kg), extraction of a bulk quantity of honey, followed by chromatographic fractionation of the extractions and isolation of the '266 Da compound' would be required in order to obtain sufficient material for structural determination using NMR methods.

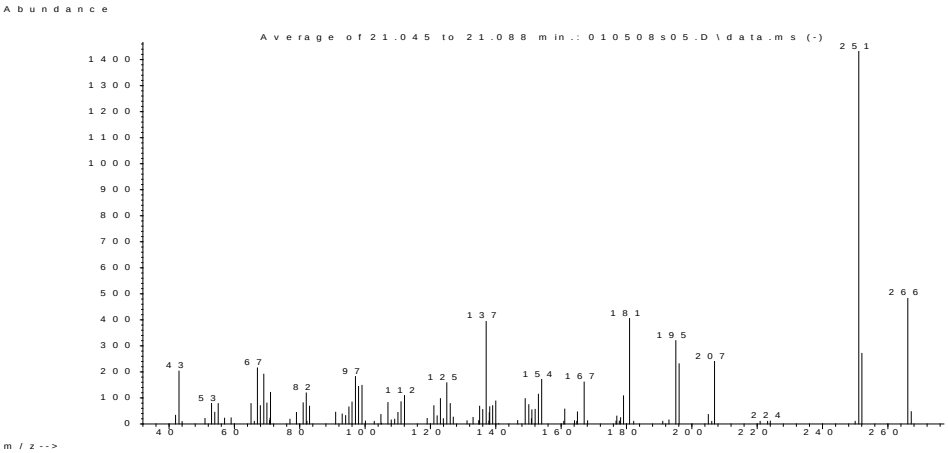


Figure 5.8. Mass spectrum of unidentified compound (peak 33) found in methylated kamahi honey extracts

5.3.3 Pohutukawa (*Metrosideros excelsa*) Honey

Due to the scarce nature of pohutukawa honey and uncertainty concerning the floral integrity of the available samples, only three samples of this honey have been examined in earlier studies. The three previous samples were found to contain relatively low levels of extractable organic substances, with only a total of nine compounds detected.[109]

Ten pohutukawa honey samples were obtained from Waitemata Honey Ltd. which sources its pohutukawa honey from Rangitoto Island in the Waitemata Harbour. Pohutukawa is ideally suited to the costal volcanic environment of Rangitoto Island and is a predominant part of the flora on the island. No supporting information such as pollen analysis data was available with these honeys; however, due to the isolated nature of the harvest site, no significant contribution from other nectars source is expected.

A representative GC-MS chromatogram from pohutukawa honey is given in Figure 5.9. Peak identifications and concentrations are listed in Table 5.8.

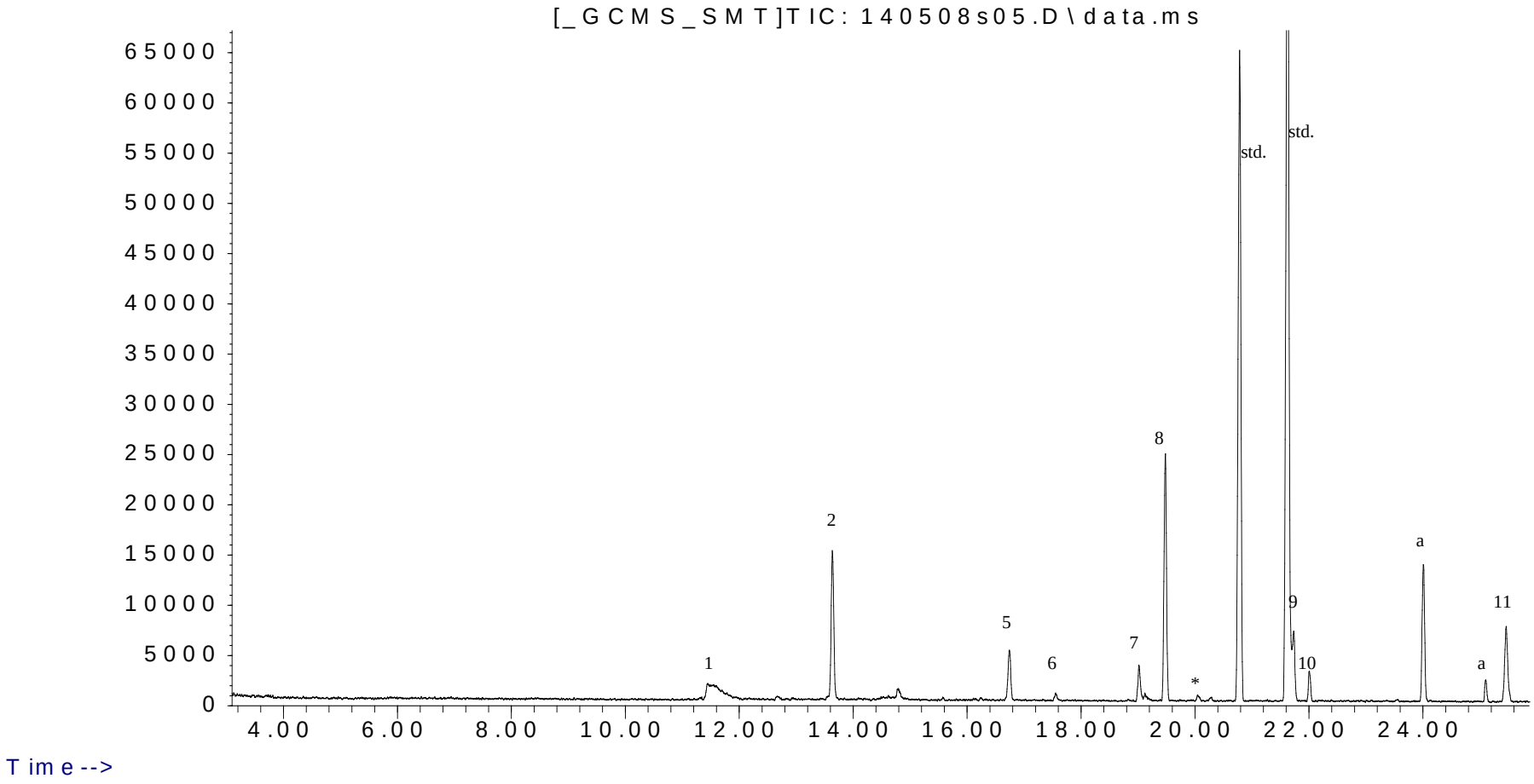


Figure 5.9 GC-MS profile of a representative methylated pohutukawa honey extract. Peak identifications are listed in Table 5.8.

* = phthalate, [a] = alkane (from wax)

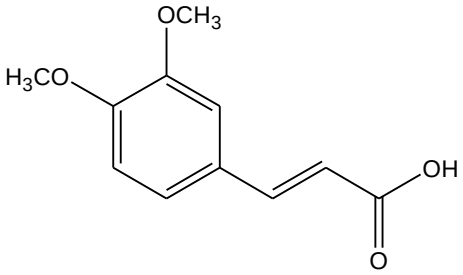
Table 5.8 Concentration of compounds detected in diethyl ether extraction of pohutukawa honey (mg/kg). Acids are quantified as the corresponding methyl ester

			Sample									
Peak	**RT**	**Compound**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	11.451	phenyllactic acid	1.0	2.8	3.4	-	1.6	-	2.2	0.6	1.2	0.3
2	13.637	lauric acid	2.6	1.7	2.0	1.3	2.0	2.1	2.7	1.0	3.8	7.2
3	15.572	decanedioic acid	-	-	0.3	-	-	-	0.2	-	0.1	-
4	16.344	decene-2-dioic acid	0.2	-	0.4	-	-	-	-	-	-	-
5	16.742	methyl syringate	0.6	1.4	1.7	0.9	1.2	1.6	1.4	0.8	1.0	1.4
6	17.559	*cis*-3,4-dimethoxycinnamic acid	0.2	0.2	0.4	-	0.1	0.2	0.3	0.4	0.4	0.6
7	19.024	*trans*-3,4-dimethoxycinnamic acid	0.3	0.6	1.2	0.6	0.6	0.9	0.9	0.4	0.3	0.4
8	19.475	palmitic acid	1.1	2.6	4.7	2.0	3.0	4.5	2.4	1.7	1.8	3.3
9	21.736	9-octadecenoic acid	-	0.7	1.7	1.1	0.9	4.7	1.2	0.6	0.8	0.9
10	22.021	stearic acid	0.3	0.3	0.4	0.4	0.3	0.9	0.4	0.3	0.3	0.6
11	25.462	pinostrobin chalcone	1.3	2.5	2.8	1.9	2.1	3.7	2.5	1.5	1.5	2.0
Total organics			8	13	19	8	12	19	14	7	11	17

- = not detected

Pohutukawa honey contains very low levels of extractable organic substances (average of 13 mg/kg). The scant extract is dominated by aliphatic fatty acids in particular lauric acid (1.0 - 7.2 mg/kg) and palmitic acid (1.1 - 4.7 mg/kg).

Both the *cis* and *trans* forms of 3,4-dimethoxycinnamic acid **(27)** (peaks 6 and 7) were detected in almost all samples. Due to the prolonged exposure to excess diazomethane it is likely that the original compound was the dihydroxy form. 3,4-Dihydroxycinnamic acid has been found in a number of different honeys including several Australian honeys and Greek cotton honey.[23, 116, 118] The mass spectrum of the *trans* isomer is given in Figure 5.10. Neither of these isomers were detected in the previous survey by Sun.[109]



(27)

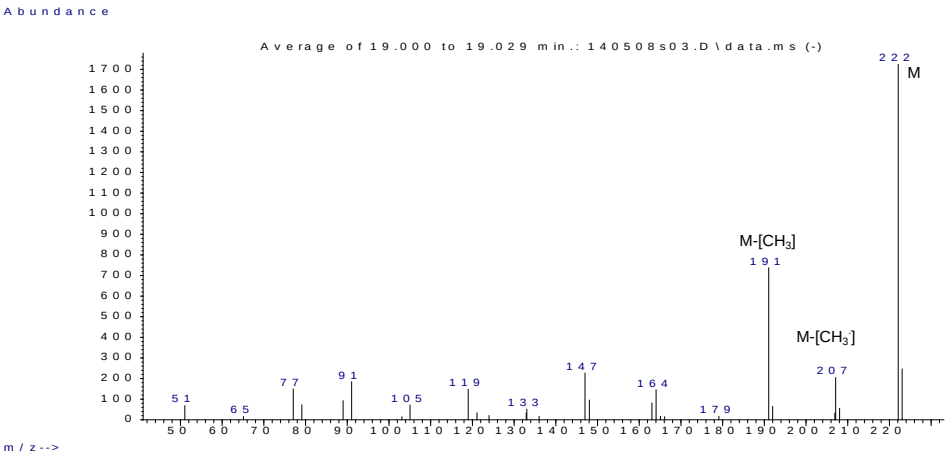
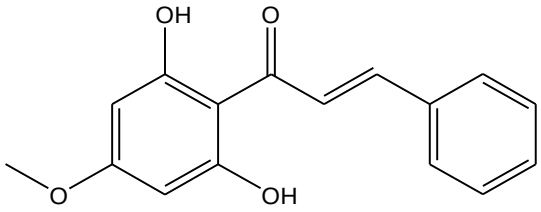


Figure 5.10 Mass spectra of methylated *trans* 3,4-dimethoxycinnamic acid found in methylated pohutukawa honey extracts

The flavonoid pinostrobin chalcone **(28)** (peak 11) was detected in all samples ranging from 1.3 - 3.7 mg/kg. The mass spectrum of pinostrobin chalcone is given in Figure 5.11, neither of the hydroxyl groups have been methylated during the derivatisation

process Pinostrobin chalcone has previously been found in cotton honey at a level considerably less than pohutukawa honey (average 0.031 mg/kg)[116] and in propolis.[119]



(28)

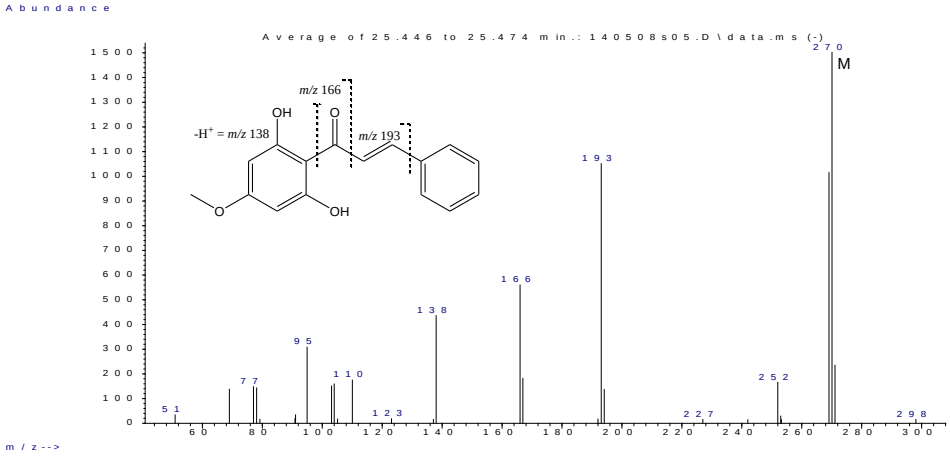


Figure 5.11 Mass spectrum of pinostrobin chalcone found in methylated pohutukawa honey extracts

5.3.4 Rata (*Metrosideros umbellata*) Honey

One survey comprising four samples has previously been conducted on rata honey.[110] Given the marked difference in the organic extracts of these samples, the purity of these honeys was questioned. This being the case, very little is known of the true organic composition of rata honey.

Ten unifloral rata honeys supplied and certified unifloral by Airborne Honey Limited were analysed in this investigation. Rata honey typically contains a proportion of kamahi honey as both species grow in the same area with an overlapping nectar flow. The consistent contribution of kamahi as a secondary nectar source in rata honey is confirmed by the pollen analysis results in Table 5.9.

Table 5.9 Rata honey pollen analysis data

Sample ID	1st Pollen type	1st Pollen (%)	2nd Pollen type	2nd Pollen (%)	3rd Pollen type	3rd Pollen (%)	Total Pollen (/10 g honey)
21894	Rata	62.5	Kamahi	32.5	Clover	1.5	201600
19706	Rata	65.6	Kamahi	28	Lotus	5.7	256600
19711	Rata	79.5	Kamahi	17.8	Clover	1.4	161600
20212	Rata	56.7	Kamahi	28.3	Lotus	10.2	379900
22294	Rata	77.5	Kamahi	19	Manuka	3.5	200000
22222	Rata	79	Kamahi	15	Manuka	2.5	197500
22224	Rata	59.5	Kamahi	31	Lotus	3.5	112500
22287	Rata	66.5	Kamahi	11	Clover	8	135000
22288	Rata	86.5	Kamahi	8	Clover	3	295000
19707	Rata	66.9	Kamahi	20.5	Lotus	5.4	259950

The GC-MS profile of a typical methylated rata honey extraction is given in Figure 5.12. Peak identifications and concentrations are listed in Table 5.10.

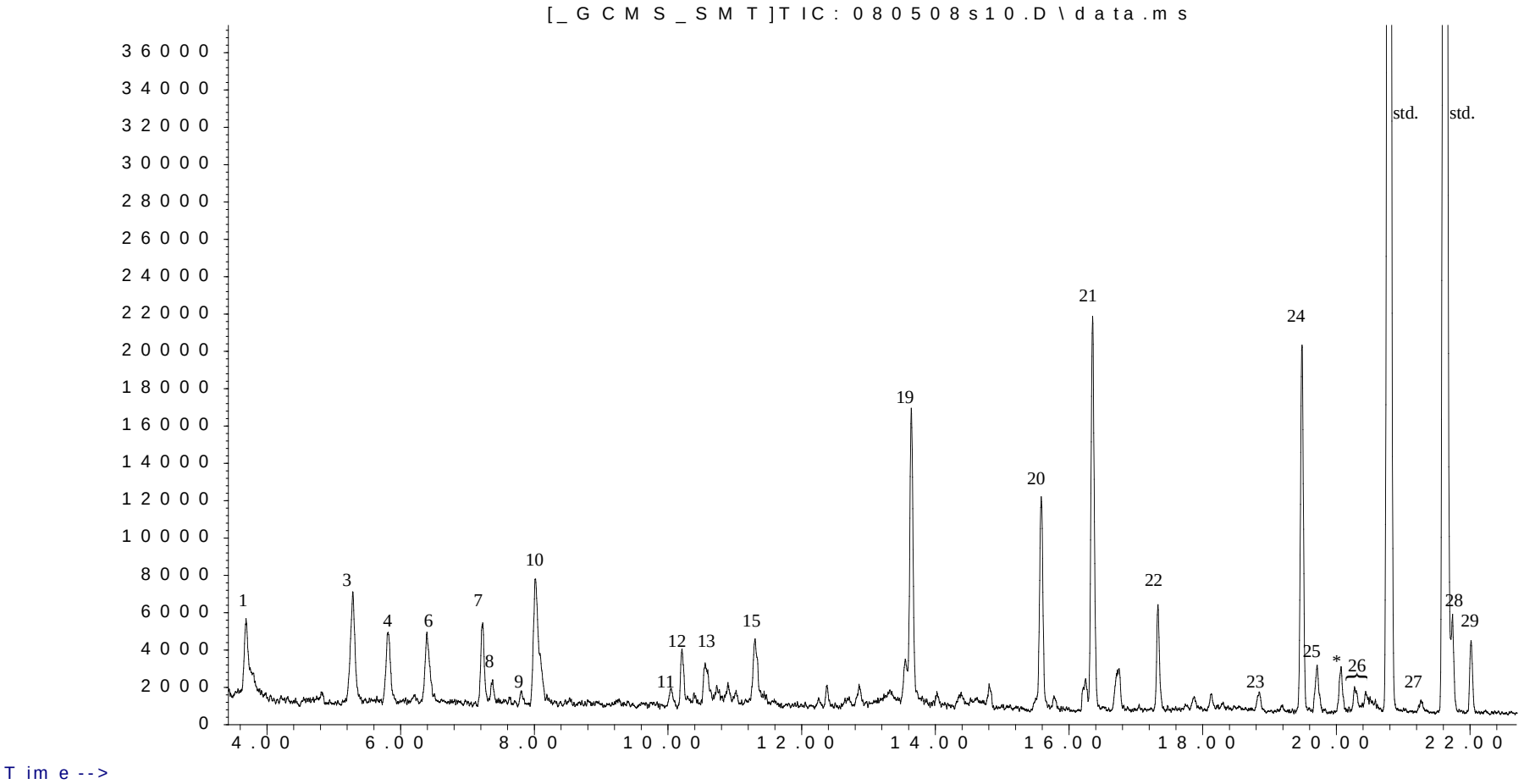


Figure 5.12 GC-MS profile of a representative methylated rata honey extract. Peak identifications are listed in Table 5.10. * = phthalate

Table 5.10 Concentration of compounds detected in diethyl ether extraction of rata honey (mg/kg). Acids are quantified as the corresponding methyl ester

			Sample									
Peak	**RT**	**Compound**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	3.685	unknown (*m/z* 45, 118)	-	-	-	2.3	0.7	1.1	1.0	2.7	2.5	0.7
2	4.41	valeric acid	-	-	-	-	-	-	1.7	1.4	-	-
3	5.284	succinic acid	-	3.3	6.3	3.1	2.6	2.4	6.3	10.8	4.4	0.9
4	5.819	methylsuccinic acid	-	3.3	2.1	3.0	1.2	1.6	1.9	1.2	0.9	0.6
5	6.221	2,2-dimethylsuccinic acid	-	0.1	0.2	-	-	0.2	0.8	1.6	0.3	-
6	6.406	benzoic acid	-	0.6	0.6	-	0.3	0.4	0.4	0.7	0.2	0.6
7	7.216	3,5,5-trimethyl-2-cyclohexene-1,4-dione	1.5	0.9	1.1	2.0	0.3	1.0	1.1	3.2	0.3	0.6
8	7.523	2-methyleneglutaric acid	-	0.1	1.3	0.4	-	-	-	0.3	-	-
9	7.801	phenylacetic acid	-	0.1	-	0.1	0.2	-	1.0	1.2	0.2	-
10	8.034	2,6-dimethylocta-3,7-diene-2,6-diol	9.8	2.1	0.7	2.0	0.7	2.7	1.3	0.5	0.7	1.3
11	10.042	unknown (*m/z* 139)	-	0.5	-	0.7	0.1	0.4	0.3	-	-	0.2
12	10.209	unknown (*m/z* 67, 82, 110, 123, 138)	-	-	-	-	-	0.7	2.2	12.0	1.0	0.3
13	10.593	unknown (*m/z* 69, 97, 101, 129, 156)	-	1.1	-	5.1	0.3	1.2	3.1	5.0	0.7	0.4
14	11.016	unknown (*m/z* 115, 143)	-	-	-	1.0	-	-	0.2	0.5	-	-
15	11.307	4-methoxybenzoic acid	-	0.4	0.4	-	-	8.0	7.2	2.9	0.4	0.5
16	11.433	phenyllactic acid	-	-	-	-	-	1.9	1.5	0.3	-	-
17	12.25	4-methoxyphenylacetic acid	-	-	-	-	-	-	0.3	3.8	0.2	-
18	12.382	octanedioic acid	-	0.2	0.2	0.3	-	-	0.2	0.8	-	-
19	13.639	lauric acid	2.9	-	-	-	-	-	-	-	-	1.0
20	15.584	decanedioic acid	-	1.1	2.0	0.9	0.7	1.5	2.4	4.9	1.1	1.3
21	16.358	decene-2-dioic acid	0.2	3.7	8.9	2.4	1.7	1.9	3.8	9.4	4.5	2.5
22	17.33	unknown (*m/z* 82, 95, 150, 210)	-	0.5	0.4	0.3	0.5	0.4	0.7	2.2	0.3	0.6
23	18.83	unknown (*m/z* 59, 121, 160, 210)	-	0.1	-	-	0.1	-	0.3	1.6	0.4	0.1
24	19.494	palmitic acid	1.4	0.5	0.7	1.1	0.9	1.1	1.3	2.2	1.4	1.2
25	19.725	meliracemoic acid	2.7	0.4	-	0.7	0.2	0.7	0.4	-	-	0.3
26	20.44	kamahines A-C	4.4	0.3	0.3	2.1	0.2	2.4	1.0	0.5	0.3	0.4
27	21.285	unknown (*m/z* 83, 127, 155, 179)	2.3	-	-	0.3	-	0.2	-	-	-	-

			Sample									
Peak	**RT**	**Compound**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
28	21.725	9-octadecenoic acid	-	0.2	0.4	1.1	-	0.3	0.4	0.7	0.2	0.3
29	22.036	stearic acid	1.2	0.1	0.1	0.2	0.2	0.3	0.2	0.3	0.3	0.2
Total organics			26	20	26	29	11	30	41	71	20	14

- = not determined

Rata honey was found to contain low levels of extractable organic substances (typically >50 mg/kg). Acids and diacids were the predominant class of compound. Palmitic acid, stearic acid and decene-2-dioic acid were present in all samples while all but sample one contained the diacids succinic acid, methyl succinic acid and decanedioic acid. The predominance of acids and diacids is typical of lighter coloured honeys.[108]

The most predominant non-aliphatic compound was the degraded carotenoid 3,3,5-trimethylcyclohex-2-ene-1,4-dione (peak 7) which was present in all samples ranging from 0.3 - 3.2 mg/kg. This compound is present in several different honeys including New Zealand ling heather and rewarewa honey and Australian yellow box honey.[11, 15, 117]

Meliracemoic acid (peak 25), a floral marker for kamahi honey was detected in the majority of rata honeys (0.2 - 2.7 mg/kg, n = 7). Given that all rata honeys were found to contain a proportion of kamahi honey (as indicated by pollen analysis results Table 5.9), meliracemoic acid almost certainly originates from the kamahi contribution.

Kamahines A-C (peak 26) were detected in low levels in all samples (0.2 - 4.4 mg/kg). In unifloral kamahi honey, kamahines are seen as a broad band due to the equilibration between A, B and C. At lower levels such as those seen in rata honey, a distinct peak followed by a broad peak occurs. This can be explained by Equation 1 where $k_r > k_f$ and $k_f' > k_r'$. At low concentrations the rate of k_r and k_f' is sufficient to maintain very low levels of B, however at higher concentrations, k_r and k_f' are unable to keep the concentration of B low, hence a large broad peak (mixture of A, B and C) is seen.

$$A \underset{k_r}{\overset{k_f}{\rightleftharpoons}} B \underset{k_r'}{\overset{k_f'}{\rightleftharpoons}} C$$

Equation 1

The substituted alkene 2,6-dimethylocta-3,7-diene-2,6-diol, a dominant constituent in kamahi honey was present in all rata honeys ranging from 0.5 - 9.8 mg/kg. 2,6-Dimethylocta-3,7-diene-2,6-diol most likely originates from the kamahi contribution in rata honey.

5.3.5 Tawari (*Ixerba brexioides*) Honey

There are no published accounts of the extractable organic substances in tawari honey. A set of ten unifloral tawari honeys was supplied by Airborne Honey Ltd. along with the corresponding pollen data (Table 5.11). Tawari pollen is extremely underrepresented in tawari honey unlike clover and kamahi pollen (a common secondary nectar source in tawari honey). Consequently even a small contribution of clover or kamahi nectar can have a marked impact on the pollen count.

Table 5.11 Tawari honey pollen analysis data

Sample ID	1st Pollen type	1st Pollen (%)	2nd Pollen type	2nd Pollen (%)	3rd Pollen type	3rd Pollen (%)	Total Pollen (/10 g honey)
22246	Tawari	2	OT	37.5	Clover	20	209950
22200	Tawari	77	Clover	10.5	Lotus	10.5	178250
22244	Tawari	34	Clover	26	OT	16	171600
22712	Tawari	4.6	Clover	80	Lotus	8.2	63300
21961	Tawari	3.5	Clover	69	Lotus	10.6	153300
22195	Tawari	5.5	Kamahi	72	Clover	11	199950
22203	Tawari	54.5	Clover	30	Lotus	9	179950
22350	Tawari	10	Clover	50.5	Lotus	31.5	78250
22253	Tawari	17	Kamahi	32	Clover	22.5	101600
22435	Tawari	2	Kamahi	48.7	OT	33.3	81600

A representative GC-MS chromatogram of the methylated tawari honey extracts is given in Figure 5.13. Peak identifications and concentrations are listed in Table 5.12.

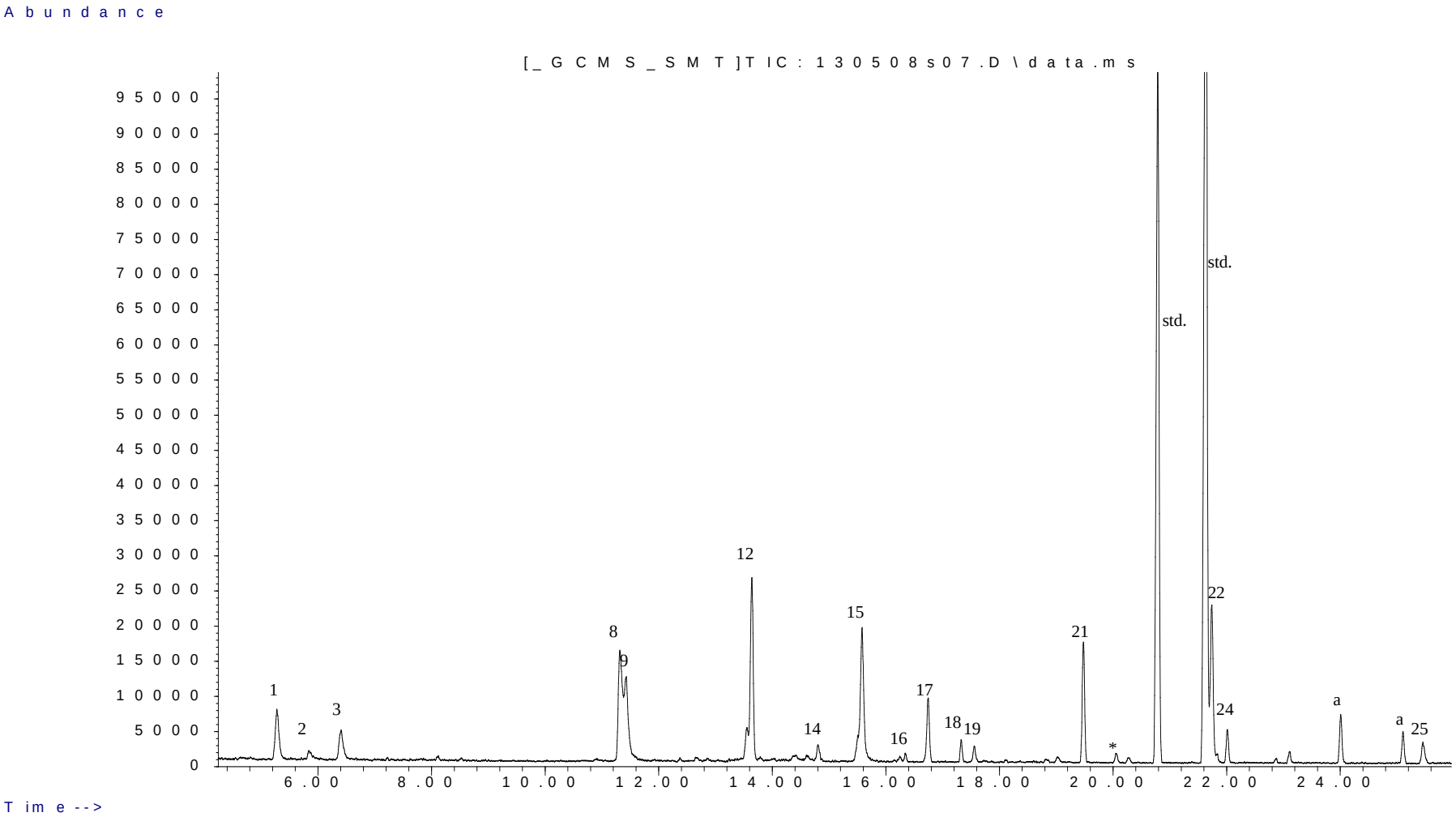


Figure 5.13 GC-MS profile of a representative methylated tawari honey extract. Peak identifications are listed in Table 5.12.

* = phthalate, [a] = alkane (wax)

Table 5.12 Concentration of compounds detected in diethyl ether extraction of tawari honey (mg/kg). Acids are quantified as the corresponding methyl ester

			Sample									
Peak	**RT**	**Compound**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	5.276	succinic acid	-	1.9	1.3	0.2	0.6	0.3	1.3	3.1	0.8	3.2
2	5.89	methylsuccinic acid	-	0.2	0.2	0.3	0.3	0.5	0.2	-	0.3	0.5
3	6.393	benzoic acid	-	0.4	0.3	0.7	1.3	1.2	0.9	0.4	0.4	0.7
4	7.203	3,5,5-trimethyl-2-cyclohexene-1,4-dione	-	0.1	-	-	-	0.6	-	-	0.4	-
5	7.363	unknown (*m/z* 59, 71, 129, 141)	-	-	-	-	-	0.4	-	-	0.5	-
6	7.801	phenylacetic acid	-	-	-	-	0.2	-	-	0.2	-	-
7	8.027	2,6-dimethylocta-3,7-diene-2,6-diol	1.2	-	-	-	-	2.1	-	-	2.6	0.9
8	11.299	4-methoxybenzoic acid	11.6	11.7	4.6	13.0	8.7	22.8	3.1	9.3	6.2	3.3
9	11.428	phenyllactic acid	-	-	8.3	3.5	4.5	6.7	1.9	-	4.2	1.1
10	12.25	4-methoxyphenylacetic acid	-	-	-	0.5	0.3	-	-	-	-	-
11	12.372	octanedioic acid	-	0.1	0.2	0.2	0.2	0.3	-	0.5	-	0.3
12	13.641	lauric acid	3.3	0.4	1.8	1.1	2.3	2.0	2.3	2.1	4.0	1.5
13	14.02	nonanedioic acid	-	0.4	-	0.1	-	0.2	-	0.6	-	-
14	14.816	3,4-dimethoxybenzoic acid	0.2	0.4	0.2	0.5	0.3	0.4	0.3	3.3	0.2	0.4
15	15.575	decanedioic acid	1.0	7.1	0.9	2.6	4.6	14.5	3.7	8.1	0.8	1.8
16	16.344	decene-2-dioic acid	1.0	0.8	-	-	1.3	1.0	0.2	5.8	1.8	-
17	16.747	methyl syringate	1.2	3.9	1.0	5.1	7.8	5.5	1.2	1.3	0.8	0.3
18	17.329	unknown (*m/z* 82, 95, 150, 210)	-	0.5	0.3	0.8	1.1	0.7	0.4	1.0	-	0.4
19	17.556	*cis*-3,4-methoxycinnamic acid	-	0.1	-	0.6	0.3	0.1	0.3	0.7	-	0.2
20	19.023	*trans*-3,4-methoxycinnamic acid	-	-	-	0.3	0.4	0.2	0.1	0.4	0.2	0.2
21	19.485	palmitic acid	1.4	0.9	1.0	2.2	2.4	1.6	1.3	1.7	1.4	2.2
22	21.775	9-octadecenoic acid	2.9	1.6	1.1	2.5	2.6	1.0	2.0	2.0	1.2	1.8
23	21.885	*cis,trans*-abscisic acid	1.0	-	0.4	0.8	2.9	-	0.1	-	-	3.2
24	22.03	stearic acid	0.5	0.2	0.2	0.5	0.5	0.4	0.3	0.5	0.3	0.5
25	25.458	pinostrobin chalcone	-	0.3	-	0.5	0.7	0.1	0.6	1.5	-	0.3
Total organics			25	31	22	36	43	62	20	43	26	23

- = not detected

Tawari honey was found to be moderately low in total extractable organics (20 - 62 mg/kg). The aromatic acid 4-methoxybenzoic acid (peak 8) was a consistently dominant feature in all honeys (3.1 - 22.8 mg/kg). The composition of the remaining extract contains a high proportion of the aliphatic acids lauric, palmitic, 9-octadecanoic and decanedioic acid.

Low levels of 3,4-dimethoxybenzoic acid (peak 14) were detected in all samples (0.2 - 3.3 mg/kg). This compound is most likely to be derived from the methylation of the dihydroxy form due to prolonged exposure to diazomethane. 3,4-Dihydroxybenzoic acid is found in similar levels in New Zealand thyme and heather honey.[11, 12]

Methyl syringate, a floral marker for manuka honey was a constant contributor in all honeys ranging from 0.3 - 7.8 mg/kg. Although manuka pollen did not feature in the pollen analysis results for these honeys, the geographical location and nectar flow for manuka is similar to tawari.

The flavonoid pinostrobin chalcone was detected in seven out of ten honeys. This flavonoid is a consistent feature of pohutukawa honey (Section 5.3.3).

While some flavonoids are detected in diethyl ether extracts, they are not considered to be exhaustively recovered using this approach. In general HPLC methods are used to quantitatively analyse flavonoids following extraction with amberlite resin.[120]

5.3.6 Summary

The following table (Table 5.13) summarises the characteristics of each honey type studied in the present investigation. Together with Table 5.1, this gives a summary of the characteristics for all major New Zealand unifloral honeys.

Table 5.13 Summary of honey characteristics

Honey	Characteristic and/or floral marker	Range (mg/kg)
Beech honeydew honey	indole-3-acetic acid	1.1 - 16.8
	4-hydroxy-3,5-dimethoxyhydrazidebenzoic acid	0.4 - 16.5
Kamahi	kamahines A-C	>6
	meliracemoic acid	>2.5
Pohutukawa	Low extractable organic substances	<20
	pinostrobin chalcone	1.3 - 3.7
	cis-3,4-dimethoxycinnamic acid	0.1 - 0.6
	trans-3,4-dimethoxycinnamic acid	0.3 - 1.2
Rata	Low extractable organic substances	≈50
	Low levels of typical kamahi compounds	
Tawari	Low extractable organic substances	≈50

5.4 Statistical Analysis

A series of statistical analysis were undertaken in order to ascertain if a unique fingerprint exists in the extractable organic substances in honey which can be related to floral origin. A matrix was constructed containing the concentration of compounds detected in each sample of all honeys as outlined in Section 2.2.9. A list of the compounds used in this matrix is given in Table 5.14. Compounds present in fewer than five honeys from a particular floral source were excluded from the statistical investigation as the focus was on floral specific compounds, not those associated with geographical location.

Table 5.14 Compounds used in the statistical analysis of honey extractives data

Compound number	Name
1	unknown (*m/z* 45, 118)
2	succinic acid
3	methylbutanedioic acid
4	methylsuccinic acid
5	2,2-dimethylsuccinic acid
6	benzoic acid
7	glutaric acid
8	3,5,5-trimethyl-2-cyclohexene-1,4-dione
9	unknown (*m/z* 59, 71, 129, 141)
10	2,6,6-trimethylcyclohexane-1,4-dione
11	phenylacetic acid

Compound number	Name
12	2,6-dimethylocta-3,7-diene-2,6-diol
13	salicylic acid
14	2-coumaranone
15	unknown (*m/z* 54, 82, 110, 151, 166)
16	2-methoxyacetophenone
17	unknown (*m/z* 139)
18	unknown (*m/z* 67, 82, 110, 123)
19	unknown (*m/z* 70, 95, 140, 154, 168)
20	2-methoxybenzoic acid
21	unknown (*m/z* 55, 71, 79, 91)
22	4-methoxybenzoic acid
23	phenyllactic acid
24	4-methoxyphenylacetic acid
25	octanedioic acid
26	4-hydroxybenzoic acid
27	4-hydroxyphenylacetic acid
28	lauric acid
29	nonanedioic acid
30	3,5-dimethoxybenzoic acid
31	3,4-dimethoxybenzoic acid
32	1-methoxy-4-propylbenzene
33	decanedioic acid
34	decene-2-dioic acid
35	methyl syringate
36	unknown (*m/z* 82, 95, 150, 210)
37	4-hydroxy-3,5-dimethoxyhydrazidebenzoic acid
38	indole-3-acetic acid
39	*cis*-3,4-methoxycinnamic acid
40	*trans*-3,4-dimethoxycinnamic acid
41	palmitic acid
42	meliracemoic acid
43	kamahines A-C
44	unknown (*m/z* 137, 181, 251, 266)
45	unknown (*m/z* 83, 127, 155, 179)
46	9-octadecanoic acid
47	*cis,trans*-abscisic acid
48	stearic acid
49	*trans,trans*-abscisic acid
50	pinostrobin chalcone

5.4.1 Data Pre-Processing

Identifying and correcting for transformable inhomogeneity in a data set can result in the simplification and increased sensitivity of subsequent statistical modelling.[99] In order to identify if a transformation of the extractives data is necessary, the mean of each compound was plotted against the standard deviation. It can be seen in Figure 5.14

that the standard deviation is proportional to the mean (an approximate linear relationship) and therefore a log transformation is appropriate (as in Section 3.4.1).

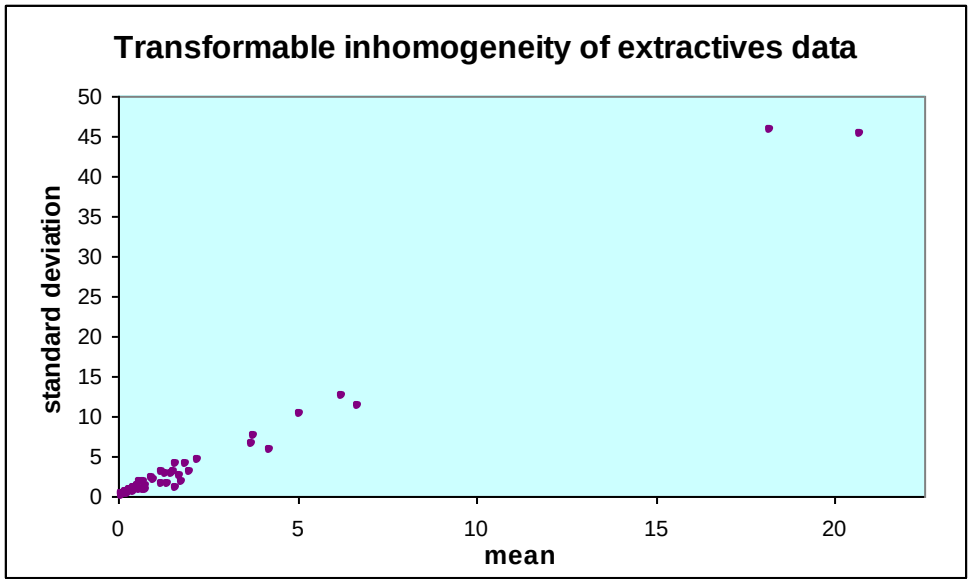


Figure 5.14 Plot of compound standard deviation vs mean of the extractives concentrations

Subsequent to a log transformation, no relationship was apparent between the mean and standard deviation. All subsequent analyses are performed on the log extractives matrix.

5.4.2 Exploratory Statistical Techniques

5.4.2.1 Cluster Analysis

Agglomerative clustering techniques were used to examine the extractives data, a definition of this analysis is given in Section 2.4.1. A range of different distance measures were used on the log matrix of the extractives concentrations using R, the results of which are summarised in Table 5.15. The least number of misclassified samples was obtained with the Ward distance measure. The resulting dendrogram from hierarchical clustering using Ward's method is given in Figure 5.15.

Table 5.15 Results of hierarchical clustering using various distance measures

Distance measure	Defined clusters	Misclassified/ ungrouped samples	Honey type (misclassified)				
			HD	K	PO	R	TW
Ward	5	2	1		1		
Single	5	4	1	2	1		
Complete	5	3	1	1	1		
Average	5	3	1	1	1		

HD = beech honeydew, K = kamahi, PO = pohutukawa, R = rata, TW = tawari

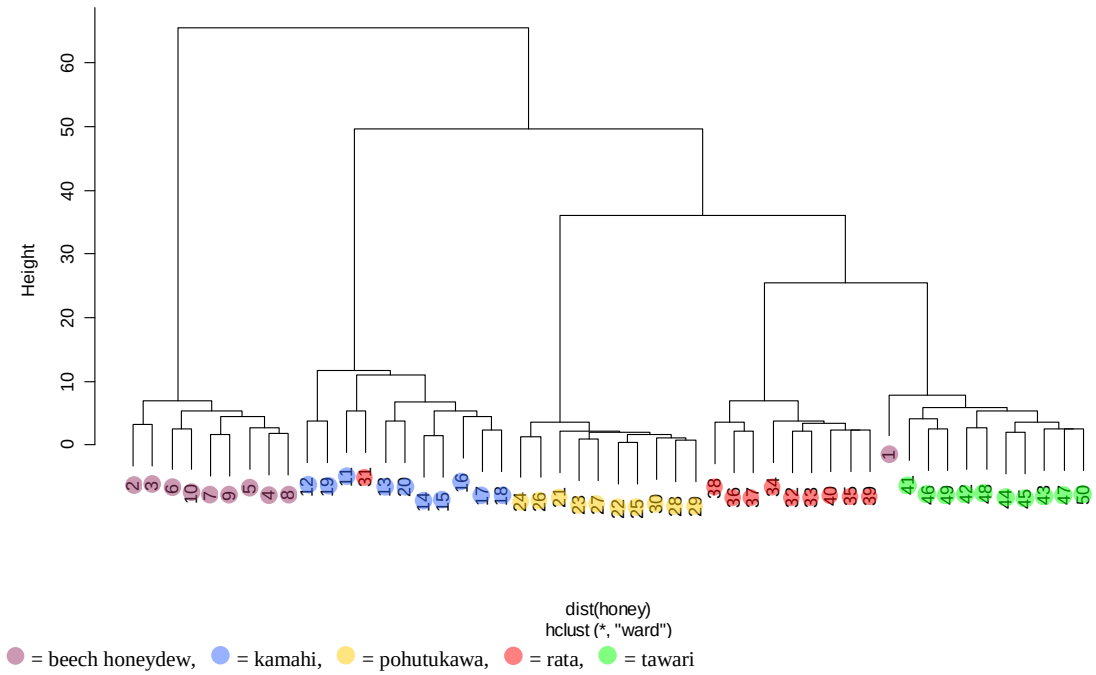


Figure 5.15 Dendrogram of the log extractives matrix using Ward's method

Agglomerative clustering techniques are used to reveal relationships and indicate where patterns of similarity lie. The dendrogram of agglomerative cluster analysis on the log extractives data using Ward's method gives an excellent degree of separation between floral types. Two samples were misclassified; a beech honeydew honey (sample 1) was grouped with tawari honey and a rata honey (sample 31) was grouped with kamahi honey.

The total extractives in sample 1 were significantly lower than other beech honeydew honey samples (71 mg/kg compared to 135 - 591 mg/kg). Sample 1 also contained a distinct 4-methoxybenzoic acid and decanedioic acid peak whereas the other beech honeydew honeys did not. It is likely that sample 1 was misclassified as tawari honey as both 4-methoxy benzoic acid and decanedioic acid are present in all tawari honeys

which also low contain a low level of total extractives. As the physiochemical properties of beech honeydew honey were well within the limits characteristic of beech honeydew honey (data supplied by Airborne Honey Ltd who characterised all the honeys), this sample was not excluded from the statistical analysis. As only 10 honeydew honeys were analysed, it can not be ruled out that the extractives profile displayed in sample 1 could not be attributed to a true beech honeydew honey.

As can be seen by the pollen analysis results for rata honey (Table 5.9), kamahi pollen and thus nectar (both species grow in the same area with a similar nectar flow over the summer months) is consistently found in all rata honeys in this study. Sample 31 contained the highest proportion of kamahi pollen (32.5%) in this study compared to other rata samples (average 21%). Rata honey has been found to contain low levels of extractable organic substances (Table 5.10) whereas kamahi contains a high proportion of extractives (Table 5.7), some of which are unique to honey originating from kamahi nectar. The levels of kamahi derived extractives in sample 31 were much higher than other rata honeys, most notably meliracemoic acid, kamahines A-C and 2,6-dimethylocta-3,7-diene-2,6-diol.

The level at which the clusters are joined along the y axis indicates how early on in the agglomerative process the groups were formed. Beech honeydew honey samples were split from the remaining floral sources first which indicates that this group has the highest degree of between group variance. Very little difference is seen in the splitting level of the individual samples of beech honeydew honey which indicates all these samples are very similar. The misclassified honeydew honey (sample 1) is joined to the tawari honey group at a level much higher than the other tawari samples which indicates that the model found this sample to be different to the remaining tawari honeys.

The level at which kamahi honeys were joined together varied more than other honey types, this is a reflection of the degree of variation seen in kamahi extractive composition. On the other hand pohutukawa honeys were joined at a very low level which indicates very little within group variance. This low level of within group variance can be expected as the pohutukawa honey came from a single location which was isolated to a degree from other nectar sources.

5.4.2.2 Principal Components Analysis

PCA was conducted using the statistics package R on the log extractives matrix as described in Section 2.4.1. No standardisation or centering of the data was performed prior to PCA. The variance is spread over a large number of components with the first 10 components accounting for ~90% of the variance (Figure 5.16). A score plot of the two principal components is shown in Figure 5.17.

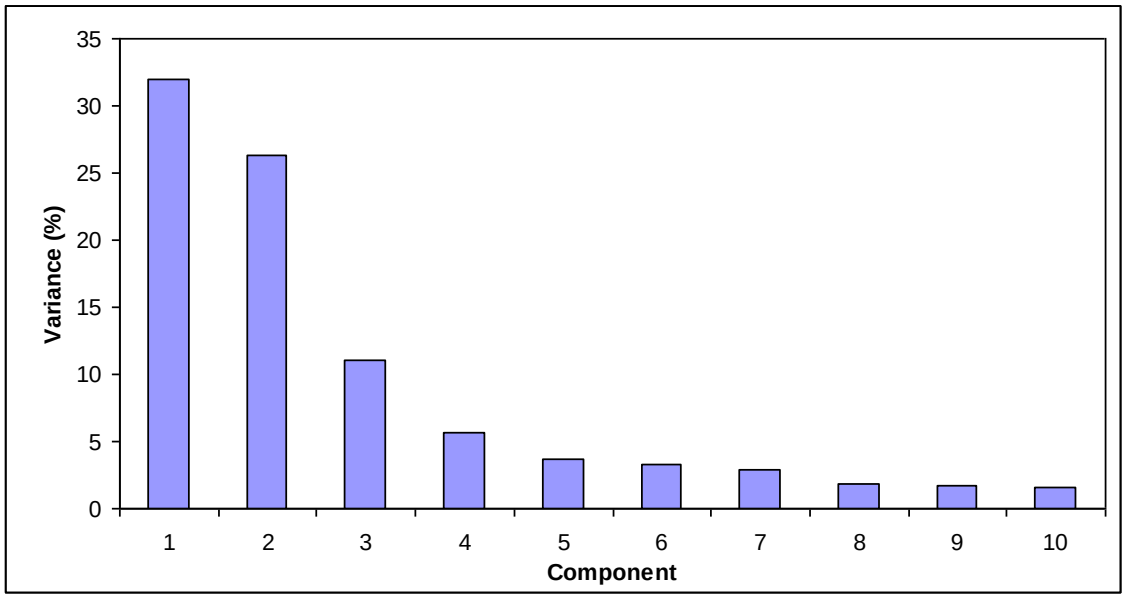
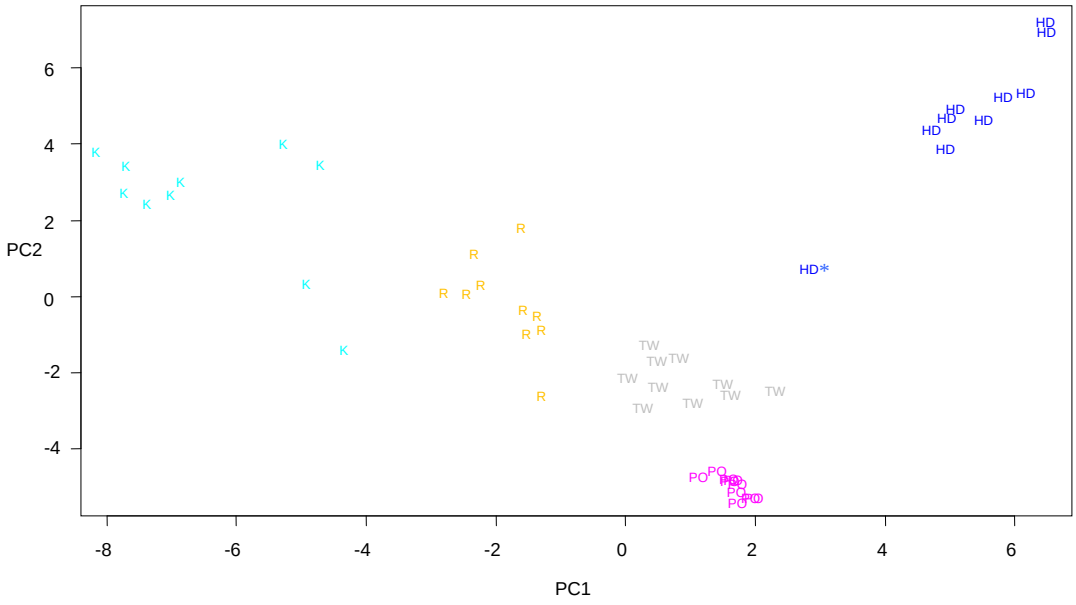


Figure 5.16 Scree plot from PCA of the log extractives matrix



K = kamahi, R = rata, TW = tawari, PO = pohutukawa, HD = beech honeydew, * = sample 1

Figure 5.17 Score plot of PC2 vs PC1 for the log extractives matrix

The score plot achieves good separation between all floral types for the first two principal components (Figure 5.17). A greater variation in composition is seen for beech honeydew honey and kamahi which is reflected in the large diffuse clusters in the score plot whereas pohutukawa honey has a much tighter cluster due to only minor differences in composition. A single beech honeydew honey (sample 1) is positioned halfway between the remaining beech honeydew honeys and tawari honey. The same sample was misclassified as tawari honey in the dendrogram using Ward's method (Section 5.4.2.1).

The first principal component achieved separation between all floral types except tawari and pohutukawa honey. The second component only separated pohutukawa honeys from the remaining floral types.

Hierarchical analysis was applied to the PCA scores using the same distance measures described above. Ward's method produced the greatest separation with just two misclassifications (Figure 5.18). As with the analysis on the log matrix data, sample 31 (rata honey) was misclassified however it was classified as a tawari honey as opposed to kamahi honey. Sample 11 (kamahi honey) was also misclassified as tawari honey.

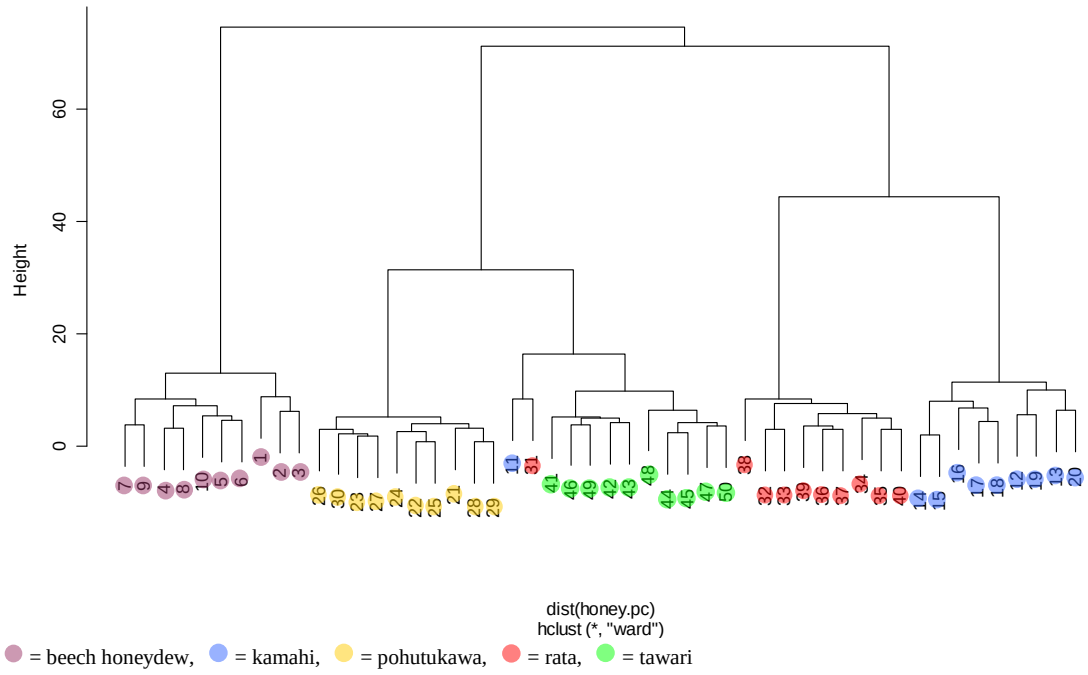
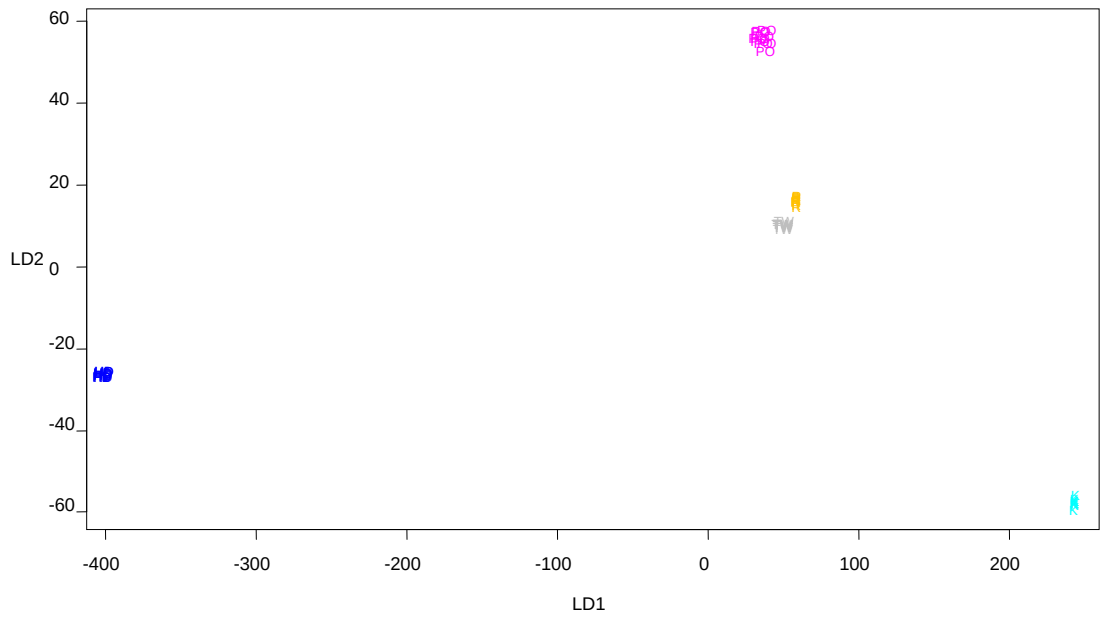


Figure 5.18 Dendrogram of PCA scores using Ward's method.

As with the dendrogram of the log extractives matrix, beech honeydew honey samples were separated from the remaining floral types very early on in the analysis. The two misclassified samples were grouped together and joined to the tawari honey group at a high level which indicates a degree of difference between these groups. The calculation of principal components appears to reduce the within group variance for kamahi honey.

5.4.2.3 Linear Discriminant Analysis

Where unsupervised techniques appear to work adequately there is often no justification for a supervised learning approach. For completeness, an examination of the log extractives data was conducted, however results should be treated with caution as overfitting can occur with small data sets. Linear Discriminant Analysis was conducted on the log matrix as described in Section 2.4.1. The score plot of the first two Linear Discriminants is given in Figure 5.19.



K = kamahi, R = rata, TW = tawari, PO = pohutukawa, HD = beech honeydew

Figure 5.19 Score plot of LD2 vs LD1 conducted on the log extractives matrix

The first Linear Discriminant successfully separated beech honeydew honey and kamahi honey from other floral types. Beech honeydew honey in particular is very far removed from the remaining floral types which is consistent with splitting levels seen in the

agglomerative clustering dendrograms. The second Linear Discriminant was required to separate the remaining floral sources. The tawari honey cluster is located in very close proximity the rata cluster. Both these floral sources contain very low levels of extractives. Neither of these floral sources contain compounds which can be identified as being specific to the floral origin. All honeys are grouped in very tight clusters which indicates a very small degree of within group variation.

The coefficients of Linear Discriminants can be used to determine the importance of each compound in the model. Compounds with coefficients of greatest magnitude are of highest importance. The coefficients of Linear Discriminants for the log extractives data are listed in Table 5.16.

Table 5.16 Coefficients of Linear Discriminants calculated on the log extractives data

Compound	**LD1**	**LD2**
unknown (*m/z* 45, 118)	0.86	6.94
succinic acid	1.33	-4.84
methylbutanedioic acid	-9.68	-3.27
methylsuccinic acid	15.11	-6.44
2,2-dimethylsuccinic acid	4.23	7.66
benzoic acid	18.22	0.88
glutaric acid	44.81	7.63
3,5,5-trimethyl-2-cyclohexene-1,4-dione	-9.46	4.81
unknown (*m/z* 59, 71, 129, 141)	-6.44	-3.87
2,6,6-trimethylcyclohexane-1,4-dione	-13.51	-6.63
phenylacetic acid	-5.27	-2.44
2,6-dimethylocta-3,7-diene-2,6-diol	15.78	-2.03
salicylic acid	-135.67	18.08
2-coumaranone	-26.05	-3.87
unknown (*m/z* 54, 82, 110, 151, 166)	8.57	-0.62
2-methoxyacetophenone	-5.25	4.46
unknown (*m/z* 139)	-3.68	9.80
unknown (*m/z* 67, 82, 110, 123)	4.84	2.45
unknown (*m/z* 70, 95, 140, 154, 168)	26.30	-1.33
2-methoxybenzoic acid	-2.75	-3.18
unknown (*m/z* 55, 71, 79, 91)	-17.55	-5.01
4-methoxybenzoic acid	4.50	0.89
phenyllactic acid	4.50	-1.60
4-methoxyphenylacetic acid	3.85	-7.80
octanedioic acid	3.66	12.40
4-hydroxybenzoic acid	71.73	-31.35
4-hydroxyphenylacetic acid	-162.61	5.21
lauric acid	-2.03	-2.62
nonanedioic acid	-0.56	-0.18
3,5-dimethoxybenzoic acid	-46.65	24.47
3,4-dimethoxybenzoic acid	30.21	-16.75

Compound	LD1	LD2
1-methoxy-4-propylbenzene	1.37	-3.56
decanedioic acid	-2.87	-3.17
decene-2-dioic acid	-0.41	-0.70
methyl syringate	21.14	0.36
unknown (*m/z* 82, 95, 150, 210)	-6.15	-7.85
4-hydroxy-3,5-dimethoxyhydrazidebenzoic acid	-42.88	-0.06
indole-3-acetic acid	78.95	-17.18
unknown (*m/z* 59, 121, 160, 210)	36.44	4.35
trans-3,4-dimethoxycinnamic acid	-17.19	8.65
palmitic acid	4.55	3.15
meliracemoic acid	2.02	-2.34
kamahines A-C	23.32	-4.71
unknown (*m/z* 137, 181, 251, 266)	87.26	-34.21
unknown (*m/z* 83, 127, 155, 179)	-21.75	-3.82
9-octadecanoic acid	3.35	-5.28
cis,trans-abscisic acid	-1.35	-1.47
stearic acid	2.43	-8.27
trans,trans-abscisic acid	-26.13	12.63
pinostrobin chalcone	27.83	-4.45

The most important Linear Discriminants are all associated with a single floral source and are present in all samples from that source; 4-hydroxyphenylacetic acid, salicylic acid and indole-3-acetic acid are found only in beech honeydew honey whereas unknown (*m/z* 137, 181, 251, 266) is only found in kamahi honey. The presence of these unique compounds in beech honeydew honey and kamahi samples can account for the large degree of separation of these floral sources in the score plot of LD1 vs LD2.

5.5 Conclusions

This survey completes the record for the extractable organic substances of the more dominant New Zealand unifloral honey types to a publishable standard.

An investigation of beech honeydew honey and kamahi honey previously undertaken[68] with the intention of completing the published extractives record for these honey types however this survey was not published for reasons outlined in Section 5.1.

Indole-3-acetic acid, previously proposed as a marker compound for beech honeydew honey[68, 110] was detected in all samples in a similar range and was not found in any

other honey type. An unusual phenolic compound, tentatively assigned as 4-hydroxy-3,5-dimethoxyhydrazidebenzoic acid was also proposed as a floral marker for beech honeydew honey.

Based on results determined in this investigation, a minimum quantity of kamahines A-C and meliracemoic acid, known floral markers of kamahi honey,[105, 106] of 6 and 2.5 mg/kg respectively should be present in unifloral kamahi honey. Trace amounts of both compounds are found in rata honey which characteristically contains a low proportion of kamahi honey as these two species are endemic to the same regions and have similar nectar flows.

Pohutukawa honey was found to be particularly low in extractable organic substances with pinostrobin chalcone (both of which are flavanoids), *cis*-3,4-dimethoxycinnamic acid and *trans*-3,4-dimethoxycinnamic common to all samples. These compounds are not however unique to pohutukawa honey. The general absence of peaks attributable to other extractable organic substances is a distinctive feature of the profiles determined for pohutukawa honey samples.

The examination of data by multivariate statistical analyses has proved valuable in differentiating between honey types. The results of the unsupervised analysis indicate that each of the five unifloral honey types in this study contains a unique fingerprint of extractives. The hierarchical cluster analysis using Ward's method is capable of differentiating between these honey types with the exception of a few extraneous samples. While unsupervised methods were on the whole very successful at separating the honey types, Linear Discriminant Analysis was undertaken in order to determine which compounds were the most important.

An examination of data produced from LDA cannot be used to directly identify marker compounds however information as to which components are most influential in obtaining separation of class types in the model can be obtained. Three compounds, 4-hydroxyphenylacetic acid, salicylic acid and indole-3-acetic acid were present exclusively beech honeydew honey and were the most important discriminants in the LDA model along with unknown (*m/z* 137, 181, 251, 266) which was found only in kamahi honey.

These four compounds can be attributed to the high degree of separation between honeydew honey and kamahi honey in relation to other floral types in the first discriminant. While the structure of the unknown kamahi associated compound which exhibited significant *m/z* 137, 181, 251, 266 ions is not known, its mass spectral features and apparent molecular weight (266 Da) are consistent with the view that is an oxygenated degraded carotenoid-like compound, of the type which Broome[111] and Ede[106] have previously isolated from kamahi honeys which have been proposed as floral source marker compounds. Kamahines A-C were found to be of moderate importance in the first discriminant, meliracemoic acid was found to be of relatively low importance.

Based on the statistical analyses results reported here for beech honeydew honey, kamahi, pohutukawa, rata and tawari honeys, it can be concluded that similar statistical analyses of the organic substances present in the diethyl ether extracts of other predominant honeys such as clover, manuka, thyme, nodding thistle, vipers' bugloss and rewarewa honeys[10-15] that were first investigated more than a decade ago would unequivocally validate the presence in extracts of those honeys of floral markers compounds. A number of compounds (summarised in Table 5.1) have been proposed as marker substance for these honey types. A limitation of the historic investigations was that the floral integrity of only a small number of samples was established via pollen analyses. Statistical methods of the type reported in this chapter should only be applied to floral source validated honey samples.

The possibility exists that regional or varietal variations may exist in the array of nectar compounds that are transferred from a plant source to hive. Statistical analyses of the extractable organic substances present in the diethyl ether extracts of honeys from known locations may or may not lead to the identification of 'regional' fingerprints which could then be superimposed on a broader suite of floral source specific marker compounds.

Chapter 6

Evaluation of Floral Origin and UMF™ Activity using NIR Spectroscopy

6.1 Introduction

Since honey is not produced in a controlled environment, the final product can vary significantly. Unifloral honey is derived predominantly from a single nectar source. The characteristics of unifloral honey are unique to the floral origin and therefore a reasonably consistent product is produced. Polyfloral honey contains honey derived from more than one predominant nectar source. The properties of polyfloral honey can therefore vary greatly. All nectar sources within a 3 km radius of a hive may be collected by bees and turned into honey. A series of compositional analyses are required to determine which nectar sources predominate. Seasonal variation and geographical origin can also have an effect on honey production.

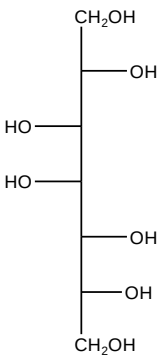
Near infrared (NIR) spectroscopy utilises electromagnetic energy in the range 12800 - 4000 cm^{-1} to produce overtones and combination bands of molecular vibrations that have fundamental vibrations in the mid-infrared region (7000 - 3000 cm^{-1}). Bonds containing hydrogen have the most influence in the NIR region. NIR spectroscopy is widely used in the agricultural, food, chemical and pharmaceutical industries in conjunction with multivariate data processing. The analysis of protein in wheat was the first commercially accepted "chemometric" NIR analysis.[121] The main advantage of NIR spectroscopy over other methods is that it requires little sample preparation and is relatively quick and inexpensive.

Honey is predominantly a mixture of glucose and fructose with lesser amounts of sucrose and maltose in water. Absorbances from the sugars and water dominate the NIR spectrum. Minor components in honey like acids and phenolics will also contribute. The composition of both the major and minor components in honey can vary substantially depending on the floral source. Differences in carbohydrate

composition will have an effect on the NIR spectra. Several New Zealand honeys are known to have unique carbohydrate profiles, for example rata honey is characterised by a higher than average glucose content and beech honeydew honey contains high levels of trisaccharides, particularly erlose.[82] Aromatics, diacids, degraded carotenoids and aliphatic fatty acids are found in varying proportions in honey depending on floral origin, and some of these have been suggested as floral markers (Section 1.2.5). It is therefore likely that the NIR absorbance of unifloral honeys will be characteristic of the floral origin.

Several preliminary studies have been conducted on the determination of floral origin by NIR and mid infrared spectroscopy (MIR). MIR has been used to determine the floral origin of clover, buckwheat, basswood, wildflower, orange blossom, carrot and alfalfa honeys from various states in the USA.[122] Principal component analysis and Canonical Variates Analysis (equivalent to Linear Discriminant Analysis) were used to develop discriminant models. These models were successfully validated (97 - 100% correct classification) using 20 samples of known origin which were not included in creating the model.

NIR analysis has been useful in determining the proportion of Israeli avocado nectar collected by bees using perseitol **(29)** as a marker compound.[123] The standard error in the prediction of perseitol in honey was very low (0.13%) however the proportion of perseitol in honey was much lower than expected. Hives were placed in an avocado orchard during flowering, however it appears that bees (some more than others) do not find avocado nectar particularly attractive, thus honey found to contain perseitol is more likely to contain only a proportion of avocado honey as opposed to being truly monofloral.



(29)

A combination of visible and NIR spectroscopy has been used to classify Uruguayan pasture and *Eucalyptus* spp. honeys.[124] Linear Discriminant Analysis and discriminant Partial Least Squares regression analysis models correctly classified more than 75% of pasture honeys and 85% of the eucalyptus honeys.

The geographical and botanical source of several European honeys has been evaluated by NIR.[125] Sufficient data for Canonical Variates Analysis was obtained from four unifloral honeys: acacia, chestnut, heather and rapeseed. Using 10 principal components, 68% of honeys from these four groups were classified correctly; correct classification increased to 81% when heather honey was removed from the data set.

To date the classification of floral origin by NIR has predominantly focused on a few European, Uruguayan and American floral sources. The main limitation of the above mentioned studies is the small number of samples analysed from each floral source (5 - 52 samples). In order to build an accurate model a large representative sample set must be used. No NIR studies have been reported on New Zealand honeys.

The aim of the following chapter is to develop a method to assess the potential of NIR to discriminate between New Zealand unifloral honey types. All main unifloral honeys will be included in the investigation and a series of statistical analyses conducted in order to evaluate potential.

Currently, the certification of unifloral honeys must be carried out using multiple techniques as no singular method exists which can unequivocally establish floral origin. Pollen analysis is the principal method used to determine floral source supported by other data such as moisture, colour, conductivity and sugar analysis. Pollen analysis is time consuming, and requires highly skilled personnel. A need exists within the industry for a rapid and inexpensive method of determining honey quality.

6.2 Experimental

6.2.1 Pre-processing and Statistical Techniques

A range of transformations and pre-processing techniques have been used to reduce spectral noise and maximise between group variance of the data. The wavelength range of the recorded spectra tends to be much wider than the final range chosen for further analysis. Different ranges of wavelengths are often evaluated separately in order to ascertain the ideal range to maximise classification.

In general most studies use averaging of spectra (where multiple spectra of the same honey are recorded) as the main pre-processing technique. The normalization of spectra by dividing the intensity of each spectra at a given wavelength by the standard deviation of the spectra has also been reported.[122]

Spectral features may be enhanced by computing the first or second derivative of the spectra. Smoothing can be combined with derivatisation using a Savitzky-Golay filter. It is however important to experiment with the window width in order to increase the signal to noise ratio without smoothing out important features. A combination of Savitzky-Golay smoothing and second order derivatisation has produced slightly higher classification rates using a discriminant Partial Least Squares regression model.[124]

Principal Component Analysis (PCA) is consistently used in all studies to reduce the number of variables while retaining a large proportion of the variance. PCA is an orthogonal linear transformation that transforms the data into a new coordinate system in order to remove autocorrelations and reduce the dimensionality of the data. The number of PCs utilised in subsequent classification models range from 10 - 20.

Almost all studies utilise Linear Discriminant Analyses (LDA) (also referred to as Canonical Variates Analysis) to build classification models. In general LDA models were validated using leave-one-out cross validation.[124, 126, 127]

Partial Least Squares analysis (PLS) has also been used to discriminate between pasture and eucalyptus honeys.[124] The PLS model on the second derivative spectra was found to be superior in classifying these two honey types compared to LDA on the first 20 PC scores.

6.2.2 Sample Sets and Methodology

The sample sets and methodology used for the evaluation of floral origin of honey by NIR spectroscopy are outlined in Section 2.3. Dataset A contained samples originating from an initial survey of 100 honeys while Datasets B-E comprised of data obtained

from a subsequent study of 323 honeys with different preprocessing methods (Table 2.7). Compositional analysis results for honeys used in the following sections are given in Appendix A4.1.

Honeys from ten different floral origins were analysed. The full names and abbreviations used throughout this chapter are given in Table 6.1.

Table 6.1 Common, Latin and abbreviated names given to honeys analysed in this study

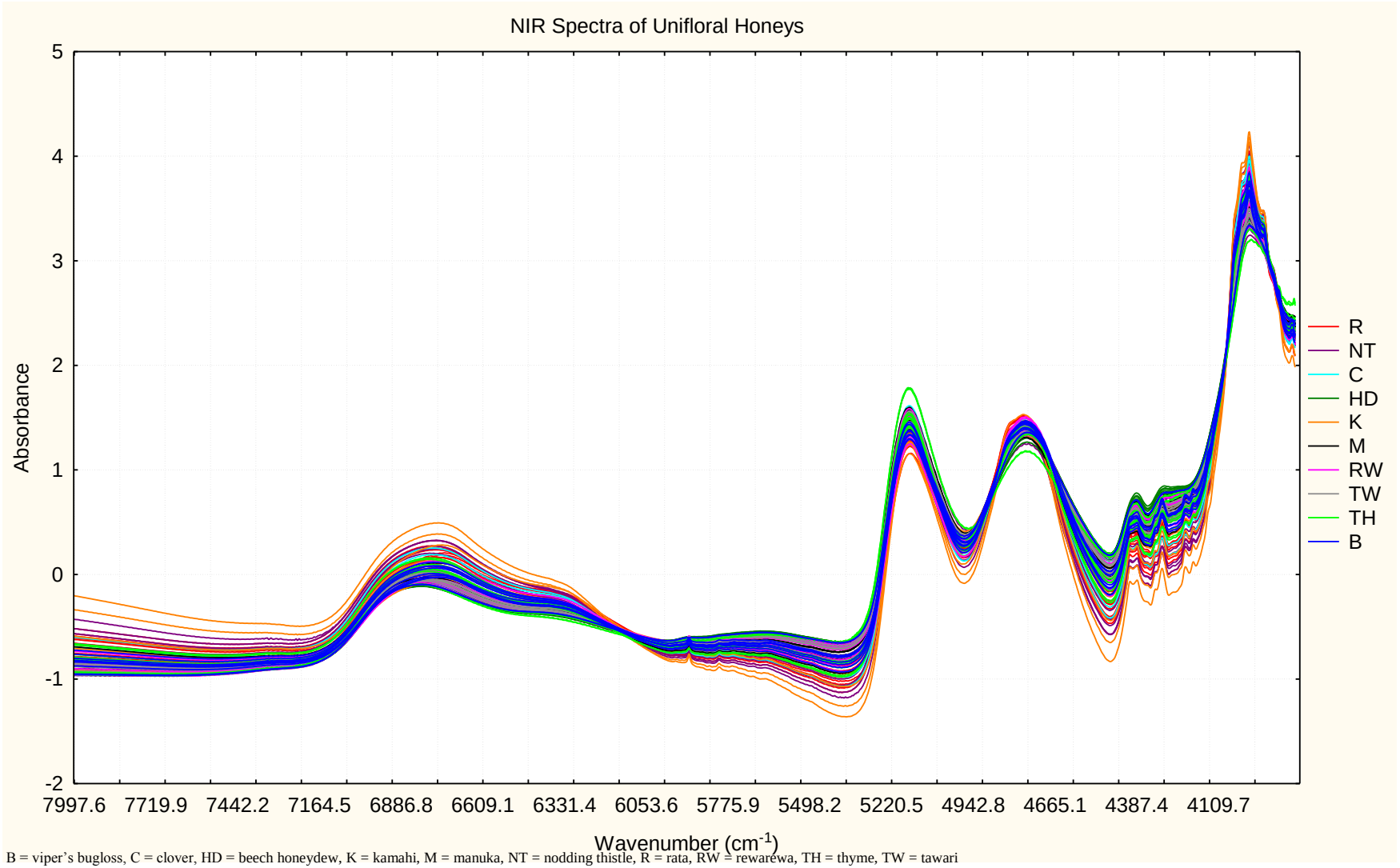
Common name	Latin name	Abbreviation
Beech honeydew honey	*Nothofagus* sp.	HD
Clover	*Trifolium repens*	C
Kamahi	*Weinmannia racemosa*	K
Manuka	*Leptospermum scoparium*	M
Nodding Thistle	*Carduus nutans*	NT
Rata	*Metrosideros* sp.	R
Rewarewa	*Knightea excelsa*	RW
Thyme	*Thymus* sp.	TH
Tawari	*Ixerba brexioides*	TW
Vipers' Bugloss	*Echium vulgare*	B

6.3 Evaluation of Floral Origin

6.3.1 Exploratory Work

6.3.1.1 Visual Inspection

The mean centered NIR spectra of each honey were visually inspected in order to ascertain if any differences between floral origin are discernable by eye. The full NIR spectra are given in Figure 6.1, expanded sections and plots containing a reduced number of floral origins are given in Appendix A4.2. While all honeys show broadly similar spectra, beech honeydew honey spectra were clearly unique. Very little variation is seen between individual beech honeydew honey samples. In the wavelength range from 4534 - 4225 cm^{-1}, beech honeydew honey displayed a higher absorbance compared to other floral types. All other honeys display a wider degree of variability than beech honeydew honey. In the spectral range from 4688 - 4534 cm^{-1}, kamahi and rata honeys generally have a much lower absorbance than vipers' bugloss and clover honeys. Both kamahi and rata honeys also have a higher absorbance compared to vipers' bugloss and clover in the band centered around 4765 cm^{-1}.



B = viper's bugloss, C = clover, HD = beech honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure 6.1 Mean centered NIR spectra of unifloral honeys

Beech honeydew honey originates from honeydew as opposed to nectar from flowers. Due to the unique origin of beech honeydew honey, its composition is markedly different. The conductivity in particular of beech honeydew honey is much higher than nectar honeys. It is therefore not surprising that beech honeydew honey has a unique NIR profile.

Based on the visual inspection of NIR spectra, wavelengths used in subsequent analysis were reduced from the original window of 8000 - 3850 cm^{-1} to 6000 - 3850 cm^{-1}. These wavelengths contain 1^{st} overtones and combination bands of fundamental vibrations from the mid-infrared region.

6.3.1.2 Cluster Analysis

Cluster analysis is used to determine if the data naturally falls into distinct groups. Cluster analysis is explained in more detail in Section 2.4.1.[72] The 100 unifloral honey set (Dataset A, Section 2.3.3) was examined by hierarchical clustering using the distance measures single, complete, average and Ward's method in R. The dendrogram constructed using Ward's method displayed a small degree of clustering between several honey types and is given in Figure 6.2.

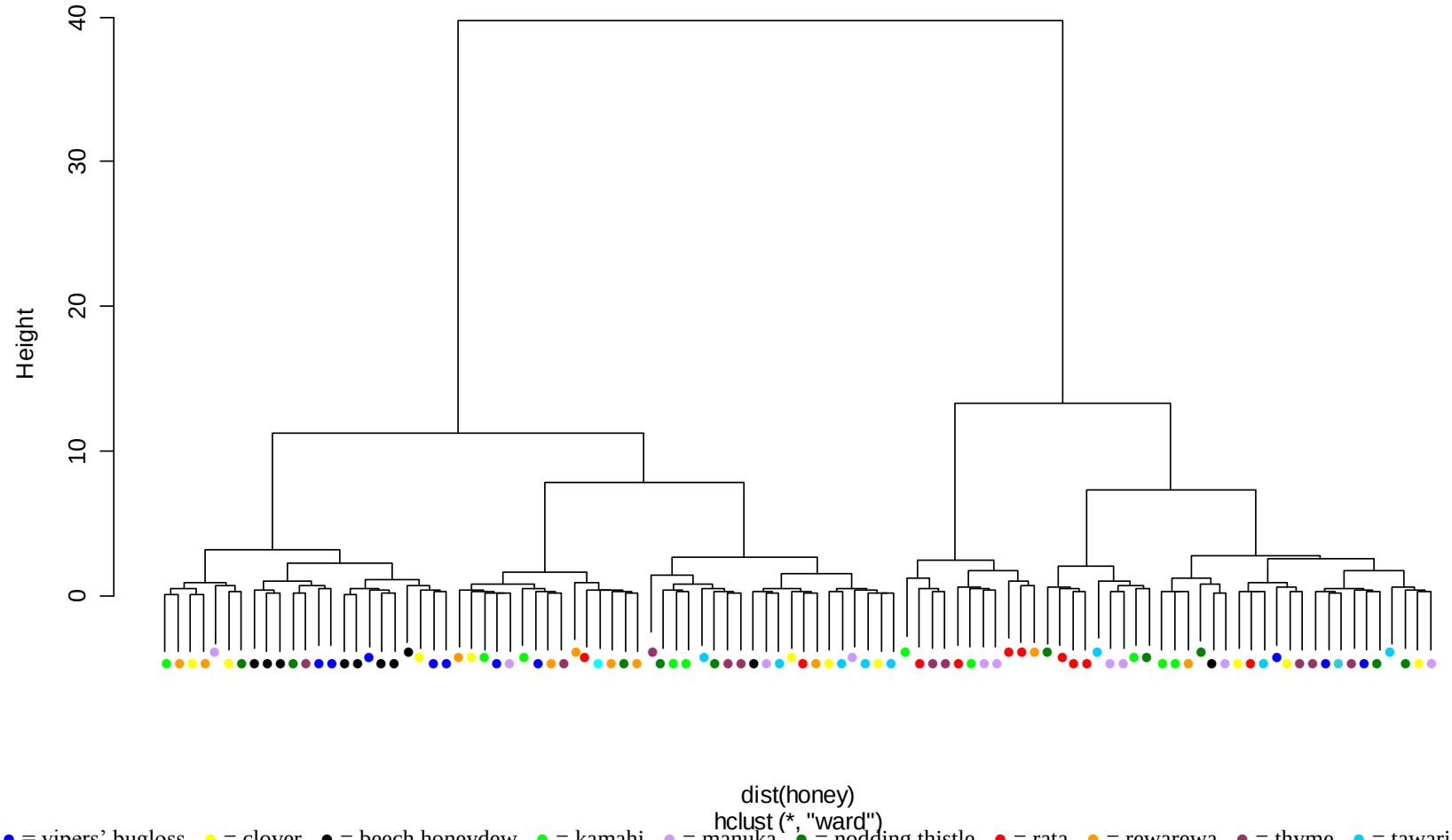


Figure 6.2 Dendrogram of 100 unifloral honeys (matrix 2) using Ward's method

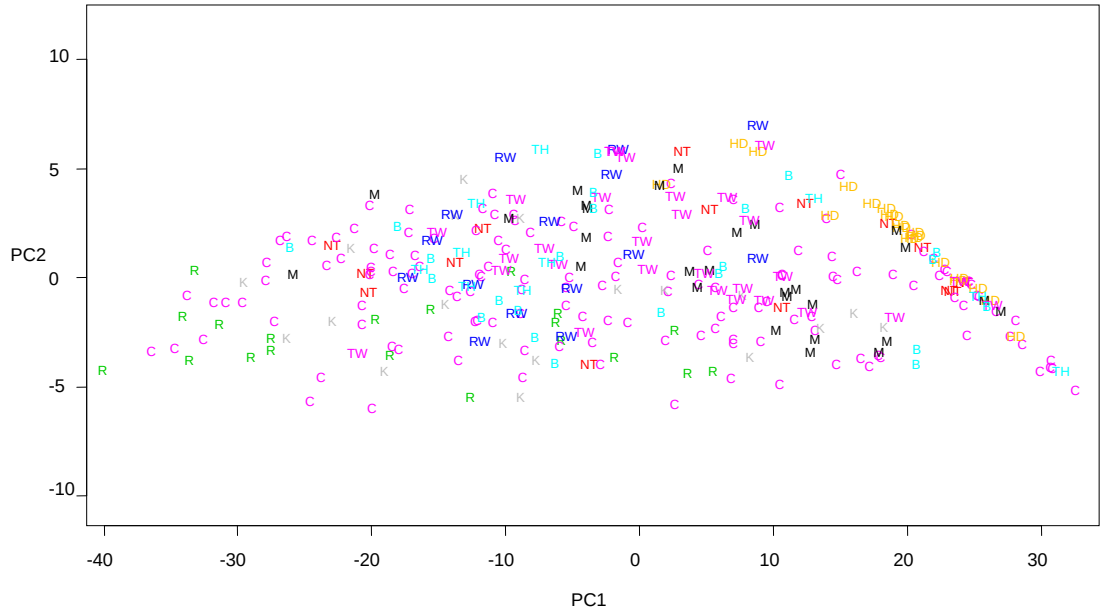
The dendrogram using Ward's method splits the honeys into two main groups. Each of these two main groups are then divided into 3 main subgroups. All beech honeydew honeys except 1 are positioned within the left arm of the dendrogram within the same subgroup. Given that the NIR spectra of honeydew honey was found to be unique with very little variation within this class, it is not surprising that these samples were clustered together.

All rata honey but two were located within the right arm within the two left subgroups. With the exception of two samples, all rewarewa honeys were located in the left arm of the dendrogram. Vipers' bugloss honeys are predominantly found within the left arm within 2 subgroups. The remaining honeys: clover, kamahi, manuka, nodding thistle, thyme and tawari were spread diffusely throughout the model.

These results suggest that the differences between floral sources with the exception of beech honeydew honey are very subtle therefore a more sophisticated model is required to separate these honeys further.

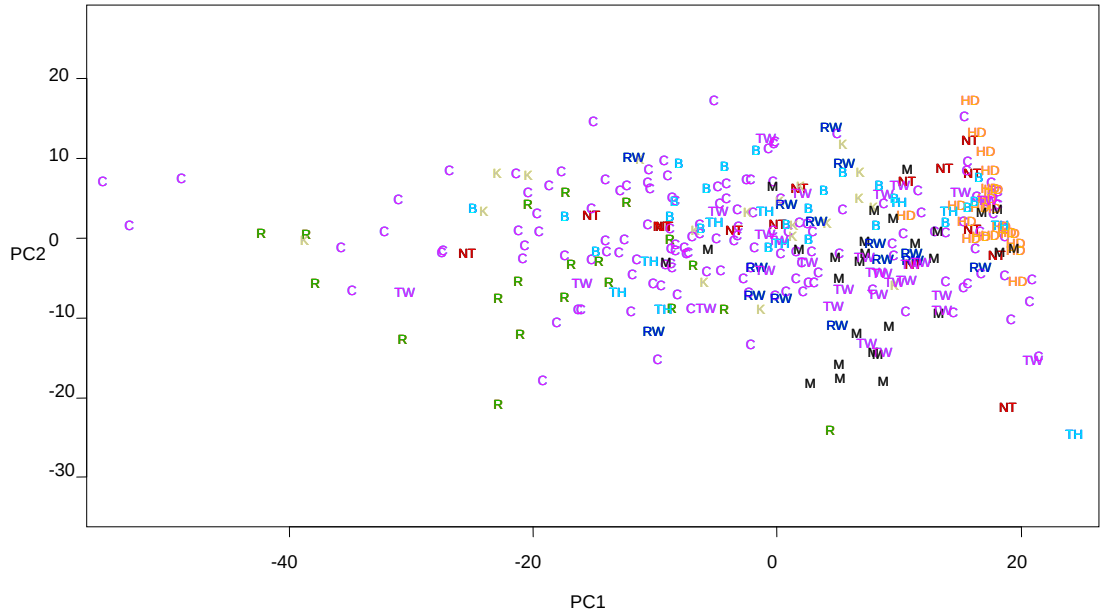
6.3.1.3 Principal Component Analysis

PCA was undertaken on Datasets B-D (Section 2.3.3), details of this analysis are given in Section 2.4.1. The score plots of the first two principal components calculated on Datasets B-D are given in Figure 6.3 - Figure 6.5. The scree plot and score plot of two further components calculated from Dataset C are given in Figure 6.6 and Figure 6.7.



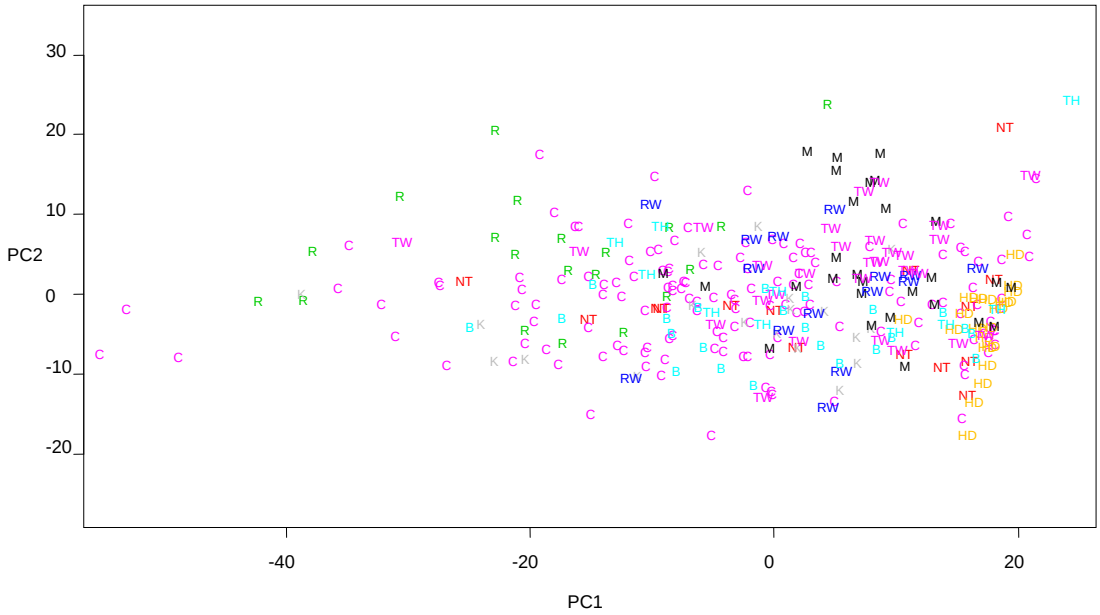
B = viper's bugloss, C = clover, HD = honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure 6.3 Score plot from PC2 vs PC1 of Dataset B



B = viper's bugloss, C = clover, HD = honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure 6.4 Score plot from PC2 vs PC1 of Dataset C



B = viper's bugloss, C = clover, HD = honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure 6.5 Score plot from PC2 vs PC1 of Dataset D

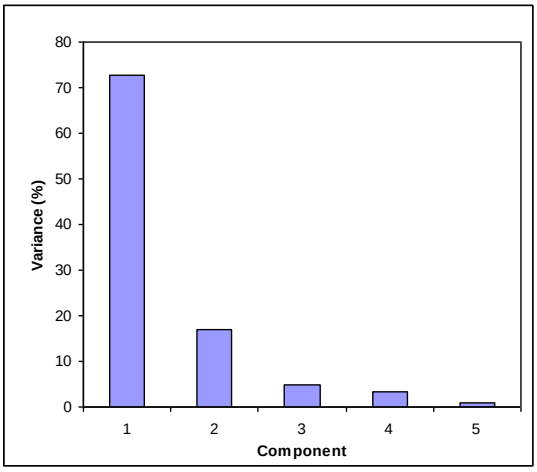
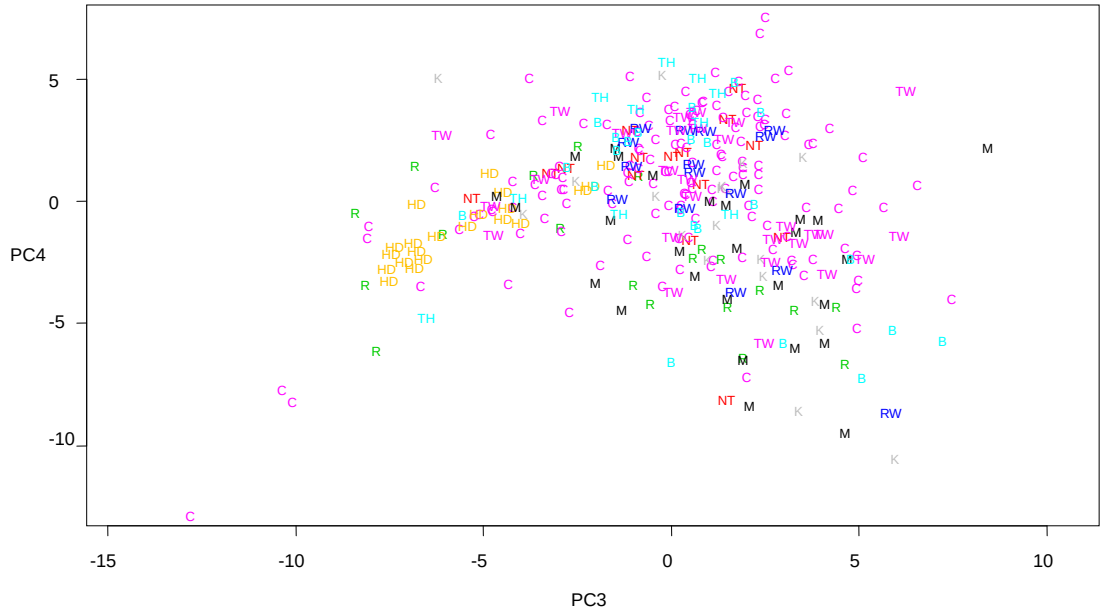


Figure 6.6 Scree plot from PCA of Dataset C



B = viper's bugloss, C = clover, HD = honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure 6.7 Score plot from PC4 vs PC3 of Dataset C

The plots were inspected visually to determine the degree of separation (if any) between clusters. The clustering pattern of PCA conducted on Dataset D was slightly different compared to Dataset C. Figure 6.6 illustrate that for Dataset C almost all of the variance (99%) can be accounted for in the first five components. An examination of the first four principal components (calculated using Dataset C) indicates only a small degree of separation between floral sources is achieved by PCA. The visual inspection of the data (Section 6.3.1.1) indicated that the differences between the honey floral types were subtle by comparison with the overall spectral variability. As PCA does not take class information into account it is not surprising that minimal separation between floral sources was obtained. In the Score plot of PC2 vs PC1, beech honeydew honey can be seen as a tight vertical band on the far right which indicates that the honey scores on PC1 alone achieve a degree of separation between this honey and the remaining floral sources. The remaining clusters are large and diffuse, however the following patterns are discernable: Manuka is located in a central position in PC2 towards the right side of PC1. Most rata honeys are shifted to the left hand side were as rewarewa honeys are more centrally located. Vipers' bugloss honeys are within a narrow horizontal band which indicates a degree of separation by PC2.

The 3rd principal component achieves a degree of clustering with beech honeydew honey which is seen as a relatively tight cluster on the left side of the Score plot (Figure 6.7). A degree of separation is achieved with thyme honey by the 4th principal component. Most nodding thistle and noddy clover samples are centered in the middle of PC3 towards the high range of PC4. Many rewarewa honeys are in the right side of PC3 within the middle of PC4. Clover, rata, kamahi, tawari and vipers' bugloss and spread diffusely throughout the model.

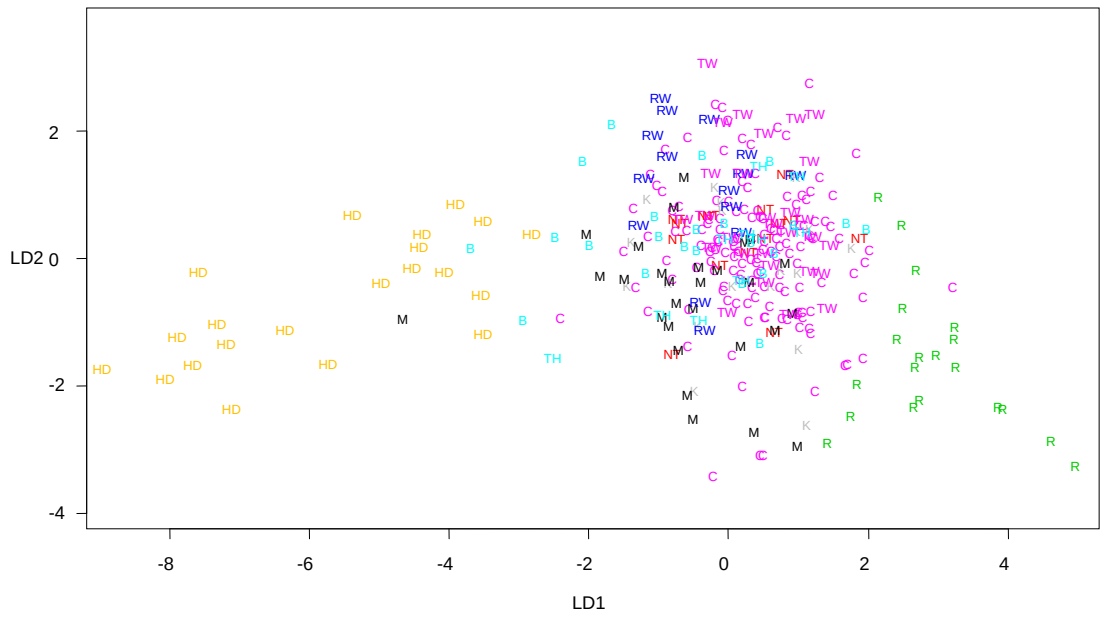
As with cluster analysis, it is apparent that while subtle differences are seen in these Score plots, gross differences in variance cannot be attributed directly to floral origin without further data processing.

6.3.1.4 Linear Discriminant Analysis of Principal Component Scores

The absorbance of each wavelength in the NIR spectra is strongly related to the absorbance of the adjacent wavelengths. This being the case, NIR data by its very nature cannot be considered as meeting the independence requirement (as outlined in Section 2.4.1) when considered as a whole spectra.

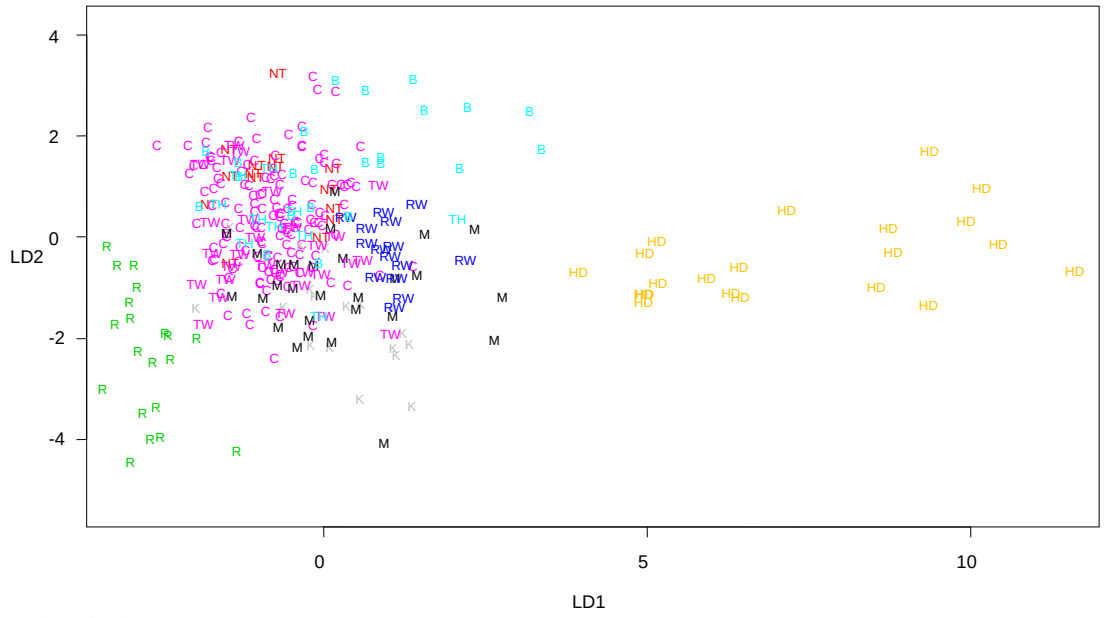
In order to overcome the independence requirement, previous studies have conducted LDA on the principal component scores which are independent.[124, 126, 127] LDA is very similar to PCA except class information (floral type) is taken into account in forming the linear functions.

The ability of LDA to discriminate between honey types using varying numbers of principal component scores was investigated. The score plots of the first two Linear Discriminants from 10, 15 and 20 components calculated using Dataset C are given in Figure 6.8 - Figure 6.10. The 15 component LDA score plot from Dataset B and D are given in Appendix A4.4.



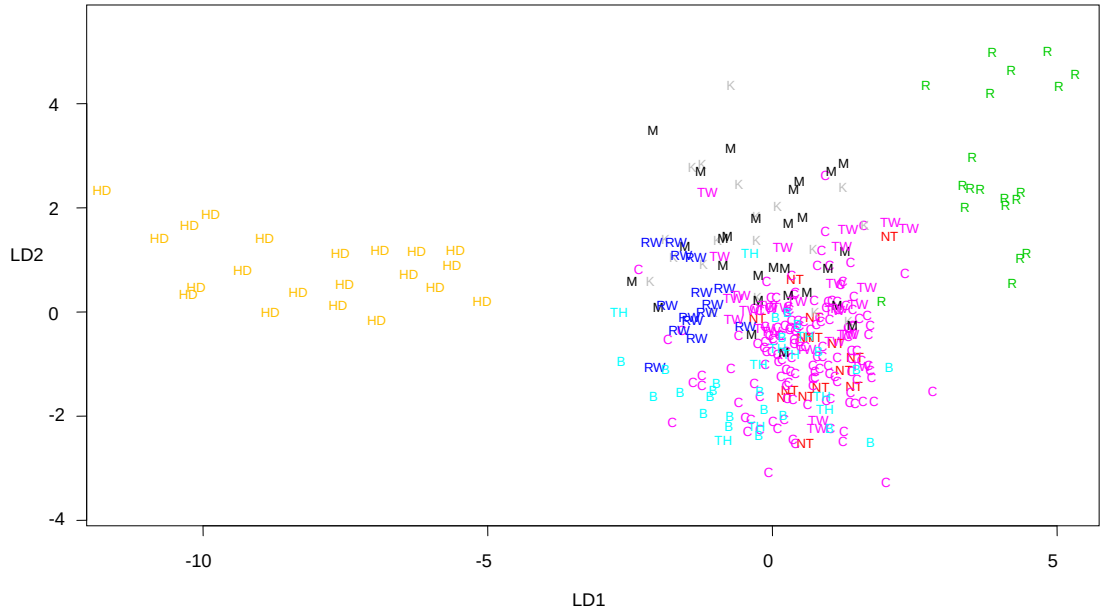
B = viper's bugloss, C = clover, HD = honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure 6.8 Score plot of LD2 vs LD1 conducted on the first 10 PC scores obtained from the analysis of Dataset C



B = viper's bugloss, C = clover, HD = honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure 6.9 Score plot of LD2 vs LD1 conducted on the first 15 PC scores obtained from the analysis of Dataset C



B = viper's bugloss, C = clover, HD = honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure 6.10 Score plot of LD2 vs LD1 conducted on the first 20 PC scores obtained from the analysis of Dataset C

Once again, from a visual inspection of the score plots, Dataset C achieved better separation between floral sources than Dataset B. While subtle differences were seen in the clustering of Dataset D, no advantage was gained over Dataset C. A discussion of results obtained using Dataset C is given below.

Most of the separation between floral types was achieved by the first Linear Discriminant. In all cases, beech honeydew honey was separated from the remaining floral sources by the first Linear Discriminant. Rata achieved full separation from other floral types in the 15 component score plot. While the remaining floral sources were not resolved, a definite degree of clustering within different areas was apparent, particularly in the 15 component model. The second Linear Discriminant separated vipers' bugloss from manuka honey and the second component combined with the first to separate nodding thistle from rewarewa honey.

While a degree of separation is apparent, it is clear that the combination of PCA followed by LDA is not sufficient to separate these honey types. The analysis of various European honeys using a combination of LDA on the PC scores was on the

whole much more successful.[124, 126, 127] These studies were however limited to a few floral sources or could not be used to distinguish all floral types surveyed.

6.3.1.5 Linear Discriminant Analysis on Selected Wavelengths

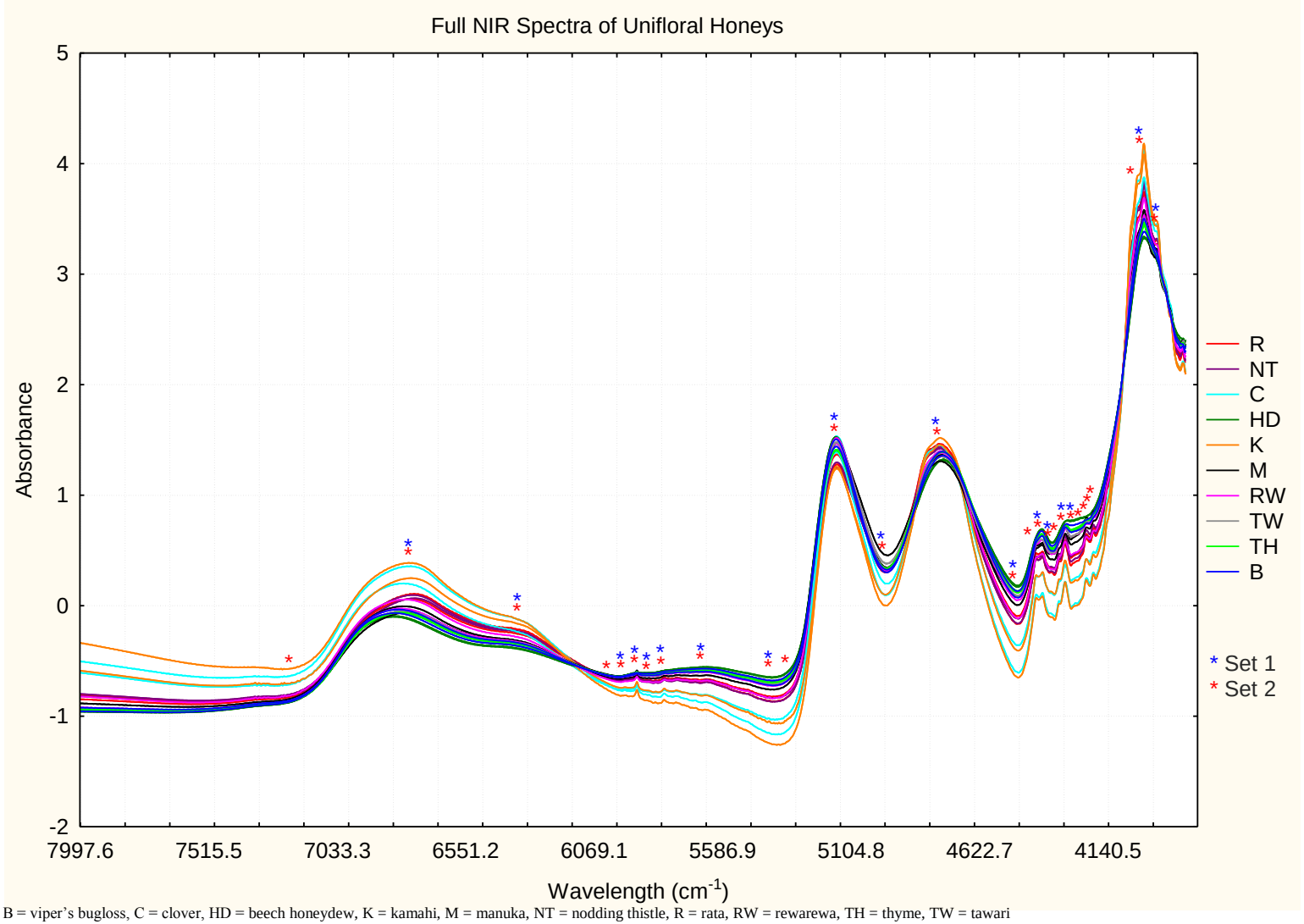
If selected wavelengths are extracted at selected points to correspond with peaks, troughs and shoulders in the spectra, adjacent data points are less likely to show strong correlation, thus eliminating the problem of redundancy encountered when considering the full spectra.

Two sets of wavelengths were selected by visual examination of the spectra, these wavelengths are given in Table 6.2 and are indicated on the NIR spectra in

Figure 6.11. LDA on these two sets of data produced the following scatter plots shown in Figure 6.12 - Figure 6.14 with the coefficients of Linear Discriminants in Figure 6.13 - Figure 6.15.

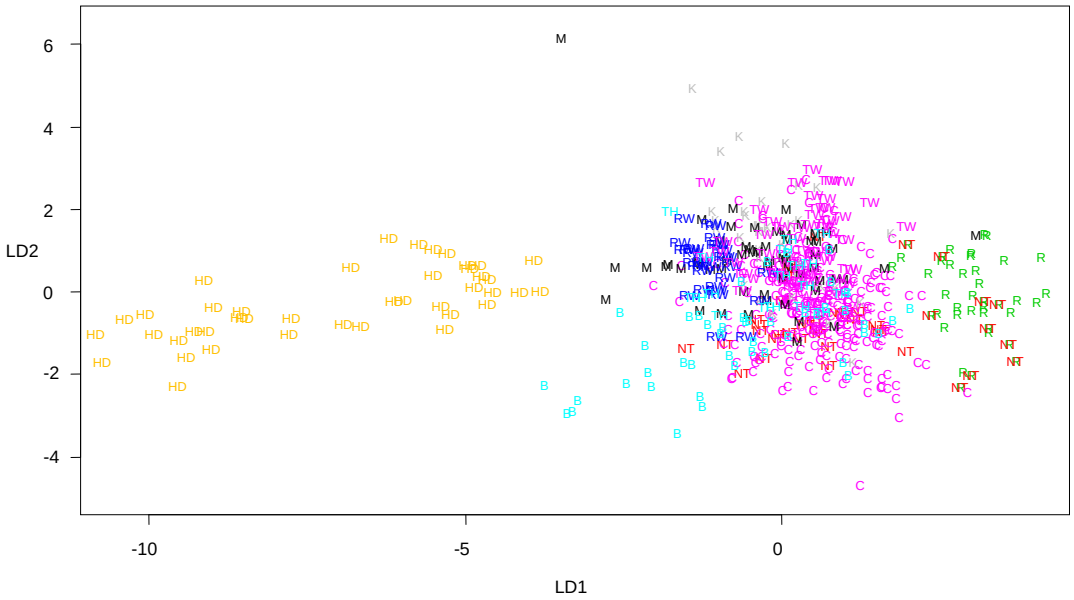
Table 6.2 Wavelengths (cm^{-1}) chosen for LDA

Set1	Set 2	Set1	Set 2
-	7234	-	4430
6815	6815	4389	4389
6356	6356	4353	4353
-	5951	-	4333
5907	5907	-	4291
5882	5882	4279	4279
5868	5868	4262	4262
5791	5791	-	4240
5602	5602	-	4224
5402	5402	-	4208
-	5381	-	4197
5163	5163	-	4185
4970	4970	4006	4006
4760	4760	3954	3954
4482	4482		



B = viper's bugloss, C = clover, HD = beech honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure 6.11 Selected wavelengths used in LDA of unifloral honey



B = viper's bugloss, C = clover, HD = honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure 6.12 Score plot of LD2 vs LD1 conducted on Dataset E (Set 1)

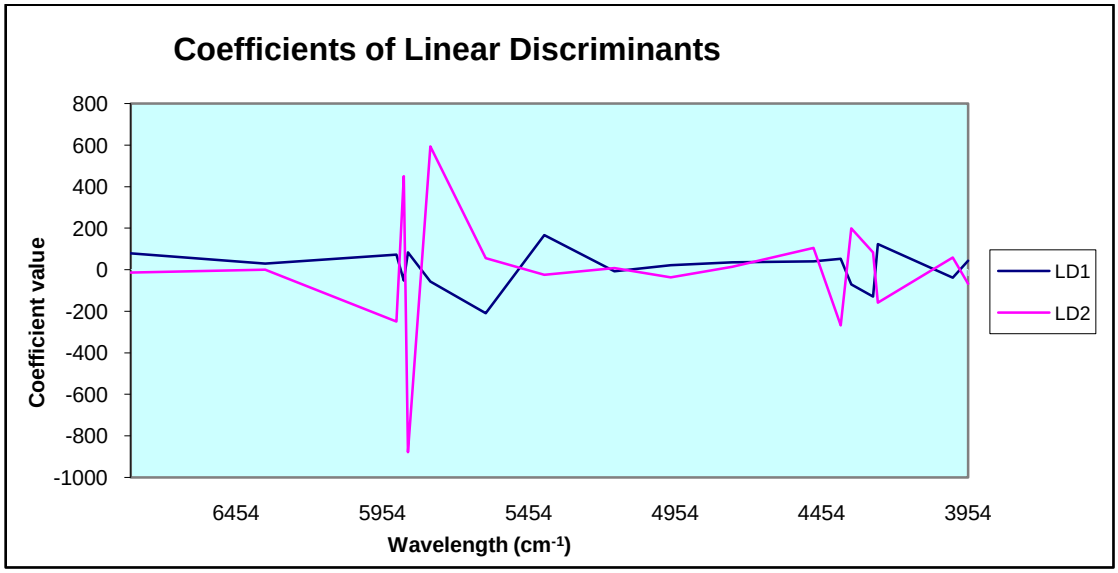
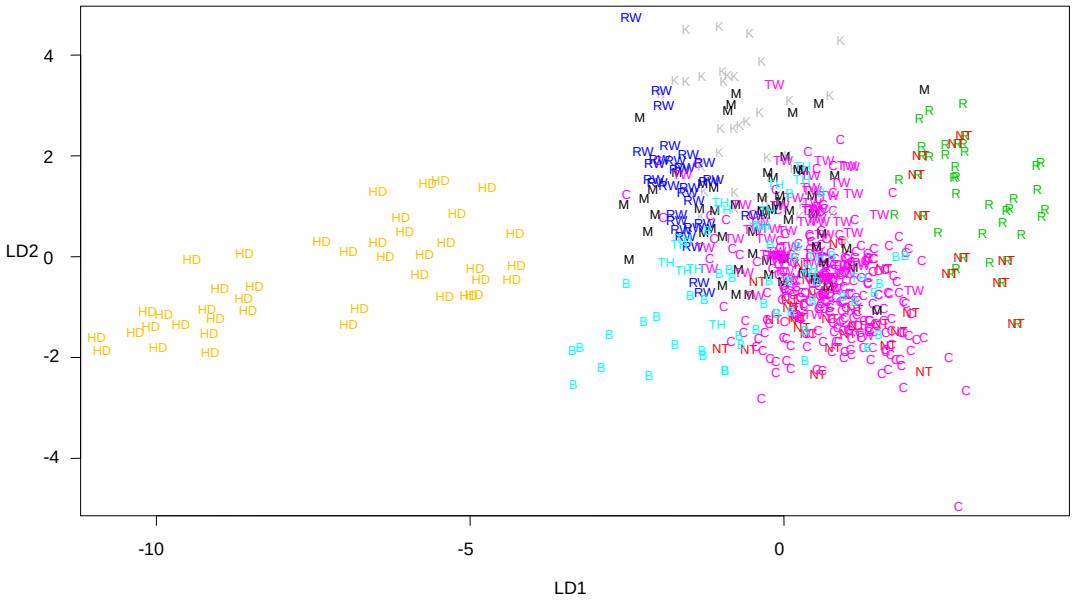


Figure 6.13 Coefficients of Linear Discriminants determined from LDA (Set 1)



B = viper's bugloss, C = clover, HD = honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure 6.14 Score plot of LD2 vs LD1 conducted on Dataset E (Set 2)

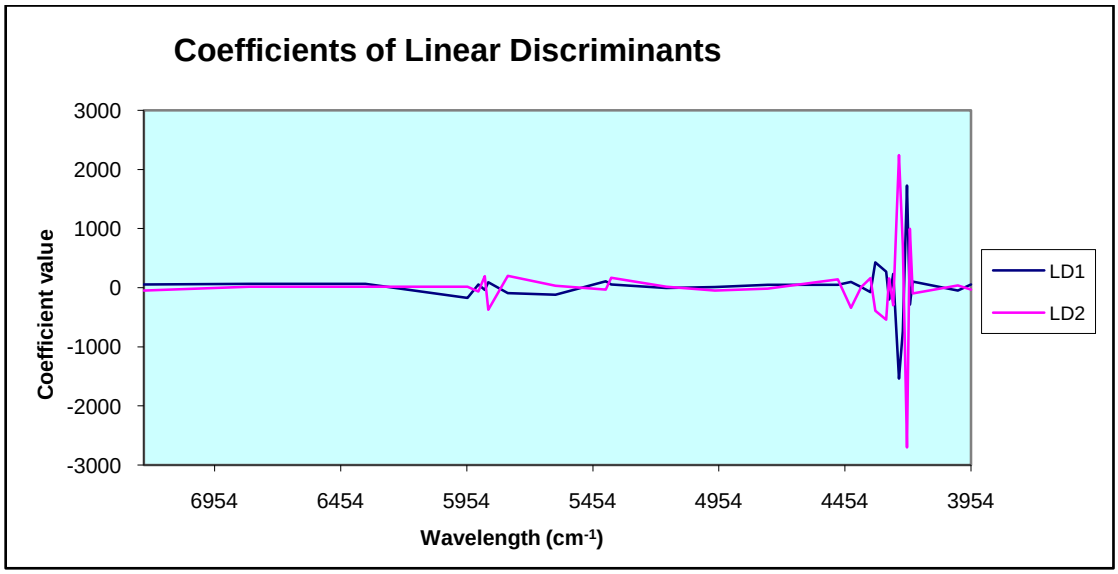


Figure 6.15 Coefficients of Linear Discriminants determined from LDA (Set 2)

The wavelengths chosen in Set 1 achieved clear separation between beech honeydew honey and the remaining honey types based on two Linear Discriminants (Figure 6.12). The first Linear Discriminant separated rata honey from all other honeys with the exception of nodding thistle honey. A tight cluster of rewarewa honeys was apparent however these were not resolved from the large diffuse cluster containing

manuka, tawari, clover and thyme honeys. A group of vipers' bugloss honeys were seen as a distinct group, however other vipers' bugloss honeys were also present in the large diffuse clover cluster. An examination of the coefficients of linear discriminants (Figure 6.13) indicates that the wavelengths 5602, 5402, 4279 and 4262 cm^{-1} were the most important in the first discriminant.

In general the clustering achieved using Set 2 (Figure 6.14) was less defined than Set 1 however several distinctions were apparent. An increased number of kamahi honeys were separated from the remaining honey types. Slightly better resolution was achieved between rata and the remaining honeys however nodding thistle honeys were still present in the rata cluster. Different coefficients were found to be more influential in the first Linear Discriminant (Figure 6.15) than in those obtained using Set 1. The four most important coefficients were found within a band from 4333 - 4208 cm^{-1}. In general the addition of more wavelengths to the model appears to decrease the models performance.

6.3.2 Classification Modelling

Classification via regression models, which used Partial Least Squares (PLS) as a base algorithm model, were evaluated using WEKA. Varying numbers of components (12-20) were evaluated, both with the original data set (Dataset C) and five resampled data sets (as described in Section 2.4.2.). The composition of the resampled data sets is given in Table 6.3.

Table 6.3 Resampled data set composition

	Sample set (random seed number)				
Class	**1**	**2**	**3**	**4**	**5**
Rata	102	83	98	95	82
Nodding thistle	82	87	78	73	85
Clover	177	186	207	169	177
Beech honeydew	73	85	79	87	87
Kamahi	84	95	82	91	95
Manuka	89	108	86	99	76
Rewarewa	93	71	78	90	87
Tawari	87	103	81	95	100
Thyme	91	69	83	88	89
Vipers' bugloss	91	82	97	82	91

6.3.2.1 Classifier Results

The test results of Dataset C produced an average correct classification from 67.0% to 79.7% (Table 6.4). An average confusion matrix for a 20 component PLS model is given in Table 6.5.

Table 6.4 Average correct classification results of the PLS model against Dataset A

Number of components	Average correct classification (%)
12	67
13	70
14	72.9
15	73.3
16	75.1
17	75.9
18	77.7
19	78.8
20	79.7

Table 6.5 Averaged confusion matrix produced from a 20 component PLS model against Dataset A

	Classified as:									
Actual Class	**R**	**NT**	**C**	**HD**	**K**	**M**	**RW**	**TW**	**TH**	**B**
R	**19**	0	1	0	0	0	0	0	0	0
NT	0	**1**	12	0	0	0	1	1	0	1
C	0	0	**135**	0	0	2	0	0	0	5
HD	0	0	0	**21**	0	0	0	0	0	0
K	0	0	2	0	**12**	2	1	2	0	0
M	0	1	3	0	1	**23**	0	0	0	0
RW	0	0	1	0	0	0	**12**	1	0	0
TW	0	1	4	0	0	0	1	**22**	0	1
TH	0	0	5	0	0	0	0	0	**4**	0
B	0	0	14	0	0	0	0	0	0	**8**

The test results of the resampled data sets produced an average correct classification ranging from 78.0% to 92.7% (Table 6.6). An averaged confusion matrix for this model is given in Table 6.7.

Table 6.6 Classification results of the PLS model against the resampled data sets

	Number of components								
Data set	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1	77.5	80.1	82.9	84.4	87.0	89.3	90.9	91.4	92.5
2	76.8	80.7	82.0	83.4	85.3	86.7	88.1	90.2	92.3
3	77.6	77.0	78.7	83.2	87.1	88.4	90.5	91.7	92.7
4	79.8	81.2	84.1	86.4	87.0	88.7	89.9	90.4	92.0
5	78.1	82.0	83.3	86.0	87.5	89.3	89.5	92.2	93.7
Average	78.0	80.1	82.2	84.7	86.8	88.4	89.8	91.2	92.7

Table 6.7 Average confusion matrix produced from the evaluation of the 20 component PLS model against the resampled datasets

	Classified as:									
Actual Class	**R**	**NT**	**C**	**HD**	**K**	**M**	**RW**	**TW**	**TH**	**B**
R	**94**	0	0	0	0	0	0	0	0	0
NT	0	**56**	20	0	0	0	0	1	0	1
C	2	4	**157**	0	0	2	1	2	4	8
HD	0	0	0	**82**	0	0	0	0	0	0
K	0	0	0	0	**88**	0	0	0	0	0
M	0	0	0	0	0	**96**	0	0	0	0
RW	0	0	0	0	0	0	**84**	0	0	0
TW	0	0	2	0	1	0	6	**82**	0	0
TH	0	0	3	0	0	0	0	0	**81**	0
B	0	1	10	0	0	0	0	0	0	**64**

6.3.2.2 Discussion

The 20 component PLS model achieved the highest correct classification results using both the Dataset C and the resampled data sets (1-5). As such a high proportion of the variance is captured within the first few components (as seen in Figure 6.6), increasing the number of components past 20 is likely to model only spectral noise and results in over fitting.

The PLS model was found to perform significantly better against the resampled data sets compared to Dataset C. This suggests that the grossly uneven distribution of classes in Dataset C has an effect on the model performance.

The confusion matrix produced from the evaluation of Dataset C produced 100% correct classification for beech honeydew honey. This result is in keeping with the gross differences seen by the visual examination of the NIR spectra of beech honeydew honey and other floral types. Excellent results were also produced for rata honey. Moderate classification results were achieved for clover, manuka, rewarewa and tawari and kamahi honey. The ability of the PLS model to correctly classify thyme, vipers' bugloss and nodding thistle honey was much lower than other honey types. A majority of these misclassified honeys were classified as clover but as clover has a similar nectar flow to these honey types, a significant contribution of clover honey in these samples is likely. The pollen analysis data given in Appendix A4.1 shows that all vipers' bugloss honeys contain a contribution of clover. The corresponding data for nodding thistle honey was not supplied however it has been demonstrated that clover nectar is a substantial contributor in nodding thistle honeys.[7]

The confusion matrix produced using the resampled data sets shows an improvement in performance for all honey types. Once again 100% correct classification was achieved for beech honeydew honey and close to 100% obtained for rata, kamahi, manuka and rewarewa honeys. Clover, tawari and thyme honeys all had high correct classification rates with much fewer samples misallocated. The performance of nodding thistle and vipers' bugloss honey was also much improved using the resampled data sets however classification rates were still significantly below other honey types.

A PLS model has previously been found to discriminate between eucalyptus and pasture honeys with 100% of all samples correctly classified.[124] This is the only reported method using PLS to distinguish between honey types using the NIR spectra. The results of the current survey on New Zealand honeys suggest that the PLS model has great potential as a screening technique for determining honey type. This is the first reported survey including all main unifloral honey types produced in a given area.

6.4 Conclusions

The NIR spectra of honey is dominated by absorbances from sugars and water, therefore it is expected that unifloral honeys which characteristically differ in these main components will be easier to distinguish by NIR spectroscopy. Due to the origin of beech honeydew honey (from sap as opposed to nectar), it was anticipated that NIR spectra would be able to distinguish between beech honeydew honey and honeys derived from nectar, which was found to be the case. Rata honey has a characteristically high glucose content, therefore this honey, as expected was relatively easy to distinguish using NIR. Other honeys, such as clover, vipers' bugloss and nodding thistle display much more subtle differences in chemical composition, these honeys as expected were much more difficult to separate using NIR spectroscopy.

A visual inspection of the NIR spectra indicates beech honeydew honey can be distinguished from nectar honeys, particularly in the regions 4534 - 4225 cm^{-1} where beech honeydew honey has a higher absorbance and 4050 - 3900 cm^{-1} where the absorbance is lower than nectar honeys (Appendix A4.2, Figure A4.2).

Principal component analysis (PCA) was no more effective at distinguishing between honey types than the visual inspection. Linear Discriminant Analysis (LDA) achieved an improvement in resolution between clusters in the scatter plot of the first two Linear Discriminants using either 15 or 17 components. Beech honeydew and rata honeys were fully separated from other honeys however full resolution of other honey

types was not achieved. The performance of the LDA model on manually selected wavelengths was diminished compared to LDA on the principal components scores.

The PLS model developed in WEKA achieved very high classification rates for all honey types (94 - 100%) with the exception of clover (87%), nodding thistle (71%) and vipers' bugloss honeys (85%).

The results presented in this chapter demonstrate that the NIR spectra of honey can reliably distinguish between all main New Zealand unifloral honey types using a PLS model. Since only a limited number of floral source validated nodding thistle, vipers' bugloss and thyme honeys were available, NIR analyses of further samples of these honeys may improve the robustness of this model in distinguishing between vipers' bugloss, nodding thistle and clover honeys.

As only a moderate number of samples have been investigated in the present survey, it is desirable that a greater number of samples (from all floral sources over several years) are added to the sample pool in order to increase the robustness of the discrimination models evaluated during the investigation reported in this thesis.

PCA and LDA, the two most common techniques used in the small number of NIR studies reported in the literature[124, 126, 127] to discriminate between honey types did not perform well when applied to New Zealand honeys. It would be of interest to evaluate how effectively the PLS technique used in this investigation discriminates between results obtained by overseas groups from the honey samples they analysed.

The greatest hurdle in creating a robust PLS model is gaining access to sufficient certified unifloral honeys. This is reflected in the low number of samples analysed in previous surveys.[122-127]

In order to reduce some of the mystique around classification, the presentation of NIR results by a commercial laboratory to beekeepers or regulatory authority using a form of cluster plot where unifloral honeys must fall within a particular region may be beneficial.

The NIR methodology that was utilised in this investigation is rapid and inexpensive, thereby making the commercial analyses accessible to both large and small suppliers. By ensuring all labelled unifloral honeys are of the highest quality, the integrity and economic value of the industry both within New Zealand and the export market will be preserved.

While it is not considered likely that an NIR method will, at least in the foreseeable future replace classical pollen analyses, it has the potential to be recognised as a reliable supplementary technique for demonstrating the floral integrity of a honey sample.

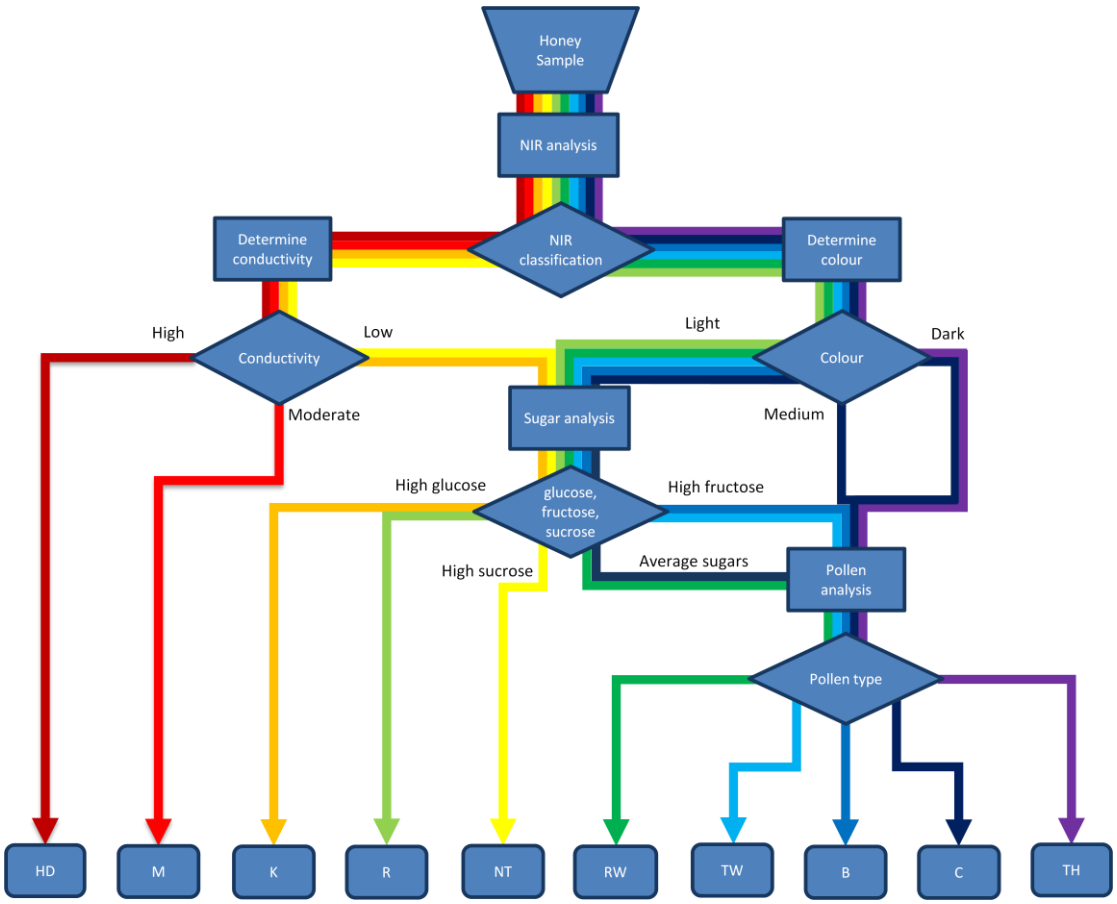
The results reported in this thesis show that NIR data can be used to determine the floral origin of honey. While chemical methods (such as organic extractives and carbohydrate profiles) and spectroscopic techniques (NIR) show an ability to differentiate unifloral honeys, from an industry point of view, what the regulatory agencies and/or trade authorities require is the principle consideration. Despite its shortcomings, pollen analyses remains the most widely accepted procedure for establishing unifloral status, followed by other parameters such as moisture content, colour and conductivity. Regulatory authorities have a preference for methods which produce a single value as opposed to complex spectra; however these methods are gradually being accepted such as the NIR analysis of protein in wheat.

The main advantage of NIR over other chemical methods (such as GC fingerprinting) is the short analyses time and low cost. In time, as more NIR surveys are conducted confirming the status of NIR as a classification tool, such a method may be viewed as a viable complimentary method to pollen analyses.

6.4.1 Proposed Multi-Technique Classification Model

The incorporation of chemical data such as the extractable organic substances with an NIR method should improve the robustness of the classification model, potentially to a level where 100% correct classification is achievable for all unifloral honey types.

A multi-technique model (Figure 6.16) using NIR as the first step and conductivity, colour, sugar analysis and pollen analysis as additional parameters may achieve 100% correct classification for all floral types. Alternatively, given the industries present dependence on pollen analysis, a multi-technique method could be developed with pollen data utilised in the first step. Industry opinions on which analyses which should be included and which should not be included in a multi-technique analyses could be computerised, with step by step outputs advising a user what subsequent analysis (if any) will be required to achieve a predetermined level of certainty.



HD = honeydew, M = manuka, K = kamahi, R = rata, NT = nodding thistle, RW = rewarewa, TW = tawari, B = vipers' bugloss, C = clover, TH = thyme

Figure 6.16 Flowchart for the multistep determination of floral source of New Zealand honeys

The analysis of extractable organic substances requires a lengthy extraction; this method as it stands is not suitable for commercial analysis. It may be possible however to develop a GC-MS method targeting specific marker compounds such as

kamahines A-C (kamahi honey), 1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethylcyclohexane-*trans*,*cis*-1,2,3-triol (thyme honey) and 1,4-hydroquinone (vipers' bugloss honey) which could then be incorporated into the proposed multistep model. A limitation of the extractable organic GC-MS data will always be that while it is capable of recognising the presence of floral marker compounds, their detection at a particular concentration level does not allow the % contribution of the floral source in question (be it greater than 95%, or perhaps as little as 65%) to be defined.

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Appendices

Appendix 1

Carbohydrate Profile of Manuka Honey

A1.1 Quantitation of Glucose and Fructose in Manuka Honey

Table A1.1 Glucose and fructose concentration of manuka honey

Sample	Weight (mg)	Calibration equation		Area		Concentration in solution (mg/mL)		% Dry weight	
		glucose	frucose	glucose	frucose	glucose	frucose	glucose	frucose
1a	15.99	134000	132000	752992	1061358	5.619	8.041	35.143	50.285
1b	15.61	134000	132000	735762	1037499	5.491	7.860	35.175	50.351
2a	15.53	134000	132000	735942	1053098	5.492	7.978	35.364	51.372
2b	16.09	134000	132000	757540	1085451	5.653	8.223	35.135	51.107
3a	14.54	134000	132000	684382	932164	5.107	7.062	35.126	48.568
3b	15.59	134000	132000	748846	1021588	5.588	7.739	35.846	49.643
4a	14.82	134000	132000	711365	939768	5.309	7.119	35.821	48.040
4b	15.41	134000	132000	730989	965497	5.455	7.314	35.400	47.465
5a	16.23	134000	132000	777232	1024792	5.800	7.764	35.738	47.835
5b	15.17	134000	132000	729465	962695	5.444	7.293	35.885	48.076
6a	15.23	134000	132000	744926	993724	5.559	7.528	36.501	49.430
6b	14.63	134000	132000	717688	956357	5.356	7.245	36.609	49.522
7a	15.27	134000	132000	757625	894041	5.654	6.773	37.026	44.355
7b	15.25	134000	132000	757650	895145	5.654	6.781	37.076	44.468
8b	14.9	133000	131000	722971	863753	5.436	6.594	36.482	44.252
8c	15.38	134000	131000	751872	898451	5.611	6.858	36.482	44.593
9a	15.51	133000	131000	734320	965947	5.521	7.374	35.598	47.541
9b	14.29	133000	131000	683694	900577	5.141	6.875	35.973	48.108
10a	15.84	133000	131000	777396	1001879	5.845	7.648	36.901	48.282
10b	14.5	133000	131000	721303	928978	5.423	7.091	37.402	48.906
11a	14.78	133000	131000	725759	904286	5.457	6.903	36.920	46.705
11b	15.11	133000	131000	747881	931823	5.623	7.113	37.215	47.076
12a	15.4	133000	131000	793736	979426	5.968	7.477	38.753	48.549
12b	15.51	133000	131000	803311	990854	6.040	7.564	38.942	48.767
13a	15.14	133000	131000	752097	869710	5.655	6.639	37.350	43.851
13b	15.43	133000	131000	762240	882059	5.731	6.733	37.143	43.638
14a	15.57	133000	131000	855289	951674	6.431	7.265	41.302	46.658
14b	14.93	133000	131000	817685	911458	6.148	6.958	41.179	46.602

Sample	Weight (mg)	Calibration equation		Area		Concentration in solution (mg/mL)		% Dry weight	
		glucose	frucose	glucose	frucose	glucose	frucose	glucose	frucose
15a	16.46	134000	132000	861149	983275	6.426	7.449	39.043	45.255
15b	16.24	134000	132000	850048	970611	6.344	7.353	39.062	45.278
16a	15.57	134000	132000	764102	973622	5.702	7.376	36.623	47.373
16b	16.48	134000	132000	809505	1030977	6.041	7.810	36.657	47.393
17a	15.91	134000	132000	848384	971325	6.331	7.359	39.794	46.251
17b	15.02	134000	132000	792951	908469	5.918	6.882	39.398	45.821
18a	15.39	134000	132000	797370	933473	5.951	7.072	38.665	45.950
18b	14.67	134000	132000	768518	900042	5.735	6.819	39.095	46.479
19a	15.47	134000	132000	774573	930567	5.780	7.050	37.365	45.570
19b	15.47	134000	132000	785805	943849	5.864	7.150	37.907	46.221
20a	14.57	134000	132000	754466	854871	5.630	6.476	38.643	44.450
20b	14.86	134000	132000	766192	866564	5.718	6.565	38.478	44.178
21a	15.36	134000	132000	790160	946955	5.897	7.174	38.390	46.705
21b	15.33	134000	132000	804638	963895	6.005	7.302	39.170	47.634
22a	15.29	134000	132000	795049	943368	5.933	7.147	38.804	46.741
22b	15.04	134000	132000	782408	928148	5.839	7.031	38.822	46.751
23a	15.98	134000	132000	831185	977107	6.203	7.402	38.816	46.322
23b	15.9	134000	132000	837359	984999	6.249	7.462	39.302	46.932
24a	14.85	134000	132000	751729	901440	5.610	6.829	37.777	45.987
24b	15.75	134000	132000	785588	943041	5.863	7.144	37.223	45.360
25a	15.75	134000	132000	771286	966564	5.756	7.322	36.545	46.492
25b	14.84	134000	132000	710710	890932	5.304	6.749	35.740	45.482
26a	14.8	133000	131000	749508	841620	5.635	6.425	38.077	43.409
26b	14.5	133000	131000	738684	829677	5.554	6.333	38.304	43.679
27a	14.66	133000	131000	727305	894043	5.468	6.825	37.302	46.554
27b	15.92	133000	131000	782532	961755	5.884	7.342	36.958	46.116
28b	14.67	133000	131000	731053	898000	5.497	6.855	37.469	46.728
28c	14.67	134000	132000	766979	943198	5.724	7.145	39.017	48.708
29a	15.8	133000	131000	808022	959411	6.075	7.324	38.452	46.353
29b	16.21	133000	131000	832662	989621	6.261	7.554	38.622	46.603

Sample	Weight (mg)	Calibration equation		Area		Concentration in solution (mg/mL)		% Dry weight	
		glucose	frucose	glucose	frucose	glucose	frucose	glucose	frucose
30a	15.62	133000	131000	795678	960867	5.983	7.335	38.301	46.958
30b	15.28	133000	131000	771133	931246	5.798	7.109	37.945	46.523
31a	14.82	133000	131000	784739	897928	5.900	6.854	39.813	46.251
31b	16.35	133000	131000	864544	990051	6.500	7.558	39.757	46.224
32a	16.04	132000	130000	828066	939662	6.273	7.228	39.110	45.063
32b	14.75	132000	130000	757633	859671	5.740	6.613	38.913	44.833
33a	15.65	134000	132000	849239	961805	6.338	7.286	40.496	46.558
33b	14.39	134000	132000	799761	905459	5.968	6.860	41.476	47.669
34a	14.09	134000	132000	791881	868988	5.910	6.583	41.942	46.723
34b	14.15	134000	132000	794932	872705	5.932	6.611	41.925	46.724
35a	15.54	134000	132000	819193	928674	6.113	7.035	39.340	45.273
35b	14.66	134000	132000	788070	892435	5.881	6.761	40.117	46.118
36a	15.5	132000	130000	873616	956691	6.618	7.359	42.699	47.478
36b	16.29	132000	130000	898507	983958	6.807	7.569	41.786	46.464
37a	14.69	132000	130000	747674	932929	5.664	7.176	38.558	48.852
37b	15.53	132000	130000	782826	976715	5.931	7.513	38.187	48.379
38a	15.59	132000	130000	746040	935288	5.652	7.195	36.253	46.148
38b	14.58	132000	130000	709087	889480	5.372	6.842	36.844	46.928

A1.2 Concentration of Myo-Inositol in Manuka Honey

Table A1.2 Concentration of myo-inositol (% freeze-dried ether extracted honey)

Honey	Inositol Conc.	Honey	Inositol Conc.
1	0.037	21	0.061
2	0.009	22	0.029
3	0.023	23	0.043
4	0.013	24	0.022
5	0.018	25	0.018
6	0.019	26	0.100
7	0.026	27	0.041
8	0.014	28	0.074
9	0.040	29	0.056
10	0.030	30	0.019
11	0.046	31	0.020
12	0.020	32	0.022
13	0.030	33	0.022
14	0.019	34	0.024
15	0.105	35	0.061
16	0.176	36	0.036
17	0.026	37	0.130
18	0.050	38	0.018
19	0.018	Average	0.041
20	0.051	Std. dev.	0.03

A1.3 Calculation of Disaccharide Response Factors

Table A1.3 Disaccharide Response Factors

Sample	Standard	Weight (s)	Weight (x)	Area (s)	Area (x)	W s/x	A s/x	RF	Average RF	R^2
1008s1	Sucrose	1.05	2.38	308.50	243.38	0.44	1.27	0.3		
1008s2	Sucrose	1.19	0.85	258.81	38.11	1.40	6.79	0.2		
1008s3	Sucrose	2.00	0.81	832.12	69.37	2.47	12.00	0.2	0.3	0.99
0806s4	α,α-Trehalose	1.17	3.02	348.3	1296.8	0.39	0.27	1.4		
0806s5	α,α-Trehalose	2.12	1.8	399.6	346.2	1.18	1.15	1.0		
0806s6	α,α-Trehalose	1.66	1.4	915	886	1.19	1.03	1.1	1.2	0.96
3105s1	Cellobiose	1.57	0.95	829.6	556.5	1.65	1.49	1.1		
3105s2	Cellobiose	2.23	2	1424.4	1313.2	1.12	1.08	1.0		
3105s3	Cellobiose	4	0.87	2424.5	557.3	4.60	4.35	1.1		
3105s4	Cellobiose	1.06	2.93	721.1	2190.1	0.36	0.33	1.1	1.1	0.99
1606s14	Laminaribose	1.11	1.07	495.93	510	1.04	0.97	1.1		
3107s5	Laminaribose	1.54	1.02	451.4	299.1	1.51	1.51	1.0	1.0	0.97
3107s3	Nigerose	1.29	1.29	542.5	556.1	1.00	0.98	1.0		
3107s4	Nigerose	0.98	1.15	238.9	263.5	0.85	0.91	0.9	1.0	0.71
0706s10	Turanose (front)	5.71	3.34	1173.7	2708.6	1.71	0.43	3.9		
0706s12	Turanose (front)	2.11	2.11	136.3	463.4	1.00	0.29	3.4	3.7	0.93
0706s10	Turanose (rear)	5.71	3.34	542.8	2708.6	1.71	0.20	8.5		
0706s12	Turanose (rear)	2.11	2.11	69.7	463.4	1.00	0.15	6.6	7.6	0.8
1606s7	Maltulose (front)	1.04	0.93	160.4	553.6	1.12	0.29	3.9		
1606s9	Maltulose (front)	0.69	0.96	239.8	1023.9	0.72	0.23	3.1	3.5	0.74
1606s7	Maltulose (rear)	1.04	0.93	262	553.6	1.12	0.47	2.4		
1606s9	Maltulose (rear)	0.69	0.96	321.9	1023.9	0.72	0.31	2.3	2.3	0.99
1606s1	Maltose	1.64	1.48	777.9	912	1.11	0.85	1.3		
1606s2	Maltose	2.58	2.03	1102.4	1492.5	1.27	0.74	1.7		
1606s3	Maltose	1.48	2.28	816.3	1421.6	0.65	0.57	1.1	1.4	0.94
3107s9	Kojibiose	1.34	1.04	448.8	450.2	1.29	1.00	1.3		
3107s11	Kojibiose	0.98	2.21	372.2	1191	0.44	0.31	1.4	1.4	0.99

Sample	Standard	Weight (s)	Weight (x)	Area (s)	Area (x)	W s/x	A s/x	RF	Average RF	R^2
0806s10	Gentiobiose	0.8	1.58	386.2	973.3	0.51	0.40	1.3		
0806s12	Gentiobiose	1.27	2.09	722.9	1440.3	0.61	0.50	1.2	1.2	0.92
1606s10	Isomaltose	0.81	1.23	270.87	354.6	0.66	0.76	0.9		
1606s11	Isomaltose	1.2	1.45	733.45	774.5	0.83	0.95	0.9	0.9	0.99

A1.4 GC-FID Chromatograms of Co-injected O-TMS Honey and Standard Disaccharides

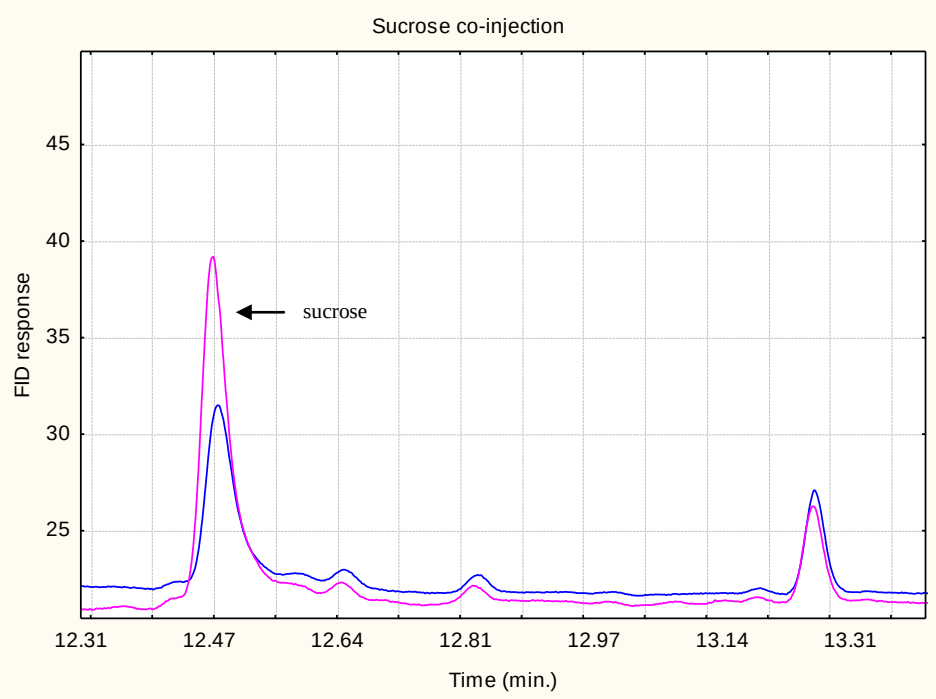

Figure 1.1 Co-injection of sucrose with honey

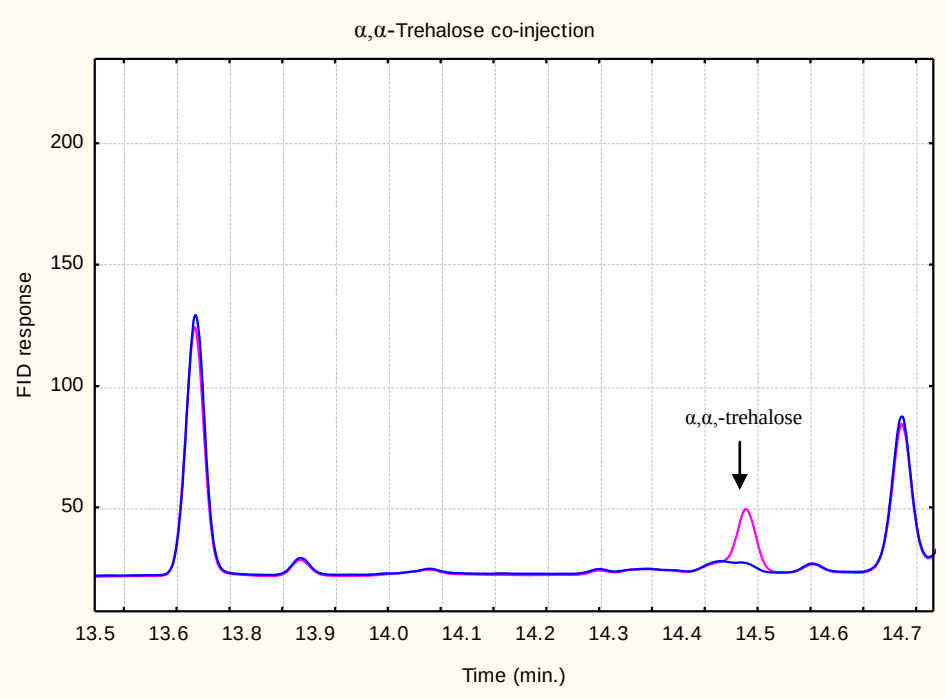

Figure A1.2 Co-injection of α,α-trehalose with honey

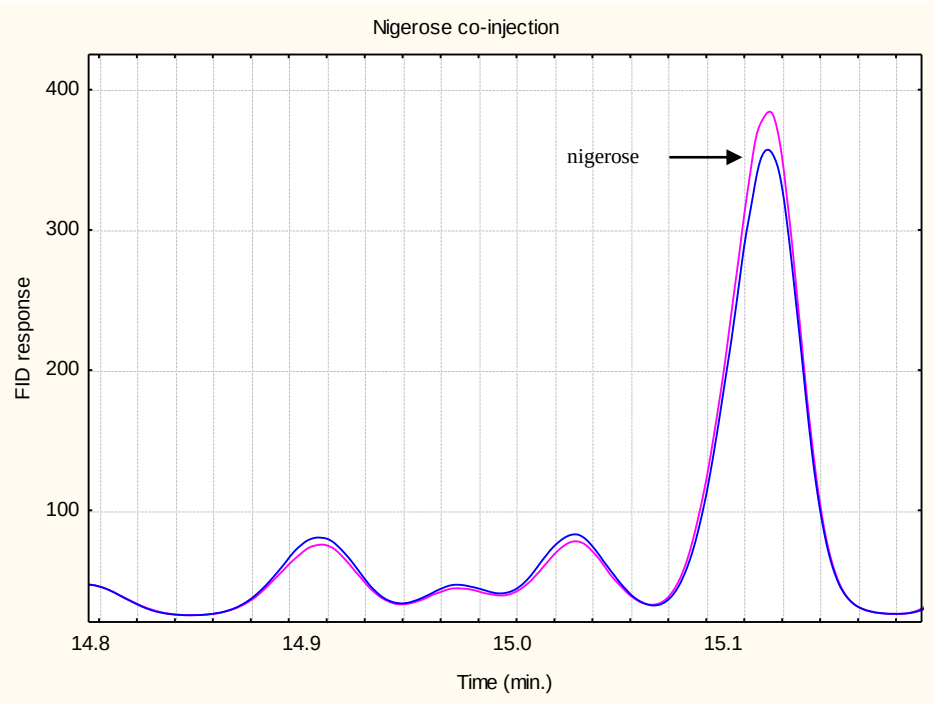

Figure A1.3 Co-injection of nigerose with honey

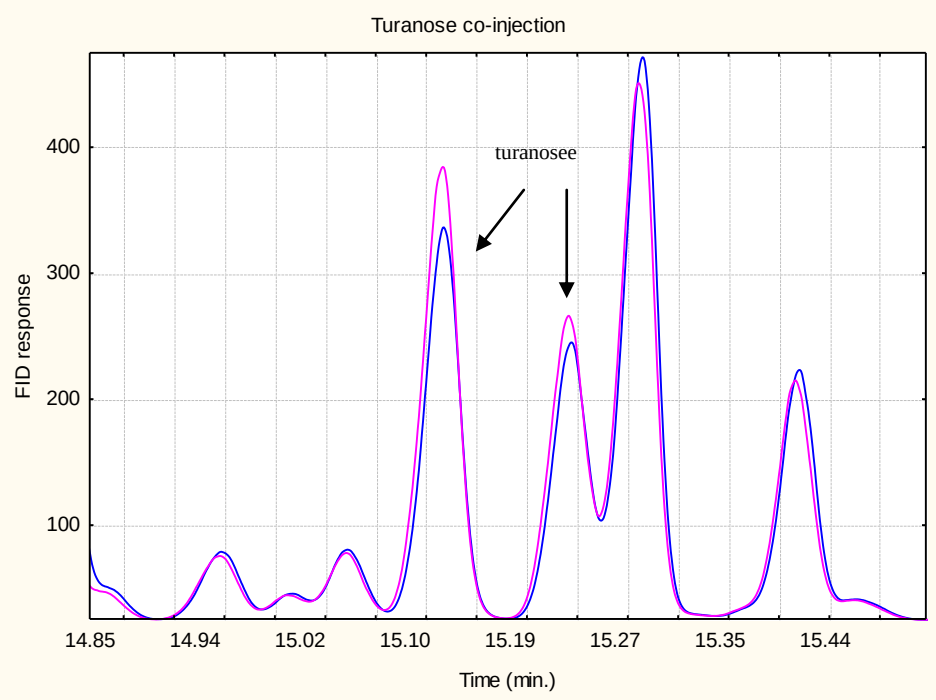

Figure A1.4 Co-injection of turanose with honey

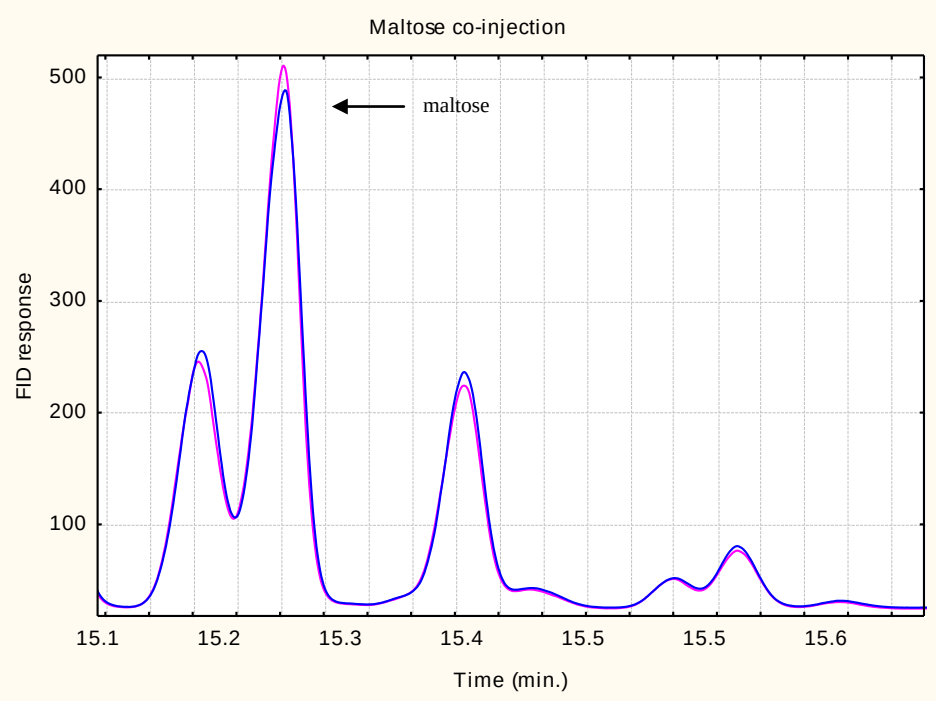

Figure A1.5 Co-injection of maltose with honey

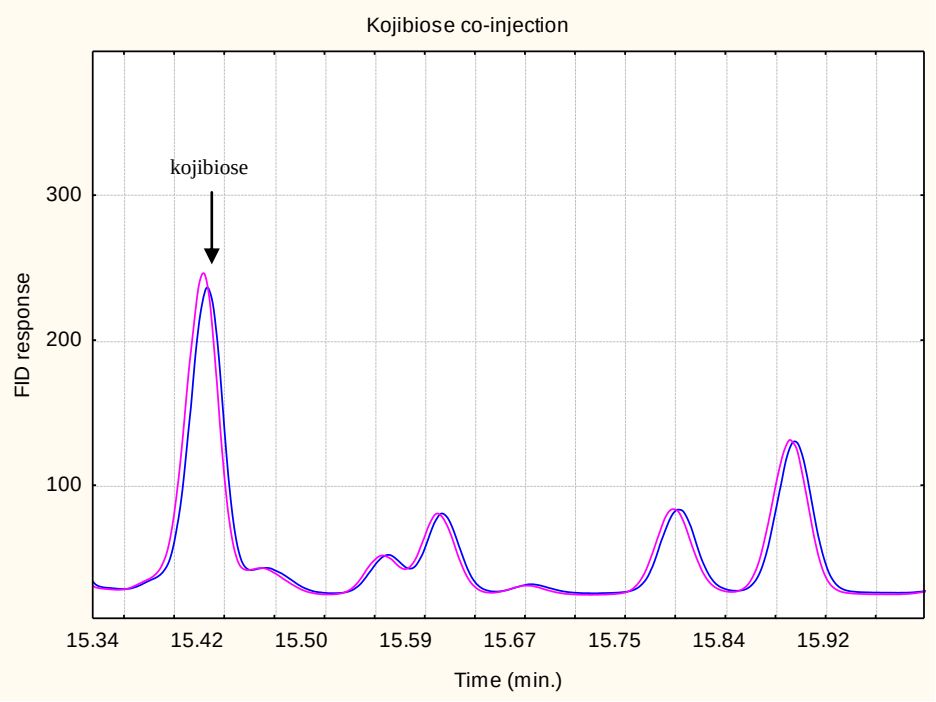

Figure A1.6 Co-injection of kojibiose with honey

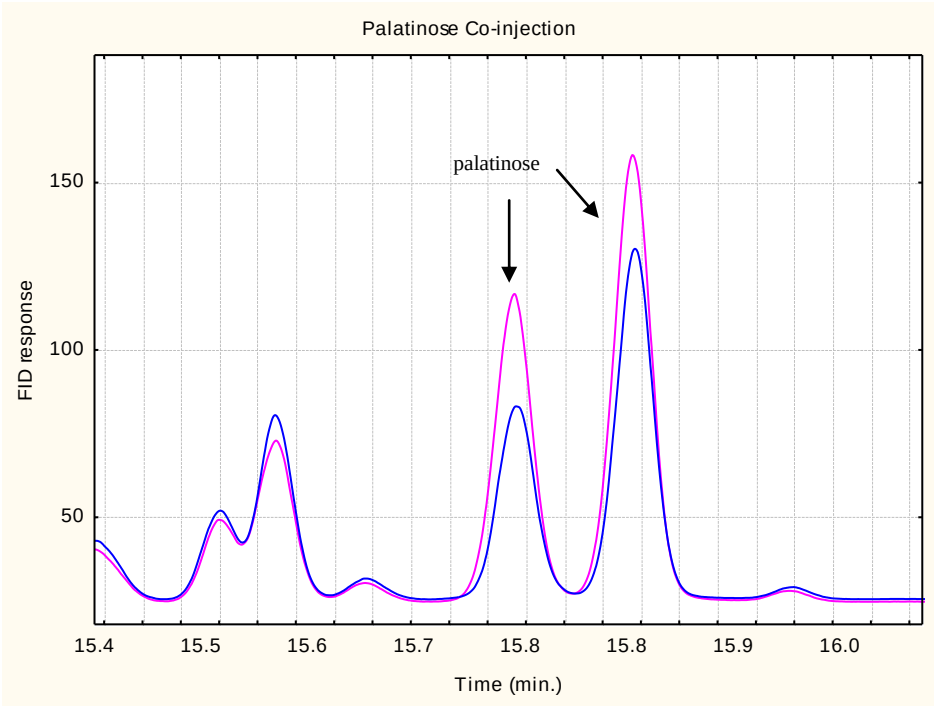

Figure A1.7 Co-injection of palatinose with honey

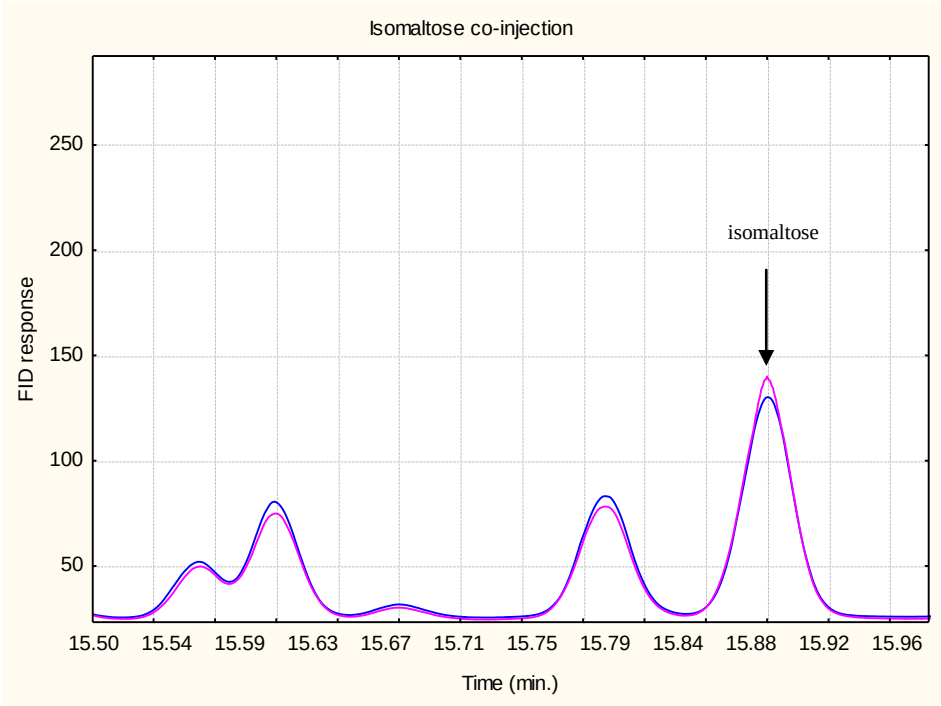

Figure A1.8 Co-injection of isomaltose with honey

A1.5 Disaccharide Content of Manuka Honey

Table A1.4 Disaccharide content of manuka honey as a percentage of freeze-dried ether extracted honey

Honey	sucrose	unknown 1	cellobiose	unknown 2	laminaribiose	unknown 3	unknown 4	nigerose	turanose	maltulose	maltose	kojibiose	unknown 5	gentibiose	unknown 6	unknown 7	isomaltose
1a	0.055	0.225	0.016	0.019	0.070	0.012	0.032	0.254	0.047	0.068	0.213	0.359	0.018	0.116	0.011	0.124	0.275
1b	0.056	0.226	0.021	0.024	0.076	0.013	0.036	0.247	0.045	0.065	0.315	0.359	0.012	0.108	0.012	0.124	0.268
1c	0.028	0.221	0.014	0.025	0.057	0.012	0.020	0.243	0.030	0.043	0.255	0.348	0.016	0.118	0.014	0.134	0.239
2a	0.042	0.144	0.029	0.040	0.168	0.033	0.106	0.178	0.020	0.034	0.226	0.363	0.020	0.156	0.015	0.137	0.340
2b	0.043	0.146	0.047	0.041	0.156	0.034	0.099	0.210	0.033	0.055	0.289	0.317	0.020	0.125	0.014	0.124	0.276
2c	0.040	0.147	0.013	0.018	0.065	0.021	0.022	0.211	0.022	0.038	0.227	0.308	0.018	0.089	0.016	0.107	0.161
3a	0.169	0.242	0.031	0.031	0.109	0.029	0.059	0.254	0.050	0.077	0.256	0.270	0.023	0.108	0.015	0.160	0.292
3b	0.153	0.216	0.028	0.037	0.145	0.026	0.087	0.238	0.051	0.079	0.677	0.304	0.025	0.183	0.014	0.188	0.439
3c	0.111	0.244	0.025	0.026	0.111	0.022	0.067	0.291	0.045	0.068	0.529	0.467	0.020	0.186	0.015	0.219	0.404
4a	0.038	0.233	0.015	0.026	0.084	0.010	0.027	0.163	0.033	0.044	0.116	0.284	0.028	0.143	0.011	0.199	0.242
4b	0.035	0.213	0.019	0.022	0.098	0.013	0.037	0.182	0.053	0.071	0.305	0.300	0.020	0.147	0.012	0.175	0.339
4c	0.021	0.158	0.015	0.021	0.087	0.013	0.027	0.168	0.033	0.045	0.220	0.276	0.021	0.143	0.012	0.180	0.281
5a	0.023	0.169	0.028	0.022	0.108	0.024	0.059	0.214	0.026	0.040	0.078	0.225	0.016	0.128	0.008	0.186	0.298
5b	0.015	0.113	0.027	0.024	0.080	0.024	0.057	0.080	0.035	0.056	0.168	0.215	0.013	0.139	0.006	0.162	0.294
5c	0.023	0.172	0.013	0.025	0.059	0.010	0.022	0.262	0.031	0.048	0.098	0.255	0.015	0.135	0.009	0.191	0.282
6a	0.055	0.164	0.064	0.045	0.182	0.050	0.082	0.149	0.039	0.048	0.499	0.361	0.018	0.141	0.015	0.145	0.242
6b	0.048	0.171	0.042	0.045	0.156	0.040	0.074	0.267	0.025	0.031	0.265	0.424	0.019	0.180	0.020	0.223	0.317
6c	0.048	0.215	0.045	0.033	0.160	0.052	0.065	0.246	0.042	0.051	0.476	0.464	0.020	0.187	0.020	0.194	0.304
7a	0.383	0.249	0.041	0.047	0.144	0.026	0.116	0.182	0.100	0.110	0.743	0.491	0.036	0.204	0.012	0.302	0.518
7b	0.450	0.235	0.051	0.048	0.136	0.032	0.108	0.078	0.092	0.101	0.562	0.427	0.025	0.212	0.015	0.264	0.414
7c	0.437	0.306	0.032	0.032	0.128	0.032	0.063	0.413	0.068	0.075	0.505	0.571	0.075	0.220	0.018	0.298	0.383
8a	0.392	0.188	0.023	0.021	0.078	0.018	0.055	0.463	0.023	0.024	0.085	0.226	0.012	0.102	0.006	0.116	0.167

Honey	sucrose	unknown 1	cellobiose	unknown 2	laminaribiose	unknown 3	unknown 4	nigerose	turanose	maltulose	maltose	kojibiose	unknown 5	gentibiose	unknown 6	unknown 7	isomaltose
8b	0.478	0.217	0.014	0.019	0.062	0.014	0.039	0.512	0.021	0.022	0.084	0.196	0.022	0.087	0.007	0.122	0.150
8c	0.229	0.153	0.020	0.016	0.073	0.018	0.040	0.347	0.020	0.021	0.571	0.290	0.020	0.119	0.009	0.130	0.226
9a	0.230	0.210	0.011	0.014	0.076	0.010	0.033	0.375	0.029	0.041	0.097	0.238	0.037	0.091	0.014	0.126	0.191
9b	0.086	0.192	0.010	0.012	0.045	0.005	0.012	0.202	0.036	0.051	0.471	0.250	0.039	0.096	0.011	0.137	0.227
9c	0.158	0.129	0.018	0.015	0.056	0.014	0.025	0.138	0.035	0.050	0.094	0.252	0.029	0.081	0.015	0.116	0.228
10a	0.299	0.161	0.034	0.075	0.186	0.063	0.139	0.079	0.057	0.079	0.777	0.514	0.024	0.198	0.020	0.176	0.386
10b	0.124	0.149	0.041	0.055	0.187	0.059	0.114	0.151	0.064	0.089	0.716	0.520	0.042	0.233	0.027	0.180	0.412
10c	0.288	0.208	0.015	0.021	0.101	0.027	0.048	0.151	0.028	0.039	0.302	0.333	0.046	0.119	0.029	0.162	0.205
11a	0.091	0.257	0.012	0.024	0.065	0.015	0.029	0.338	0.035	0.047	0.256	0.448	0.020	0.214	0.010	0.255	0.463
11b	0.090	0.248	0.013	0.024	0.074	0.010	0.047	0.300	0.031	0.042	0.174	0.405	0.017	0.170	0.011	0.218	0.372
11c	0.097	0.271	0.013	0.016	0.069	0.015	0.042	0.354	0.029	0.039	0.051	0.295	0.014	0.121	0.011	0.176	0.233
12a	0.236	0.162	0.040	0.032	0.100	0.033	0.073	0.150	0.044	0.056	0.580	0.376	0.013	0.133	0.010	0.135	0.309
12b	0.238	0.169	0.036	0.035	0.102	0.026	0.083	0.178	0.039	0.049	0.567	0.374	0.013	0.124	0.008	0.132	0.282
12c	0.238	0.165	0.026	0.025	0.086	0.023	0.056	0.170	0.023	0.029	0.307	0.292	0.015	0.091	0.012	0.118	0.187
13a	0.581	0.192	0.013	0.024	0.090	0.016	0.073	0.247	0.033	0.035	0.563	0.376	0.021	0.188	0.010	0.219	0.360
13b	0.337	0.209	0.018	0.017	0.070	0.023	0.031	0.391	0.054	0.057	0.602	0.375	0.071	0.214	0.023	0.263	0.368
13c	0.648	0.203	0.010	0.023	0.086	0.019	0.074	0.275	0.042	0.044	0.497	0.344	0.017	0.154	0.011	0.207	0.273
14a	0.062	0.158	0.023	0.027	0.143	0.043	0.065	0.143	0.051	0.069	0.458	0.438	0.018	0.186	0.023	0.176	0.430
14b	0.059	0.148	0.023	0.030	0.160	0.046	0.087	0.138	0.024	0.033	0.274	0.389	0.022	0.154	0.017	0.179	0.352
14c	0.060	0.156	0.014	0.020	0.137	0.029	0.084	0.151	0.047	0.064	0.239	0.327	0.013	0.107	0.021	0.181	0.269
15a	0.037	0.249	0.011	0.032	0.110	0.029	0.046	0.258	0.035	0.054	0.233	0.670	0.062	0.252	0.016	0.260	0.702
15b	0.044	0.242	0.025	0.046	0.122	0.034	0.062	0.298	0.070	0.106	0.214	0.590	0.056	0.227	0.013	0.238	0.605
15c	0.107	0.619	0.020	0.021	0.139	0.026	0.054	0.936	0.040	0.061	0.002	0.574	0.025	0.156	0.019	0.247	0.342
16a	0.044	0.268	0.016	0.021	0.088	0.030	0.039	0.216	0.080	0.096	0.285	0.451	0.013	0.223	0.017	0.253	0.540
16b	0.043	0.297	0.019	0.021	0.080	0.023	0.067	0.244	0.027	0.033	0.002	0.305	0.020	0.100	0.025	0.178	0.278
16c	0.124	0.385	0.007	0.012	0.083	0.027	0.046	0.339	0.033	0.040	0.002	0.304	0.013	0.111	0.018	0.204	0.295

Honey	sucrose	unknown 1	cellobiose	unknown 2	laminaribiose	unknown 3	unknown 4	nigerose	turanose	maltulose	maltose	kojibiose	unknown 5	gentibiose	unknown 6	unknown 7	isomaltose
17a	0.109	0.177	0.009	0.013	0.093	0.020	0.065	0.235	0.022	0.027	0.374	0.366	0.011	0.101	0.009	0.123	0.255
17b	0.125	0.311	0.005	0.007	0.043	0.002	0.004	0.562	0.020	0.024	0.229	0.310	0.007	0.079	0.010	0.115	0.159
17c	0.110	0.177	0.012	0.023	0.107	0.031	0.083	0.214	0.045	0.055	0.411	0.393	0.009	0.101	0.012	0.138	0.304
18a	0.064	0.146	0.017	0.021	0.101	0.029	0.069	0.082	0.049	0.054	0.421	0.458	0.012	0.112	0.013	0.129	0.364
18b	0.067	0.160	0.023	0.024	0.092	0.022	0.058	0.229	0.020	0.022	0.095	0.333	0.018	0.092	0.015	0.103	0.196
18c	0.138	0.311	0.015	0.026	0.105	0.025	0.066	0.138	0.027	0.029	0.023	0.290	0.011	0.091	0.007	0.124	0.204
19a	0.216	0.302	0.016	0.029	0.074	0.029	0.061	0.468	0.019	0.022	0.002	0.237	0.013	0.103	0.016	0.128	0.200
19b	0.151	0.230	0.011	0.025	0.081	0.026	0.060	0.284	0.052	0.059	0.044	0.304	0.017	0.106	0.013	0.157	0.277
19c	0.148	0.226	0.010	0.021	0.090	0.024	0.068	0.127	0.036	0.041	0.298	0.386	0.011	0.145	0.012	0.158	0.337
20a	0.094	0.324	0.021	0.027	0.094	0.017	0.064	0.482	0.055	0.077	0.165	0.405	0.020	0.126	0.008	0.151	0.357
20b	0.028	0.298	0.015	0.020	0.070	0.017	0.027	0.480	0.060	0.084	0.461	0.473	0.024	0.182	0.013	0.195	0.489
20c	0.123	0.309	0.019	0.030	0.130	0.017	0.069	0.472	0.038	0.053	0.002	0.440	0.024	0.181	0.009	0.167	0.438
21a	0.015	0.232	0.009	0.012	0.038	0.004	0.006	0.015	0.078	0.117	0.246	0.559	0.064	0.293	0.017	0.300	0.730
21b	0.012	0.223	0.009	0.011	0.049	0.010	0.033	0.082	0.070	0.106	0.222	0.498	0.059	0.244	0.004	0.264	0.625
21c	0.069	0.358	0.040	0.172	0.079	0.025	0.079	0.066	0.029	0.044	0.001	0.331	0.009	0.144	0.009	0.179	0.364
22a	0.021	0.287	0.008	0.007	0.037	0.005	0.012	0.331	0.036	0.062	0.083	0.253	0.022	0.149	0.014	0.263	0.281
22b	0.044	0.213	0.009	0.010	0.037	0.011	0.024	0.182	0.066	0.111	0.170	0.376	0.027	0.240	0.016	0.287	0.602
22c	0.049	0.240	0.010	0.010	0.057	0.009	0.026	0.212	0.037	0.062	0.002	0.356	0.025	0.168	0.009	0.277	0.431
23a	0.027	0.183	0.014	0.011	0.083	0.020	0.076	0.394	0.031	0.062	0.061	0.298	0.020	0.191	0.008	0.241	0.439
23b	0.036	0.176	0.016	0.021	0.074	0.022	0.068	0.364	0.026	0.052	0.036	0.398	0.025	0.291	0.009	0.277	0.656
23c	0.029	0.161	0.015	0.015	0.081	0.022	0.083	0.358	0.019	0.037	0.021	0.317	0.018	0.188	0.009	0.229	0.396
24a	0.146	0.290	0.027	0.023	0.068	0.025	0.029	0.241	0.091	0.093	0.386	0.571	0.031	0.221	0.022	0.279	0.571
24b	0.135	0.267	0.031	0.032	0.090	0.022	0.067	0.230	0.072	0.074	0.332	0.423	0.032	0.189	0.018	0.211	0.476
24c	0.152	0.237	0.037	0.047	0.115	0.023	0.084	0.210	0.037	0.038	0.191	0.542	0.028	0.191	0.017	0.218	0.472
25a	0.029	0.239	0.017	0.021	0.109	0.030	0.100	0.335	0.027	0.042	0.180	0.465	0.052	0.274	0.023	0.306	0.643
25b	0.028	0.241	0.025	0.023	0.119	0.034	0.124	0.270	0.084	0.130	0.309	0.609	0.028	0.345	0.017	0.371	0.990

Honey	sucrose	unknown 1	cellobiose	unknown 2	laminaribiose	unknown 3	unknown 4	nigerose	turanose	maltulose	maltose	kojibiose	unknown 5	gentibiose	unknown 6	unknown 7	isomaltose
25c	0.029	0.242	0.022	0.017	0.108	0.019	0.049	0.388	0.076	0.118	0.002	0.615	0.064	0.345	0.023	0.385	0.976
26a	0.075	0.251	0.020	0.032	0.096	0.021	0.109	0.470	0.052	0.081	0.379	0.648	0.061	0.346	0.016	0.339	0.910
26b	0.085	0.253	0.023	0.032	0.103	0.032	0.075	0.401	0.036	0.057	0.302	0.689	0.051	0.336	0.016	0.373	0.882
26c	0.140	0.354	0.031	0.041	0.136	0.020	0.127	0.858	0.036	0.056	0.002	0.748	0.062	0.343	0.032	0.382	0.809
27a	0.093	0.246	0.025	0.034	0.092	0.029	0.056	0.260	0.071	0.101	0.232	0.415	0.026	0.180	0.011	0.210	0.426
27b	0.150	0.223	0.027	0.035	0.106	0.026	0.090	0.211	0.062	0.088	0.190	0.415	0.023	0.172	0.010	0.198	0.431
27c	0.151	0.231	0.030	0.037	0.105	0.026	0.090	0.215	0.023	0.033	0.132	0.337	0.019	0.140	0.012	0.165	0.328
28a	0.115	0.248	0.024	0.031	0.084	0.031	0.048	0.517	0.042	0.066	0.061	0.427	0.041	0.209	0.017	0.246	0.396
28b	0.054	0.177	0.031	0.026	0.113	0.031	0.147	0.289	0.015	0.023	0.002	0.371	0.032	0.152	0.021	0.170	0.323
28c	0.056	0.171	0.034	0.030	0.139	0.038	0.158	0.308	0.048	0.076	0.030	0.429	0.037	0.183	0.015	0.177	0.407
29a	0.099	0.209	0.028	0.023	0.102	0.029	0.127	0.184	0.056	0.062	0.329	0.312	0.018	0.121	0.006	0.131	0.272
29b	0.092	0.180	0.026	0.030	0.114	0.033	0.123	0.201	0.023	0.026	0.280	0.318	0.013	0.106	0.005	0.126	0.226
29c	0.096	0.186	0.030	0.029	0.116	0.038	0.119	0.169	0.053	0.059	0.373	0.452	0.015	0.145	0.008	0.134	0.332
30a	0.119	0.213	0.034	0.026	0.091	0.019	0.058	0.204	0.047	0.032	0.251	0.261	0.010	0.106	0.009	0.152	0.239
30b	0.123	0.236	0.034	0.027	0.090	0.020	0.069	0.141	0.067	0.046	0.140	0.299	0.011	0.139	0.007	0.165	0.336
30c	0.126	0.225	0.039	0.031	0.102	0.024	0.064	0.122	0.050	0.034	0.178	0.289	0.012	0.137	0.009	0.161	0.297
31a	0.086	0.158	0.027	0.023	0.093	0.020	0.056	0.252	0.022	0.026	0.100	0.249	0.022	0.116	0.014	0.135	0.239
31b	0.122	0.281	0.035	0.021	0.101	0.016	0.058	0.239	0.060	0.072	0.195	0.276	0.019	0.116	0.013	0.162	0.337
31c	0.079	0.152	0.038	0.038	0.114	0.023	0.070	0.104	0.055	0.066	0.103	0.357	0.020	0.167	0.015	0.154	0.430
32a	0.137	0.292	0.068	0.055	0.205	0.037	0.124	0.428	0.075	0.090	0.238	0.631	0.034	0.271	0.024	0.325	0.662
32b	0.144	0.210	0.025	0.023	0.070	0.018	0.020	0.414	0.073	0.087	0.213	0.618	0.078	0.276	0.018	0.325	0.670
32c	0.107	0.206	0.072	0.043	0.134	0.039	0.109	0.144	0.074	0.089	0.393	0.595	0.045	0.285	0.021	0.251	0.661
33a	0.044	0.144	0.025	0.031	0.141	0.042	0.092	0.152	0.048	0.063	0.343	0.472	0.029	0.210	0.015	0.165	0.445
33b	0.047	0.144	0.040	0.032	0.139	0.041	0.098	0.073	0.041	0.054	0.286	0.461	0.019	0.182	0.012	0.139	0.392
33c	0.038	0.130	0.040	0.052	0.145	0.051	0.096	0.123	0.040	0.053	0.066	0.393	0.022	0.166	0.013	0.126	0.366
34a	0.046	0.142	0.034	0.024	0.095	0.012	0.019	0.192	0.042	0.040	0.166	0.350	0.047	0.144	0.016	0.157	0.360

Honey	sucrose	unknown 1	cellobiose	unknown 2	laminaribiose	unknown 3	unknown 4	nigerose	turanose	maltulose	maltose	kojibiose	unknown 5	gentibiose	unknown 6	unknown 7	isomaltose
34b	0.075	0.264	0.055	0.039	0.156	0.031	0.090	0.481	0.023	0.022	0.019	0.320	0.024	0.110	0.017	0.139	0.227
34c	0.037	0.132	0.056	0.069	0.166	0.048	0.124	0.076	0.042	0.040	0.198	0.398	0.028	0.166	0.016	0.127	0.355
35a	0.033	0.279	0.012	0.042	0.133	0.046	0.082	0.425	0.062	0.116	0.478	0.748	0.069	0.358	0.037	0.306	0.898
35b	0.024	0.259	0.015	0.029	0.104	0.029	0.047	0.471	0.063	0.118	0.274	0.751	0.073	0.353	0.027	0.297	0.883
35c	0.057	0.283	0.039	0.054	0.163	0.051	0.084	0.709	0.048	0.090	0.002	0.619	0.071	0.219	0.035	0.276	0.517
36a	0.773	0.180	0.059	0.035	0.147	0.025	0.097	0.638	0.054	0.073	0.196	0.258	0.021	0.087	0.013	0.133	0.161
36b	0.206	0.145	0.034	0.039	0.119	0.033	0.050	0.329	0.046	0.063	0.741	0.399	0.011	0.182	0.015	0.144	0.328
36c	0.211	0.134	0.058	0.002	0.114	0.039	0.072	0.299	0.041	0.056	0.678	0.399	0.017	0.157	0.012	0.122	0.291
37a	0.041	0.219	0.010	0.034	0.068	0.028	0.044	0.331	0.019	0.029	0.208	0.541	0.018	0.330	0.010	0.313	0.700
37b	0.075	0.352	0.033	0.048	0.144	0.045	0.140	1.097	0.062	0.093	0.233	0.508	0.018	0.237	0.017	0.338	0.509
37c	0.081	0.268	0.038	0.021	0.147	0.047	0.122	1.035	0.038	0.057	0.002	0.476	0.034	0.196	0.029	0.276	0.429
38a	0.056	0.149	0.013	0.002	0.026	0.013	0.026	0.285	0.044	0.066	0.002	0.318	0.022	0.140	0.011	0.193	0.376
38b	0.109	0.362	0.016	0.002	0.038	0.010	0.040	0.580	0.027	0.041	0.002	0.237	0.017	0.116	0.008	0.156	0.272
38c	0.117	0.276	0.028	0.002	0.056	0.020	0.046	0.521	0.035	0.053	0.002	0.294	0.014	0.126	0.010	0.176	0.273
μ	0.125	0.222	0.025	0.029	0.102	0.026	0.067	0.294	0.044	0.059	0.248	0.400	0.027	0.172	0.015	0.198	0.395
σ	0.131	0.07	0.014	0.019	0.037	0.012	0.034	0.189	0.018	0.026	0.198	0.127	0.017	0.07	0.006	0.07	0.187

μ = arithmetic mean, σ= standard deviation (population)

A1.6 GC-FID Chromatograms of Co-injected O-TMS Honey and Standard Trisaccharides

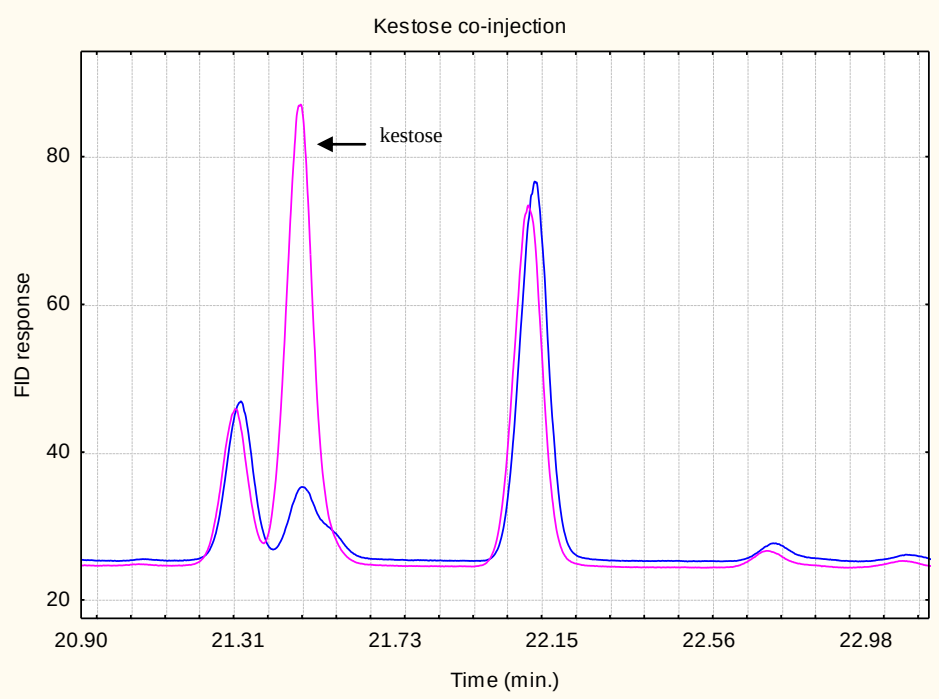

Figure A1.9 Co-injection of kestose with honey

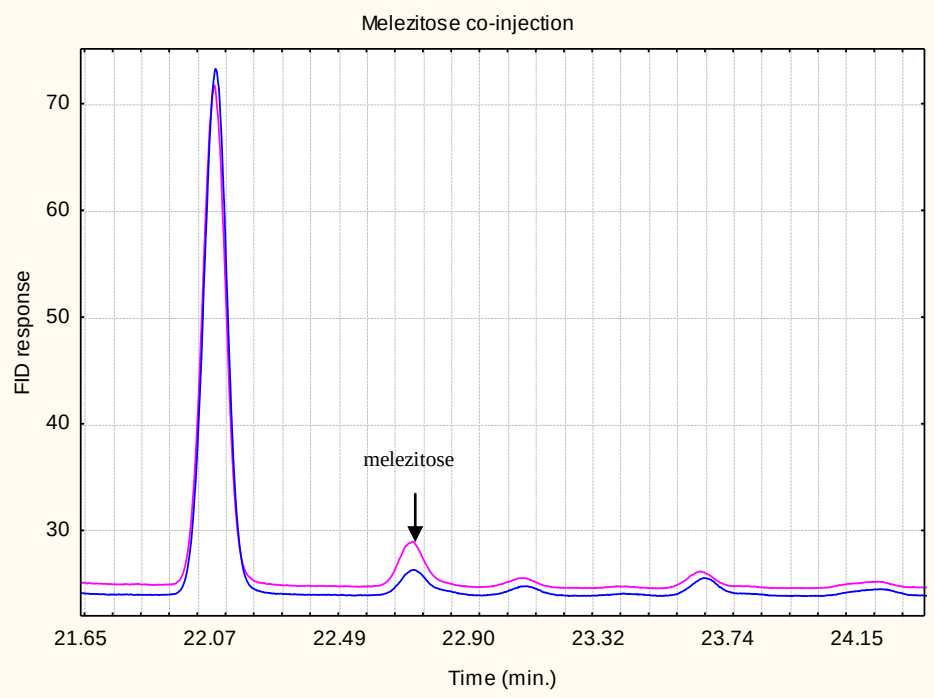

Figure A1.10 Co-injection of melezitose with honey

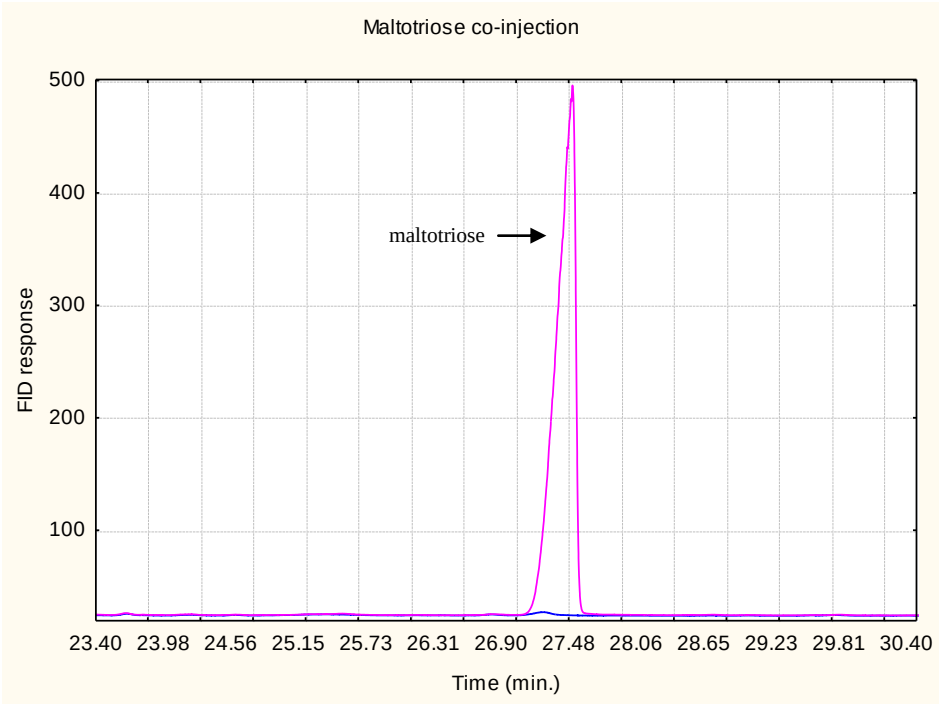

Figure A1.11 Co-injection of maltotriose with honey

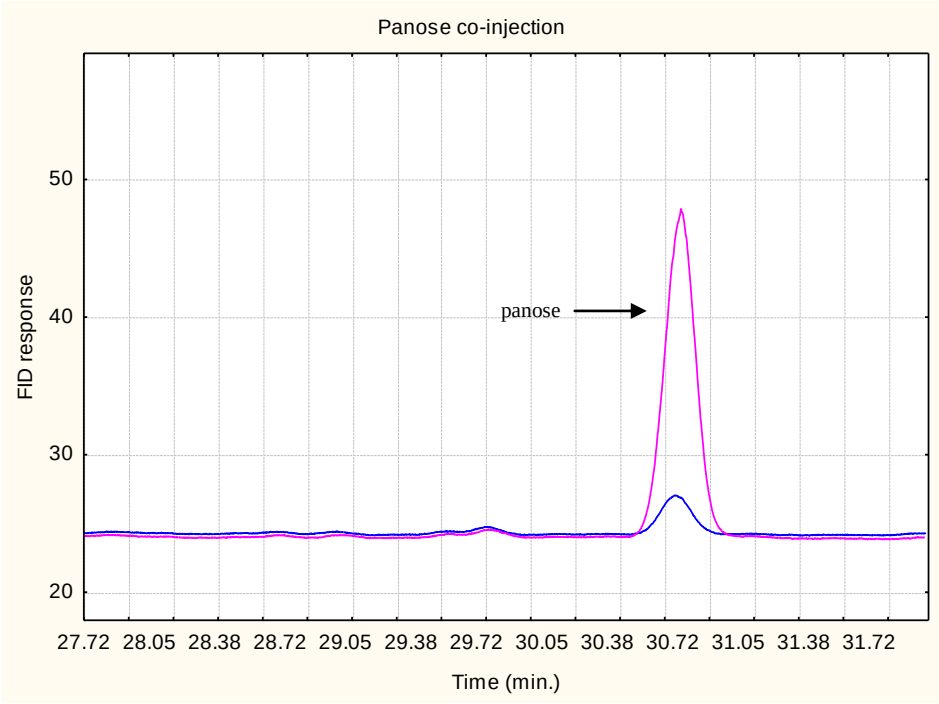

Figure A1.12 Co-injection of panose with honey

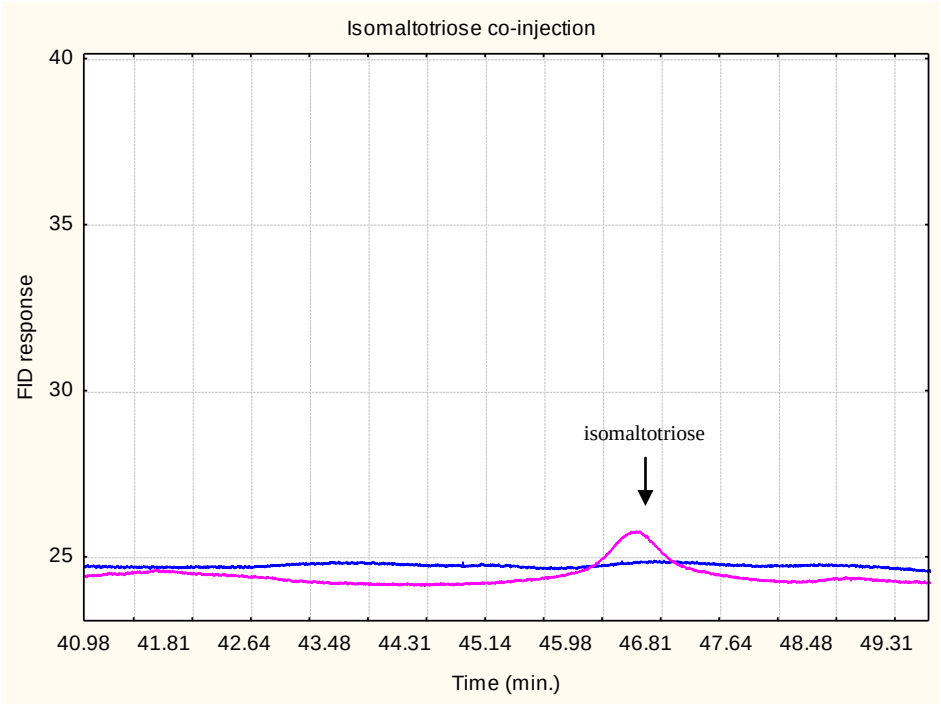

Figure A1.13 Co-injection of isomaltotriose with honey

Appendix 2

Identification of Disaccharides in Honey by GC-MS-SIM

A2.1 Intensity of Selected Ions of *O*-Trimethylsilyl Disaccharide Alditols by GC-MS

Table A2.1 Intensity of selected ions in *O*-trimethylsilyl disaccharide alditols relative to *m/z* 217 ion

Disaccharide	Intensity (% *m/z* 217 ion)													
	103	**104**	**205**	**206**	**307**	**308**	**319**	**320**	**409**	**410**	**595**	**596**	**685**	**686**
sucrose	23.9	2.3	10.2	3.2	1.1	0.3	11.3	4.5	0.03	0.01	0.011	0.006	0.003	0.002
trehalose	37.2	3.6	22.7	6.9	2.3	0.6	25.6	8.3	0.14	0.05	0.005	0.003	0.003	0.002
cellobiose	21.8	10.8	74.6	44.4	3.2	8.6	16.8	9.0	0.34	0.52	0.018	0.586	0.017	0.466
laminaribiose	17.0	3.8	57.7	35.1	6.0	2.0	12.0	10.6	0.20	0.13	0.009	0.151	0.009	0.228
nigerose	24.7	5.1	53.4	30.3	8.7	3.2	16.6	14.0	0.59	0.38	0.012	0.064	0.013	0.170
turanose	43.1	5.4	101.6	56.6	16.5	5.9	31.5	21.5	0.88	0.56	0.012	0.089	0.002	0.228
	51.6	6.2	102.4	52.8	17.1	6.5	26.4	16.9	0.69	0.67	0.017	0.165	0.017	0.127
maltulose	31.4	7.4	40.7	42.5	2.9	9.6	16.7	8.5	0.26	0.27	0.016	0.087	0.009	0.096
	26.3	8.5	54.6	38.9	3.7	17.3	23.0	11.6	0.41	1.02	0.016	0.060	0.023	0.113
maltose	21.7	10.4	44.0	36.4	4.8	18.9	23.2	15.2	0.47	1.09	0.017	0.063	0.019	0.123
kojibiose	41.4	11.1	64.8	22.4	22.0	9.1	54.7	62.3	0.42	0.18	0.016	0.230	0.005	0.105
gentibiose	31.7	17.5	82.9	73.8	5.4	21.1	14.2	78.9	0.49	0.97	0.021	0.049	0.226	0.165
palatinose	41.2	15.6	63.0	80.0	5.5	27.0	18.6	173.4	0.96	0.98	0.034	0.082	0.077	0.056
	43.2	17.0	71.6	81.6	5.7	35.9	17.2	129.2	0.69	2.00	0.027	0.031	0.065	0.053
isomaltose	33.4	17.9	61.5	71.9	5.8	31.2	14.2	113.7	0.63	1.78	0.025	0.031	0.065	0.052

Table A2.2 Intensity of selected ions in *O*-trimethylsilyl disaccharide alditols relative to *m/z* 361 ion

Disaccharide	Intensity (% *m/z* 361 ion)													
	103	**104**	**205**	**206**	**307**	**308**	**319**	**320**	**409**	**410**	**595**	**596**	**685**	**686**
sucrose	13.8	1.3	5.9	1.9	0.6	0.2	6.5	2.6	0.01	0.01	0.014	0.033	0.154	0.112
trehalose	11.6	1.1	7.1	2.2	0.7	0.2	8.0	2.6	0.04	0.02	0.002	0.001	0.001	0.001
cellobiose	21.0	10.5	72.0	42.9	3.1	8.3	16.2	8.7	0.33	0.50	0.018	0.566	0.016	0.450
laminaribiose	14.7	3.3	49.8	30.3	5.2	1.7	10.3	9.1	0.17	0.11	0.008	0.131	0.008	0.197
nigerose	12.8	2.6	27.8	15.8	4.5	1.6	8.6	7.3	0.31	0.20	0.006	0.033	0.007	0.089
turanose	14.4	1.8	34.0	19.0	5.5	2.0	10.5	7.2	0.29	0.19	0.004	0.030	0.001	0.076
	15.5	1.9	30.7	15.9	5.1	1.9	7.9	5.1	0.21	0.20	0.005	0.049	0.005	0.038
maltulose	20.1	4.7	26.0	27.2	1.9	6.2	10.7	5.4	0.17	0.17	0.010	0.056	0.006	0.061

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Disaccharide	Intensity (% *m/z* 361 ion)													
	103	**104**	**205**	**206**	**307**	**308**	**319**	**320**	**409**	**410**	**595**	**596**	**685**	**686**
	13.6	4.4	28.1	20.0	1.9	8.9	11.9	5.9	0.21	0.53	0.008	0.031	0.012	0.058
maltose	9.6	4.6	19.5	16.1	2.1	8.4	10.3	6.7	0.21	0.48	0.007	0.028	0.008	0.055
kojibiose	25.9	7.0	40.6	14.1	13.8	5.7	34.3	39.0	0.26	0.12	0.010	0.144	0.003	0.066
gentibiose	21.5	11.9	56.4	50.2	3.7	14.4	9.7	53.6	0.34	0.66	0.014	0.033	0.154	0.112
palatinose	21.4	8.1	32.8	41.6	2.8	14.1	9.6	90.1	0.50	0.51	0.017	0.042	0.040	0.029
	26.7	10.5	44.3	50.5	3.5	22.2	10.7	79.9	0.43	1.24	0.017	0.019	0.040	0.033
isomaltose	23.1	12.4	42.6	49.8	4.0	21.6	9.8	78.7	0.44	1.23	0.017	0.021	0.045	0.036

Table A2.3 Intensity of selected ions in *O*-trimethylsilyl disaccharide alditols relative to *m/z* 73 ion

Disaccharide	Intensity (% *m/z* 73 ion)													
	103	**104**	**205**	**206**	**307**	**308**	**319**	**320**	**409**	**410**	**595**	**596**	**685**	**686**
sucrose	24.4	2.3	10.4	3.3	1.1	0.3	11.5	4.6	0.03	0.01	0.011	0.006	0.003	0.002
trehalose	28.9	2.8	17.6	5.4	1.8	0.5	19.9	6.5	0.11	0.04	0.004	0.003	0.002	0.002
cellobiose	22.4	11.1	76.7	45.7	3.3	8.8	17.2	9.3	0.35	0.53	0.019	0.602	0.017	0.479
laminaribiose	24.7	5.6	84.1	51.1	8.8	2.9	17.4	15.4	0.29	0.19	0.013	0.221	0.013	0.332
nigerose	28.7	5.9	62.1	35.3	10.1	3.7	19.3	16.3	0.69	0.44	0.014	0.075	0.015	0.198
turanose	32.0	4.0	75.4	42.0	12.3	4.3	23.4	15.9	0.65	0.42	0.009	0.066	0.001	0.170
	31.9	3.8	63.2	32.6	10.6	4.0	16.3	10.4	0.43	0.41	0.011	0.102	0.010	0.078
maltulose	35.9	8.5	46.6	48.6	3.3	11.0	19.1	9.7	0.30	0.31	0.018	0.100	0.010	0.110
	29.3	9.5	60.8	43.3	4.1	19.2	25.6	12.9	0.46	1.14	0.018	0.067	0.025	0.125
maltose	25.5	12.2	51.6	42.7	5.6	22.1	27.3	17.8	0.55	1.28	0.019	0.074	0.022	0.145
kojibiose	33.3	9.0	52.1	18.1	17.7	7.3	44.1	50.2	0.34	0.15	0.012	0.185	0.004	0.085
gentibiose	22.2	12.3	58.1	51.8	3.8	14.8	9.9	55.3	0.35	0.68	0.015	0.034	0.158	0.116
palatinose	28.1	10.6	42.9	54.4	3.7	18.4	12.6	117.9	0.66	0.67	0.023	0.056	0.053	0.038
	29.2	11.5	48.4	55.2	3.8	24.3	11.7	87.3	0.47	1.35	0.018	0.021	0.044	0.036
isomaltose	26.4	14.2	48.6	56.9	4.6	24.7	11.2	89.9	0.50	1.41	0.020	0.024	0.051	0.041

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A2.2 Reproducibility

Table A2.4 Integrated area of five consecutive runs of selected ions in *O*-trimethylsilyl cellobiitol

Ion (*m/z*) Area							
73	**205**	**206**	**307**	**308**	**361**	**319**	**320**
337720719	189300337	118220296	4012099	10678957	84247440	18897509	9932521
330576030	186130856	115995408	4040974	10769559	87328361	19134667	10133679
325618035	183167044	114155730	4027288	10940241	91183170	19674193	10833649
313685783	179465956	112308244	4062593	11348121	94625526	20825740	11193905
306609583	178821965	112930612	4173944	11731716	93263288	21438557	11342154

Table A2.5 Reproducibility of the relative intensity of selected ions in *O*-trimethylsilyl disaccharide cellobiitol

	Intensity (% 73 *m/z* ion)				Intensity (% 361 *m/z* ion)	
	307	308	319	320	205	206
	1.2	3.2	5.6	2.9	224.7	140.3
	1.2	3.3	5.8	3.1	213.1	132.8
	1.2	3.4	6.0	3.3	200.9	125.2
	1.3	3.6	6.6	3.6	189.7	118.7
	1.4	3.8	7.0	3.7	191.7	121.1
μ	1.3	3.4	6.2	3.3	204.0	127.6
σ	0.1	0.3	0.6	0.3	14.8	8.9
%CV	5.4	7.9	9.5	9.7	7.3	7.0

μ = arithmetic mean, σ = standard deviation, CV = coefficient of variation

Appendix 3

Extractable Organic Substances from New Zealand Honeys

A3.1 Calibration Graphs of Class Compound Standards

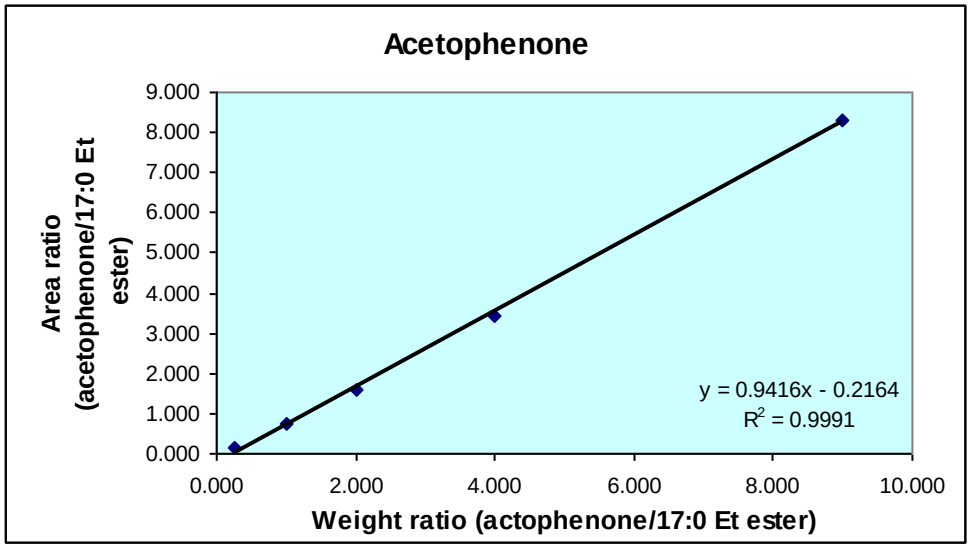


Figure A3.1 Calibration graph of acetophenone relative to heptadecanoic acid ethyl ester

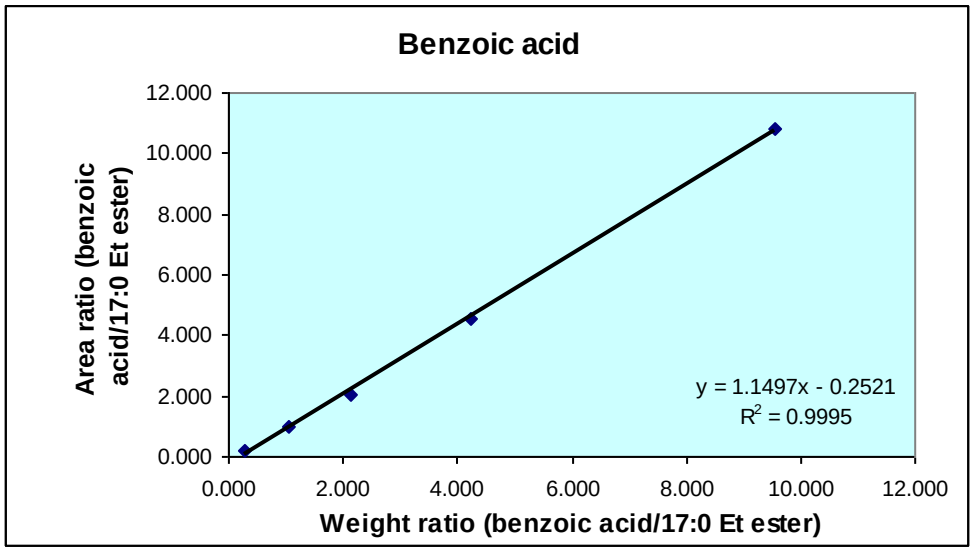


Figure A3.2 Calibration graph of benzoic acid relative to heptadecanoic acid ethyl ester

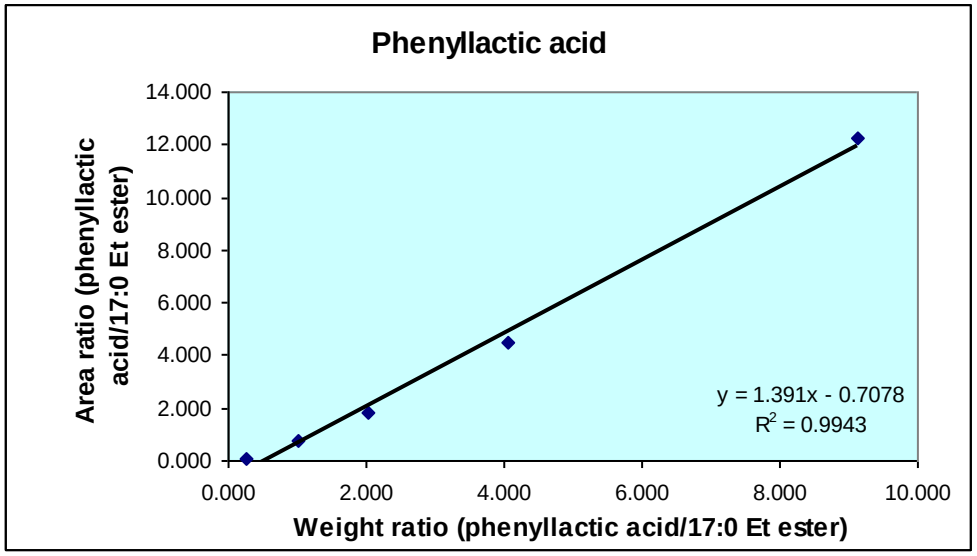


Figure A3.3 Calibration graph of phenyllactic acid relative to heptadecanoic acid ethyl ester

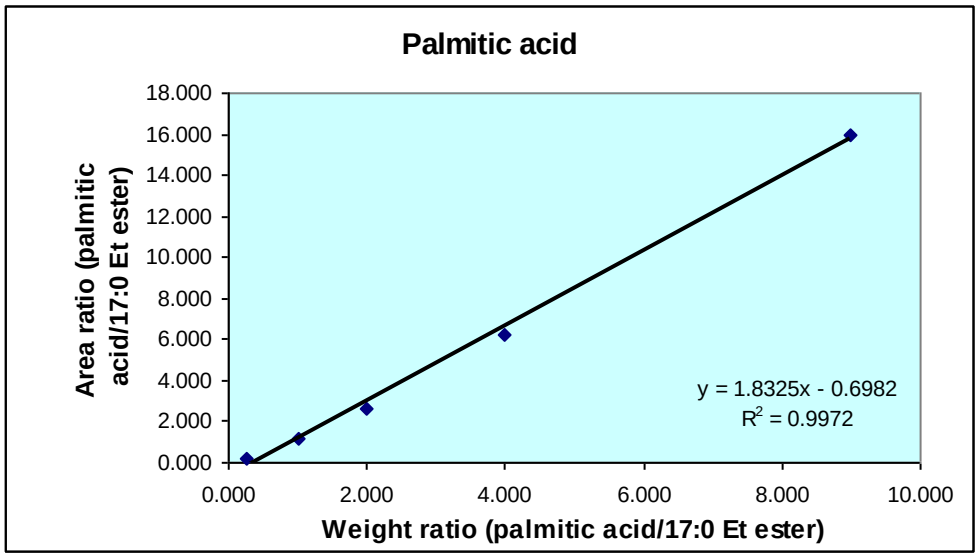


Figure A3.4 Calibration graph of palmitic acid relative to heptadecanoic acid ethyl ester

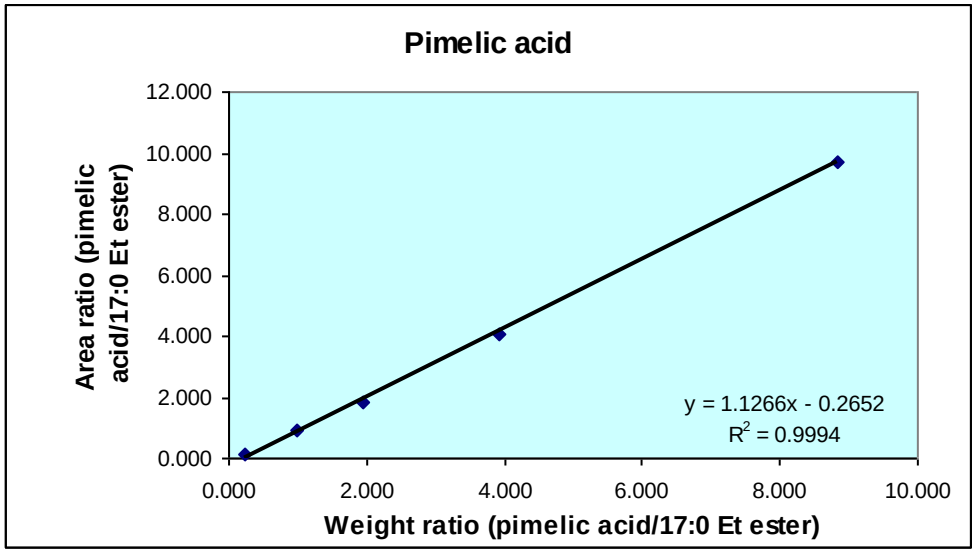


Figure A3.5 Calibration graph of pimelic acid relative to heptadecanoic acid ethyl ester

A3.2 Mass Spectrum of Identified Compound

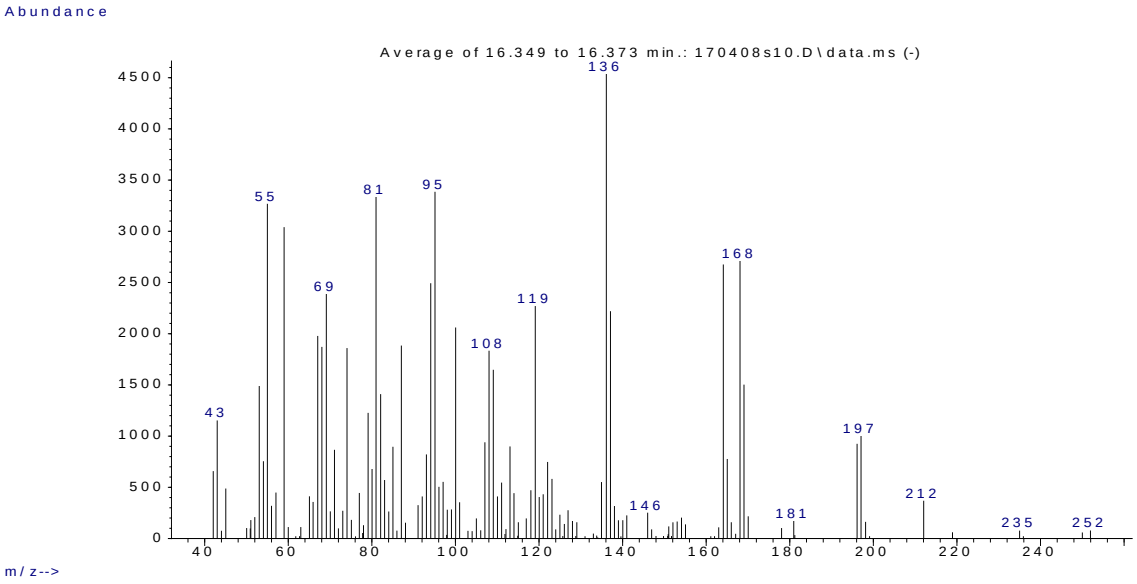


Figure A3.6 Mass spectrum of methylated decene-2-dioic acid identified by comparison to the spectrum reported by Tan[3]

[3] Tan, S.-T. A chemical investigation of some New Zealand honeys. Ph.D. Thesis, University of Waikato, Hamilton, 1989.

Appendix 4

Evaluation of Floral Origin by NIR

A4.1 Floral Origin Composition Data

Table A4.1 Pollen, colour, moisture, carbohydrate and HMF data of 100 unifloral honey set supplied by Airborne Honey Ltd.

Sample no.	Honey	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})	Main Pollen	Pollen %	Total Pollen	Fructose %	Glucose %	Sucrose %	Maltose %	HMF %
17470	RW	91	14.5		RW	2.40	323,250	36.89	30.07	1.86	4.51	0.27
17329	RW	85	15.7		RW	30.80	56,600	28.99	23.88	6.38	3.03	1.00
17503	RW	84	15.8		RW	17.80	78,300	35.19	28.70	2.23	4.02	0.74
17367	RW	81	16.2		RW	16.40	188,350	33.23	27.05	4.01	2.17	1.00
17616	RW	77	16.7		RW	4.20	229,900	35.46	29.27	0.75	3.37	0.89
17512	RW	90	15.6		RW	2.60	139,950	34.91	28.85	1.70	3.55	0.89
19777	RW	78	17.0		RW							
19594	RW	83	16.9		RW							
18042	RW	84	16.4		RW	14.10	343,300	34.94	28.34	0.54		0.42
18038	RW	85	17.2		RW	7.20	268,250					
18994	TW	15	17.9		TW	7.70	29,950	40.61	31.56	0.72	2.05	0.01
18995	TW	19	17.5		TW	2.50	53,250	41.36	32.61	0.71	3.18	0.01
18996	TW	20	17.6		TW	2.80	26,600	41.61	32.05	1.18	2.36	0.01
19119	TW	24	19.8		TW	10.10	64,900	38.27	29.89	0.16	1.40	0.01
19382	TW	25	17.4		TW	12.20	81,600					
19381	TW	28	17.4		TW	10.00	101,600					
16401	TW	41	17.7		TW	2.20	356,600	40.58	33.64	0.01	1.03	
20048	TW	40	16.9		TW							
18236	TW	43	16.9		TW							2.01
19125	TW	27	18.5		TW	8.90	44,950	38.64	30.06	0.12	2.39	0.01
18968	K	24	17.7		K	74.10	143,250	37.03	33.08	6.83	2.79	0.01
19113	K	36	18.5		K	78.50	114,900	37.04	30.69	0.45	3.28	0.01
19112	K	29	18.1		K	83.60	139,950	35.50	30.28	2.07	3.24	0.01
17411	K	39	17.1		K	23.70	219,950	36.38	32.69	2.57	1.75	1.00
17423	K	33	17.1		K	70.80	246,600	33.08	28.58	6.22	4.02	0.21
17407	K	34	17.2		K	24.10	243,250	37.28	33.22	2.20	3.66	0.01
17602	K	24	16.7		K	70.00	156,600	36.19	33.12	0.49	1.16	0.58
17513	K	22	17.1		K	75.70	231,600	35.12	33.10	0.01	1.21	0.44

Sample no.	Honey	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})	Main Pollen	Pollen %	Total Pollen	Fructose %	Glucose %	Sucrose %	Maltose %	HMF %
17489	K	25	17.7		K	66.80	279,950	36.58	33.62	0.01	1.12	0.61
19702	K	37	16.8		K	66.20	174,950	41.98	36.42	0.22	2.41	
19036	M	71	18.7	6.97	M	82.00	321,600	38.00	29.70	0.61	3.09	0.69
19037	M	71	18.7	6.94	M	92.90	458,250	38.00	30.73	0.65	2.76	1.23
19786	M	85	17.9	6.21	M	86.10	586,600					
19589	M	84	18.3	5.27	M	82.80	546,600					
19131	M	86	17.5	6.39	M	74.60	466,600	38.28	28.04	0.15	2.98	0.98
19026	M	66	17.3	7.46	M	44.60	176,600	38.71	29.83	0.01	2.79	
19943	M	86	19.2	5.12	M	88.50	599,950					
20036	M	72	17.3		M	92.60	558,250					
19986	M	87	17.8		M	78.60	933,250					
18344	M	81	19.7	5.92	M	79.30	599,950	36.64	28.50	0.01	2.14	0.01
20073	HD	90	16.1	12.46	N/A		358,250					
18445	HD	64	17.3	8.53	N/A		163,300	34.68	24.84	0.01	3.85	0.79
17404	HD	95	14.7	12.52	N/A		159,950	34.14	22.43	0.50	4.73	1.00
17557	HD	82	16.5	11.61	N/A			33.84	21.85	4.51	4.51	0.01
17555	HD	96	15.2	10.77	N/A		168,300	34.16	25.44	0.01	1.30	0.01
18661	HD	72	18.6	10.55	N/A		109,950	35.14	23.51	0.17	4.30	0.91
18712	HD	71	16.6	12.25	N/A		204,600	32.24	19.42	0.71	4.09	0.83
18327	HD	78	15.8	10.73	N/A		189,950	34.44	19.29	0.57	3.84	0.69
17931	HD	91	15.8	9.48	N/A		116,600	34.64	24.70	0.23	6.89	0.01
18957	C	19	18.1		C	81.90	71,600	37.68	32.80	1.31	2.45	0.01
19100	C	25	16.2		C			38.78	31.09	2.30	3.24	1.46
19064	C	12	17.7		C	83.40	124,950	38.50	32.35	1.83	2.95	0.01
19068	C	3	16.6		C	91.80	188,300	39.03	31.91	3.47	3.43	0.01
18666	C	15	16.8		C	70.40	103,250	38.24	30.67	0.85	3.32	1.44
17836	C	12	18.4		C	86.20	86,600	35.23	29.81	0.71	2.39	1.69
19104	C	26	17.6		C			38.01	31.97	0.24	2.75	0.20
19135	C	38	17.2		C	61.30	288,300	38.05	29.60	0.62	3.04	0.01
19252	C	5	16.4		C	90.30	91,600	39.56	32.22	0.91	3.28	0.01

Sample no.	Honey	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})	Main Pollen	Pollen %	Total Pollen	Fructose %	Glucose %	Sucrose %	Maltose %	HMF %
19303	C	29	17.9		C			39.05	29.86	0.01	2.49	0.01
17431	TH	67	16.5		TH	14.40	306,600	37.05	28.86	0.01	3.59	0.21
17438	TH				TH	9.70	346,600	35.84	28.90	0.01	2.93	0.01
17437	TH				TH	6.10	469,950	35.41	31.78	0.01	2.07	0.01
18915	TH	59	16.1		TH	10.40	138,250					0.12
18916	TH	55	16.1		TH	17.20	93,300					0.23
18923	TH				TH	16.20	46,600					
19818	TH	64	16.2		TH							
18477	TH	77	16.2		TH			38.98	32.31	0.01	2.91	0.91
16144	TH	58	16.9		TH	41.40		33.60	26.78	0.01	1.17	
16976	TH	87	18.0		TH	15.60	134,950					
19778	B	25	17.3		B	87.80	164,950	33.61	27.14	5.56	4.05	
18967	B	14	16.8		B	56.10	88,250	38.50	31.74	5.13	3.15	1.69
18966	B	13	17.1		B	77.90	83,250	36.68	30.25	7.68	3.08	0.92
19470	B	18	16.8		B	62.00	229,950	36.17	38.67	4.30	3.14	
19520	B	19	17.0		B	87.00	94,950	32.93	25.91	8.55	3.34	
19430	B	23	16.6		B	51.80	46,600	36.49	29.47	3.84	3.99	
19799	B	21	15.5		B	78.70	98,250	31.93	26.45	10.47	4.04	
18175	B	11	17.8		B	55.90	76,600	34.98	29.06	4.82	2.98	0.96
19293	B	27	17.3		B	53.50	71,600	37.73	31.01	1.30	3.45	4.98
19968	B	28	16.7		B		111,600	36.45	30.02	3.68	3.70	
19258	NT	2	17.5		NT			37.01	29.23	4.20	3.04	1.41
19264	NT	8	18.4		NT			38.78	29.90	5.78	3.94	2.41
19292	NT	2	20.1		NT							0.01
17804	NT	14	17.5		NT	1.10	14,900	37.97	28.83	3.57	2.62	3.00
17802	NT	19	16.7		NT	0.50	26,600	38.24	31.01	1.67	3.00	2.35
19472	NT	7	17.1		NT			38.76	31.20	1.24	2.71	
19567	NT	7	19.0		NT			38.19	30.67	1.94	2.77	
19260	NT	8	17.1		NT			36.73	28.33	4.70	2.59	2.22
17787	NT	16	16.4		NT			38.09	29.49	1.22	2.19	16.96

Sample no.	Honey	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})	Main Pollen	Pollen %	Total Pollen	Fructose %	Glucose %	Sucrose %	Maltose %	HMF %
19308	NT	8	18.6		NT			38.12	30.19	2.28	2.84	3.47
19186	R	13	18.1		R		144,950	36.34	31.55	1.54	1.59	0.01
18431	R	11	18.4		R	77.50	164,900	38.59	35.86	0.01	0.95	1.05
18429	R	11	18.9		R	66.30	148,300	38.03	36.47	0.03	0.81	1.38
19712	R	19	18.9		R	84.00	14,160	39.70	38.12	1.48	0.46	
18289	R	13	17.7		R	31.80	89,950					
19453	R	20	17.9		R							
19647	R	14	18.1		R							
18174	R	12	19.0		R	76.00	131,600	35.42	35.10	0.60	1.32	2.83
18291	R	17	17.9		R	23.90	201,600					0.36
19611	R	19	17.9		R	53.60	224,950					
19445	R	21	19.4		R	63.50	109,900	40.22	36.68	0.44	1.22	

RW = rewarewa, TW = tawari, K = kamahi, M = manuka, HD = honeydew, C = clover, TH = thyme, B = vipers' bugloss, NT = nodding thistle, R = rata

Table A4.2 Pollen, colour, moisture, carbohydrate and HMF data of 345 unifloral honey set supplied by Airborne Honey Ltd.

Sample no.	Honey	1st Pollen	1st Pollen%	2nd Pollen	2nd Pollen %	3rd Pollen	3rd Pollen %	Total Pollen	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})
19317	B	B	72.6	MG	15.4	C	7	99950	25	15.6	
19409	B	B	64.5	C	32.6	OT	1.4	56600	7	16.9	
19410	B	B	69.4	C	14.2	MG	8.3	61600	8	17.4	
19411	B	B	76.6	C	20.9	OT	2.5	59950	8	17.4	
19412	B	B	75.4	C	15.9	MG	3.6	64900	8	17.2	
19430	B	B	51.8	C	32.4	OT	5.9	46600	23	16.6	
19431	B	B	57.3	C	24.3	MG	11	94950	22	16.4	
19470	B	B	62	C	36.8	OT	1.2	229950	18	16.8	
19520	B	B	87	MG	6.1	C	4.6	94950	19	17	
19521	B	B	87.9	C	4.7	MG	4	119900	17	16.9	

Sample no.	Honey	1st Pollen	1st Pollen%	2nd Pollen	2nd Pollen %	3rd Pollen	3rd Pollen %	Total Pollen	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})
19522	B	B	93	C	2.3	MG	2	174900	21	17.2	
19523	B	B	92.9	C	3.6	M	2.4	139950	20	17.4	
19531	B	B	50.6	C	47.1	OT	1.2	199950	11	17	
19566	B	B	57.3	C	24.3	MG	11	94950	22	16.4	
19602	B	B	55.4	C	39.2	MG	1	166600	17	16.3	
21286	B	B	84.5	C	10		0	159950	16	17.2	
22164	B	B	54	C	41.5	W	1.5	62500	15	16.1	
22165	B	B	71	C	29		0	15000	15	16.4	
22337	B	B	54.5	C	42.3	OT	3.2	93300	18	16	
22406	B	B	70.3	C	14.4	OT	12.7	76600	18	15.8	
22407	B	B	72.5	C	22.5	OT	3.3	53300	16	16.9	
22408	B	B	79.3	C	14.3	W	2.5	74950	14	16.5	
19516	CA	CA	2.4	C	25.4	OT	22.8	263250	66	17.2	
19596	CA	CA	20.9	L	27.3	M	23.3	399950	97	18.5	
19170	CD	C	65.7	M	18.3	OT	12	209900	45	17.8	
19172	CD								37	17.8	
19296	CD								61	17	
19299	CD								41	17.5	
19300	CD								37	17.6	
19318	CD								44	19.1	
19327	CD								39	19.2	
19328	CD								35	18.6	
19333	CD								36	18.5	
19334	CD								49	17.5	
19388	CD								45	16.9	
19390	CD								37	16.8	
19391	CD								36	16.8	
19393	CD								38	17.7	
19402	CD								38	17.9	
19403	CD								39	17.9	

Sample no.	Honey	1st Pollen	1st Pollen%	2nd Pollen	2nd Pollen %	3rd Pollen	3rd Pollen %	Total Pollen	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})
19404	CD								43	17.6	
19439	CD								36	17.8	
19462	CD	C	88.5	OT	6.6	K	2.5	36600	58	18	
19463	CD	C	72.2	OT	15.7	L	7.4	61600	66	18.5	
19517	CD								37	16.4	
19599	CD								35	18	
19610	CD								36	17.1	
19628	CD								36	17	
19629	CD								37	17.1	
19630	CD								36	17	
19648	CD								40	17	
19651	CD								41	17.1	
19652	CD								40	17	
19655	CD								42	17	
19656	CD								41	17.1	
19657	CD								51	16.7	
19659	CD								37	16.7	
19660	CD								54	16.9	
19661	CD								48	17	
19684	CD							48300	37	16	
19691	CD								36	18.4	
19692	CD								36	18.4	
19693	CD								37	18.4	
19695	CD								36	18.2	
19696	CD								35	18.4	
19699	CD								41	18.4	
19727	CD	C	37.8	M	31.5	OT	14.7	299950	88	16.9	
19728	CD	C	72.4	OT	13.3	M	6.7	268250	77	17.3	
22826	CD								42	16.8	
19185	CL	C	72.8	K	22.8	M	2.5	103250	12	17.5	

Sample no.	Honey	1st Pollen	1st Pollen%	2nd Pollen	2nd Pollen %	3rd Pollen	3rd Pollen %	Total Pollen	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})
19191	CL	C	68.4	M	28.8	OT	1.8	106600	13	17.2	
19192	CL	C	70.5	M	25.5	OT	2.7	88250	10	17.1	
19269	CL	C	91.1	OT	5.2	L	1.5	146600	19	17.8	
19311	CL	C	88.4	K	4.4	OT	4.4	98300	17	16.8	
19315	CL	C	75.2	W	12.4	OT	6.6	223250	15	17.6	
19345	CL	C	88.1	OT	4.6	B	3.7	51600	14	19.2	
19477	CL	C	81.3	OT	8.6	K	7.2	113300	17	17.5	
19488	CL	C	93.3	OT	2.4	DN	1.4	126600	16	17.1	
19530	CL							134950	16	16.8	
19556	CL								18	18.1	
19557	CL								19	18.1	
19570	CL								12	16	
19572	CL	C	70	B	28.1	MG	0.7	246600	14	16.3	
19573	CL	C	67.7	B	14.2	OT	9.5	104900	17	16.7	
19574	CL	C	72.8	B	26.1	OT	1.1	158250	13	16.3	
19607	CL								10	15.7	
19624	CL	C	93.7	OT	2.4	NT	0.8	36600	15	16.9	
20242	CL	C	79.7	BA	14.3	OT	5.3	129950	14	16.4	
21636	CL							169900	15	16	
21952	CL	C	88.3	B	5.3	W	1.9	149950	17	18	
22336	CL	C	75.2	B	21.9	OT	2.9	111600	14	15.9	
22778	CL	C	96.3	OT	1.9	L	0.6	34950	15	16.2	
19173	CM	C	77.9	BA	7.2	OT	7.2	76600	29		
19283	CM								24	16.6	
19301	CM								25	16.2	
19302	CM								33	16.3	
19316	CM								27	17.6	
19326	CM								34	18.9	
19329	CM								28	18.1	
19330	CM								31	18	

Sample no.	Honey	1st Pollen	1st Pollen%	2nd Pollen	2nd Pollen %	3rd Pollen	3rd Pollen %	Total Pollen	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})
19341	CM								23	16.2	
19343	CM								25	18.3	
19344	CM								27	18.7	
19346	CM								23	17.9	
19386	CM								31	16.2	
19389	CM								31	16.9	
19392	CM								33	17	
19394	CM								28	17.8	
19405	CM								29	17.1	
19446	CM								34	17.8	
19447	CM								29	17.4	
19448	CM								29	17.3	
19449	CM								34	16.9	
19454	CM								23	17.3	
19455	CM								25	17.4	
19480	CM								20	16.5	
19481	CM								33	17.4	
19482	CM								33	17.3	
19483	CM								33	17.8	
19484	CM								28	17.7	
19485	CM								26	17.8	
19486	CM								27	17.2	
19487	CM								26	17.2	
19490	CM								24	16.5	
19527	CM	C	65.7	B	14.5	W	9.5	244950	24	16	
19528	CM							178280	20	16.6	
19529	CM	C	65.2	W	15.2	OT	8.2	158250	23	16.5	
19555	CM								29	18.4	
19569	CM								20	17.4	
19597	CM								26	17.4	

Sample no.	Honey	1st Pollen	1st Pollen%	2nd Pollen	2nd Pollen %	3rd Pollen	3rd Pollen %	Total Pollen	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})
19598	CM								21	16.7	
19600	CM								26	16.6	
19604	CM								25	17.3	
19605	CM								22	16.9	
19606	CM								27	17	
19627	CM								38	16.7	
19631	CM								34	17.2	
19732	CM								33	17.6	
19733	CM								33	17.6	
19734	CM								33	17.8	
19735	CM								31	17.7	
21399	CM	C	82.7	K	8.4	OT	4.5	173250	23	18.1	
21639	CM								20	15.8	
21641	CM								33	15.9	
21717	CM	C	80.9	OT	8.1	L	4.4	353250	28	18	
21947	CM	C	87.5	BA	9.7	OT	2.1	143250	23	17.2	
22594	CM		0					0	20	17.9	
19184	CX	C	77.1	K	16.9	OT	4.5	78300	7	17	
19189	CX	C	82.2	M	16.4	L	0.7	74950	4	16.6	
19193	CX							73250	9	16.6	
19194	CX	C	96.4	OT	2.2	NT	0.7	53300	5	16.7	
19195	CX	C	91.4	RC	3.4	BA	2.3	56600	9	16.7	
19196	CX	C	95.6	OT	1.6	RC	1.1	74900	4	16.5	
19197	CX	C	94	OT	3	L	1.5	71600	3	16.5	
19271	CX	C	84.3	L	12.6	DN	2.4	131600	8	18.2	
19342	CX	C	86.5	B	4.8	OT	4.3	108250	9	17	
19471	CX	C	89.8	OT	5.6	B	4.6	83250	6	17.2	
19576	CX	C	91.5	B	3.8	OT	2.3	64900	4	17.8	
19592	CX								3	16.3	
19593	CX								7	15.4	

Sample no.	Honey	1st Pollen	1st Pollen%	2nd Pollen	2nd Pollen %	3rd Pollen	3rd Pollen %	Total Pollen	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})
19662	CX								5	14.6	
19663	CX								6	14.3	
19664	CX								5	15	
19683	CX	C	89	B	8.8	OT	2.2	68300	7	18.4	
21872	CX	C	92.2	BA	3.5	A	2.6	138300	7	17.1	
22693	CX	C	81.5	L	12.7	M	2.1	169950	5	16.5	
22694	CX	C	83.4	L	10.6	DN	10	233250	8	17.1	
19181	HD							68250	71	14.8	10.81
19609	HD								77	16.5	8.78
19686	HD								67	16.5	11.45
19687	HD								61	17.1	9.67
20166	HD							51600	89	16.1	11.89
20677	HD								92	16.6	8.7
20678	HD								91	16.9	9.81
20679	HD								87	17.1	10.24
20680	HD							203300	91	17.6	9.78
20681	HD								79	17.4	9.55
20682	HD								72	17.1	9.94
20684	HD								91	17.3	10
20686	HD								93	17.4	10.3
21583	HD	C	52.4	M	23.2	OT	22	39950	82	15.8	11.55
21918	HD							96600	90	15.3	11.78
21919	HD							93250	87	15.1	12.71
22308	HD							71600	85	15.9	11.81
22561	HD							99950	74	17.4	8.65
22591	HD							91600	90	16.4	10.98
22592	HD							138300	90	16.8	11.31
22803	HD								87	16.4	11.32
19374	K	K	26	L	27	OT	26.5	228250	58	16.4	
19375	K	K	35.2	OT	23.1	C	19.2	284950	48	16.9	

Sample no.	Honey	1st Pollen	1st Pollen%	2nd Pollen	2nd Pollen %	3rd Pollen	3rd Pollen %	Total Pollen	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})
19376	K	K	57.1	OT	15.8	L	11.2	171600	48	16.6	
19407	K	K	63.7	M	14.9	C	12.6	89950	67	18.1	
19703	K	K	44.6	R	50.8	QU	0.8	218250	23	16.2	
19705	K	K	51	R	45.2	QU	1.9	239950	32	16.7	
19773	K	K	73.1	C	14.9	OT	8.5	223250	41	16.8	
19774	K	K	83.3	OT	7.2	C	5	199900	43	17	
19775	K	K	78.3	W	9.2	OT	6.6	168300	45	17.6	
20233	K	K	74	R	13.5	C	4	235000	32	16.9	
20249	K	K	73.8	R	7.2	C	7.2	208300	20	16.5	
20256	K	K	85.1	C	6.5	OT	5.5	218250	26	16.4	
20318	K	K	58.8	QU	32.2	M	3.9	213300	52	19	
21398	K	K	75.1	C	18.8	M	5.2	229900	41	16.9	
21900	K	K	71.7	M	13.2	QU	5.7	189950	31	16.9	
21901	K	K	77.5	QU	18.6	C	2	129900	28	17	
22462	K	K	74.6	R	14.4	QU	2.5	184900	38	17.6	
19450	M	M	70.5	C	15.6	B	4.6	388300	83	17	7.23
19451	M	M	75.8	C	10.9	MG	4.2	463250	88	16.1	6.97
19558	M	M	74.5	B	10.5	C	10	336600	59	18	
19559	M	M	70.8	C	14.8	B	8.1	373250	62	18.5	
19638	M	L	61.3	C	16.8	K	13	506600			
19639	M	M	46.7	L	40.6	C	10	459950			
19640	M	M	47.8	L	34.4	C	14.4	319900			
19641	M	M	43.1	L	33.5	C	13.3	398300			
19642	M	M	41.5	C	39.9	L	16.9	566600			
19643	M	M	51.6	C	32.3	L	15.2	394950			
19644	M	M	29.9	C	36.2	L	33.2	606600			
20223	M	M	68.6	K	22.8	HD	3.4	211600	77	18.1	7.64
20246	M	M	84.4	C	7.3	HD	3.1	189950	76	19.1	9.2
20247	M	M	82.6	C	9.5	HD	3.3	448250	73	20	7.08
20248	M	M	91.7	C	4.5	HD	1.6	539950	83	19.7	5.8

Sample no.	Honey	1st Pollen	1st Pollen%	2nd Pollen	2nd Pollen %	3rd Pollen	3rd Pollen %	Total Pollen	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})
20288	M	M	75.5	K	18.3	C	3.4	459950	84	17.4	
20427	M	M	88.3	B	3.7	OT	3	523250	74	18.2	6.45
20428	M	M	89.4	B	3	HD	1	619950	72	17.8	6.69
20429	M	M	92.5	C	2.6	OT	2	649900	71	18.3	5.98
21584	M	M	81.8	C	6.8	MG	4.8	416600	97	18	7.26
21619	M	M	62.8	L	19.3	C	10.8	309900	74	19.5	
22211	M	M	82.5	B	9.5	C	5.5	340000	85	20.9	4.49
22220	M	M	92.5	C	2	HD	1.5	605000	92	17.5	5.62
22303	M	M	92	C	3.1	OT	2.2	386600	97	18	6.12
22504	M	M	85.6	C	9	MG	2	369900	90	17.5	7.33
22580	M	M	46.6	L	15.5	C	14.7	836600	100	18	
22687	M	M	91.5	OT	4.1	C	3.7	673300	95	18.5	5.81
19384	MF								97	16	
19385	MF								93	17.2	
19729	MF								126	18	
20697	MF										
19253	NC								5	16.7	
19254	NC								6	16.4	
19255	NC								12	17.2	
19256	NC								4	17.4	
19257	NC								2	17.5	
19261	NC								9	17.7	
19669	NC								12	18.9	
19670	NC								11	18.1	
19671	NC								9	18.2	
19672	NC								5	17.9	
19673	NC								8	18	
19674	NC								5	18.2	
19675	NC								9	19.1	
22304	NC	NT	0.01	C	91	B	5.4	24900	10	17.4	

Sample no.	Honey	1st Pollen	1st Pollen%	2nd Pollen	2nd Pollen %	3rd Pollen	3rd Pollen %	Total Pollen	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})
22324	NC	NT	0.8	C	91.8	B	2.5	48250	13	18.7	
22392	NC	NT	0.01	C	82.8	OT	11.7	44900	7	17.6	
19259	NT								4	17.3	
19260	NT								8	17.1	
19262	NT								12	17.7	
19264	NT								8	18.4	
19287	NT								5	17.4	
19292	NT								2	20.1	
19362	NT								11	18.9	
19472	NT								7	17.1	
19567	NT								7	19	
20298	NT								16	17.8	
21018	NT								37	16.2	
21019	NT								30	15.7	
21020	NT								33	16.4	
21021	NT								29	16.3	
21029	NT								28	17.1	
21030	NT								24	16.8	
19186	R	R	47.5	K	28.5	C	9.5	144950	13	18.1	
19187	R	R	55.8	K	17.2	C	16.7	131600	13	18.2	
19188	R	R	45.7	K	27.1	C	12.6	144900	15	18.3	
19445	R	R	63.5	K	13	L	10.4	109900	21	19.4	
19706	R	R	65.6	K	28	L	5.7	256600	17	17.8	
19707	R	R	66.9	K	20.5	L	5.4	259950	19	18.7	
19708	R	R	79.1	K	14.2	L	6.7	134950	9	17.5	
19709	R	R	85.5	K	8.3	OT	2.1	131600	8	17.5	
19710	R	R	74.7	K	18.2	L	3.2	169950	11	18.7	
19711	R	R	79.5	K	17.8	C	1.4	161600	13	18.8	
20212	R	R	56.7	K	28.3	L	10.2	379900	11	17.7	
21894	R	R	62.5	K	32.5	C	1.5	201600	21	17.6	

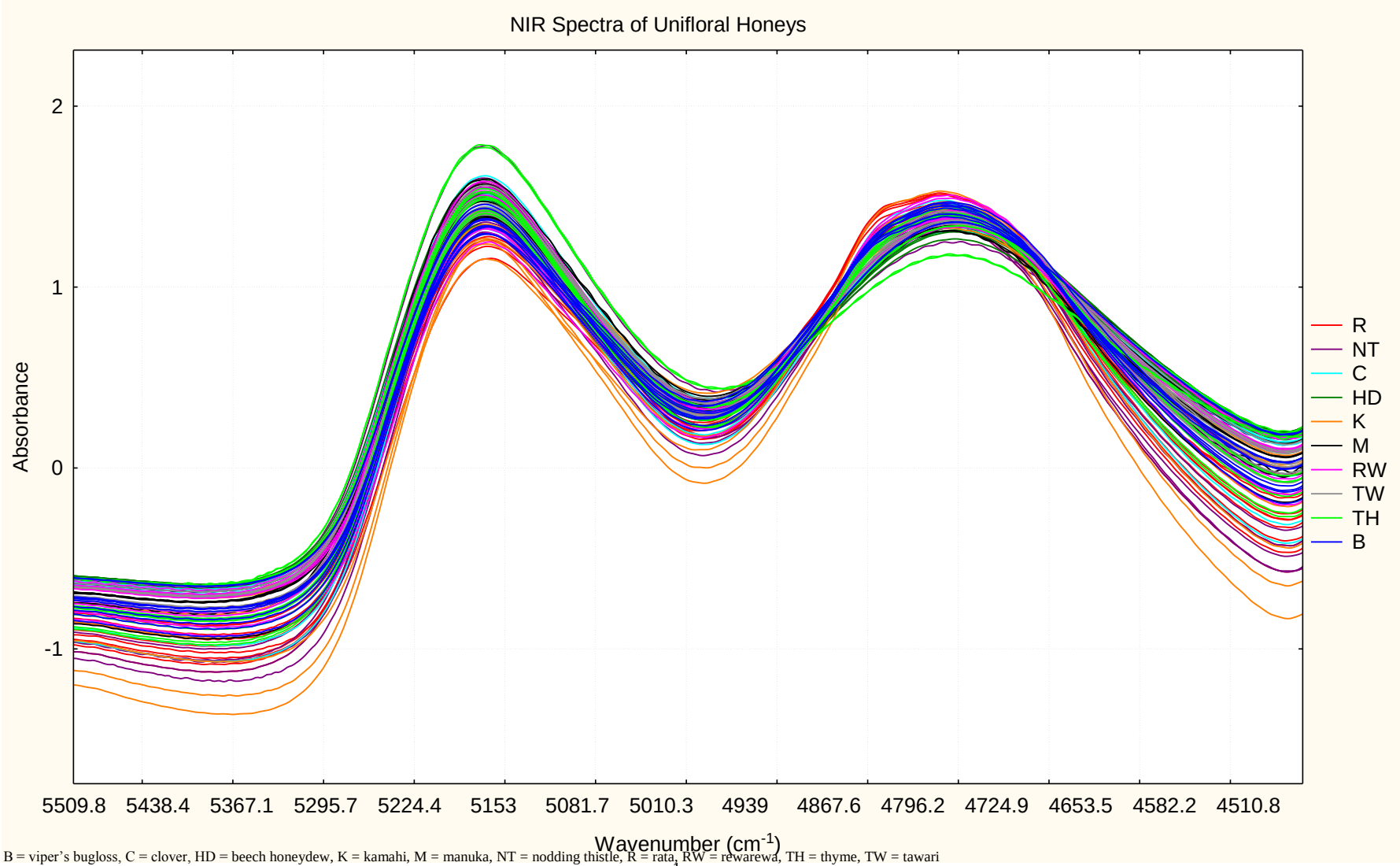
Sample no.	Honey	1st Pollen	1st Pollen%	2nd Pollen	2nd Pollen %	3rd Pollen	3rd Pollen %	Total Pollen	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})
21896	R	R	67.7	K	25	OT	3.2	176600	15	17.5	
21897	R	R	82.8	K	12.4	L	2.4	134950	12	20.1	
22222	R	R	79	K	15	M	2.5	197500	12	16.8	
22223	R	R	92	K	7	L	0.5	115500	18	18.5	
22224	R	R	59.5	K	31	L	3.5	112500	23	17.1	
22287	R	R	66.5	K	32.5	M	11	135000	23	20.2	
22288	R	R	86.5	K	8	C	3	295000	13	19.7	
22294	R	R	77.5	K	19	M	3.5	200000	12	18.3	
21017	RW	RW	2.6	C	55.2	L	33.6	709900	82	15.6	
21074	RW	RW	6.4	L	58.4	C	14.4	149900	85	16.6	
21328	RW				0		0		76	15.8	
21614	RW	RW	7.6	L	39.8	K	21.2	91600	87	18.1	
21615	RW	RW	11.1	L	44.4	C	22.9	91600	86	17.6	
21616	RW	RW	5.5	L	69.2	C	11	154900	93	17	
21617	RW	RW	6	K	52.2	M	17.2	108250	83	17.4	
21618	RW	RW	4.92	L	39.5	K	42	84950	88	17.6	
21817	RW	RW	11.6	OT	26.7	C	25.6	139900	80	17.4	
21821	RW	RW	5.4	C	37.8	L	27	228250	83	17.3	
21829	RW	RW	6.5	K	41.9	M	14.5	236600	75	17.3	
21830	RW	RW	2.1	K	51	OT	14.6	153250	86	17.3	
21833	RW								82	17.3	
21836	RW	RW	6.6	L	29.1	C	21.9	271600	74	17.9	
21850	RW	RW	38.3	OT	22.4	K	20.6	83250	88	16.2	
19622	TH								72	17	
20239	TH	TH	14.6	B	38	C	23.9	431600	77	17.7	
20240	TH	TH	7	C	43.3	B	31.2	239900	72	18.5	
20241	TH	TH	10.2	B	29.7	C	23.7	53300	70	17.8	
21883	TH	T	20.9	MG	54.3	B	11.6	81600	101	23.1	
22699	TH								82	16.9	
22696	TH								82	16.6	

Sample no.	Honey	1st Pollen	1st Pollen%	2nd Pollen	2nd Pollen %	3rd Pollen	3rd Pollen %	Total Pollen	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})
22697	TH								82	16.7	
22698	TH								85	16.9	
22699	TH								82	16.9	
19366	TW	TW	11.1	OT	43.6	C	17.1	209950	32	15.4	
19367	TW	TW	12.4	OT	45.3	C	20.4	178250	39	17.9	
19368	TW	TW	5.8	L	29.1	C	28	171600	36	17.2	
19369	TW	TW	8.2	C	35.4	OT	18.4	63300	30	17.9	
19370	TW	RW	10.4	OT	39.6	K	14.1	153300	82	16.4	
19371	TW	OT	36.8	RW	12	TW	12	199950	81	16.5	
19373	TW	L	27	OT	25	K	15.5	179950	73	17.5	
19380	TW	TW	8.4	OT	60	K	8.4	78250	27	17.4	
19381	TW	TW	10	OT	69.2	K	9.2	101600	28	17.4	
19382	TW	TW	12.2	OT	65.3	K	8.2	81600	25	17.4	
19612	TW								36	17.9	
21961	TW	TW	3.5	C	69	L	10.6	93250	28	18.9	
22195	TW	TW	5.5	K	72	C	11	13750	32	17	
22197	TW	TW	8	C	49	M	23	31250	23	17.9	
22199	TW	TW	52	C	30	L	11	73750	13	16.3	
22200	TW	TW	77	C	10.5	L	10.5	27500	10	16.7	
22201	TW	TW	42.5	C	46	K	7.5	22500	13	17	
22203	TW	TW	54.5	C	30	L	9	35625	11	16.6	
22243	TW	TW	1.5	OT	44	OT	20		33	17.8	
22244	TW	TW	34	C	26	OT	16	62500	38	17.3	
22246	TW	TW	2	OT	37.5	C	20	115750	29	18.2	
22248	TW	TW	18	C	24.5	OT	21.5	105000	17	19.5	
22253	TW	TW	17	K	32	C	22.5	65000	29	18.8	
22254	TW	TW	5.5	C	57.6	OT	27.4	79950	28	18.9	
22256	TW	TW	10.9	C	37.5	OT	29.7	66600	27	18.3	
22350	TW	TW	10	C	50.5	L	31.5	26250	52	17	
22435	TW	TW	2	K	48.7	OT	33.3	61600	35	19.9	

Sample no.	Honey	1st Pollen	1st Pollen%	2nd Pollen	2nd Pollen %	3rd Pollen	3rd Pollen %	Total Pollen	Colour (mm)	Moisture (%)	Conductivity (ohms/cm x 10^{-4})
22700	TW				0		0		35	16	
22712	TW	TW	4.6	C	80	L	8.2	96600	26	19.1	

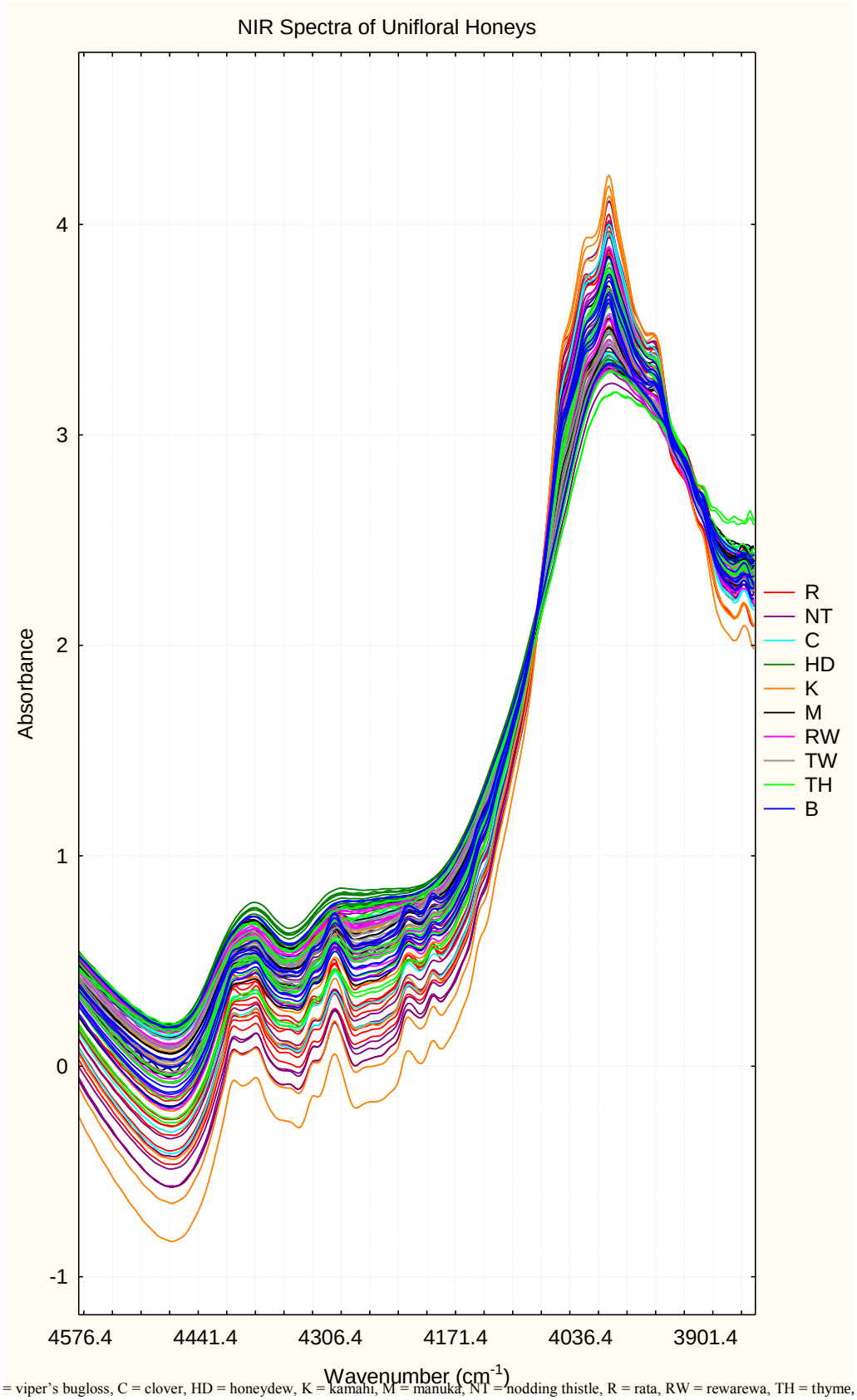
B = vipers' bugloss, CA = calluna, C = clover (divided into dark (D), medium (M), light (L) and extra light (X)), HD = beech honeydew, K = kamahi, M = manuka, MF = manufacturing grade, NC = noddy clover, NT = nodding thistle, OT = other type, R = rata, RW = rewarewa, T = thyme, TW = tawari

A4.2 NIR Spectra



B = viper's bugloss, C = clover, HD = beech honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure 4.1 NIR spectra of unifloral honeys from 5510 – 4510 cm^{-1}



B = viper's bugloss, C = clover, HD = honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure A4.2 NIR spectra of unifloral honeys from 4576 – 3900 cm^{-1}

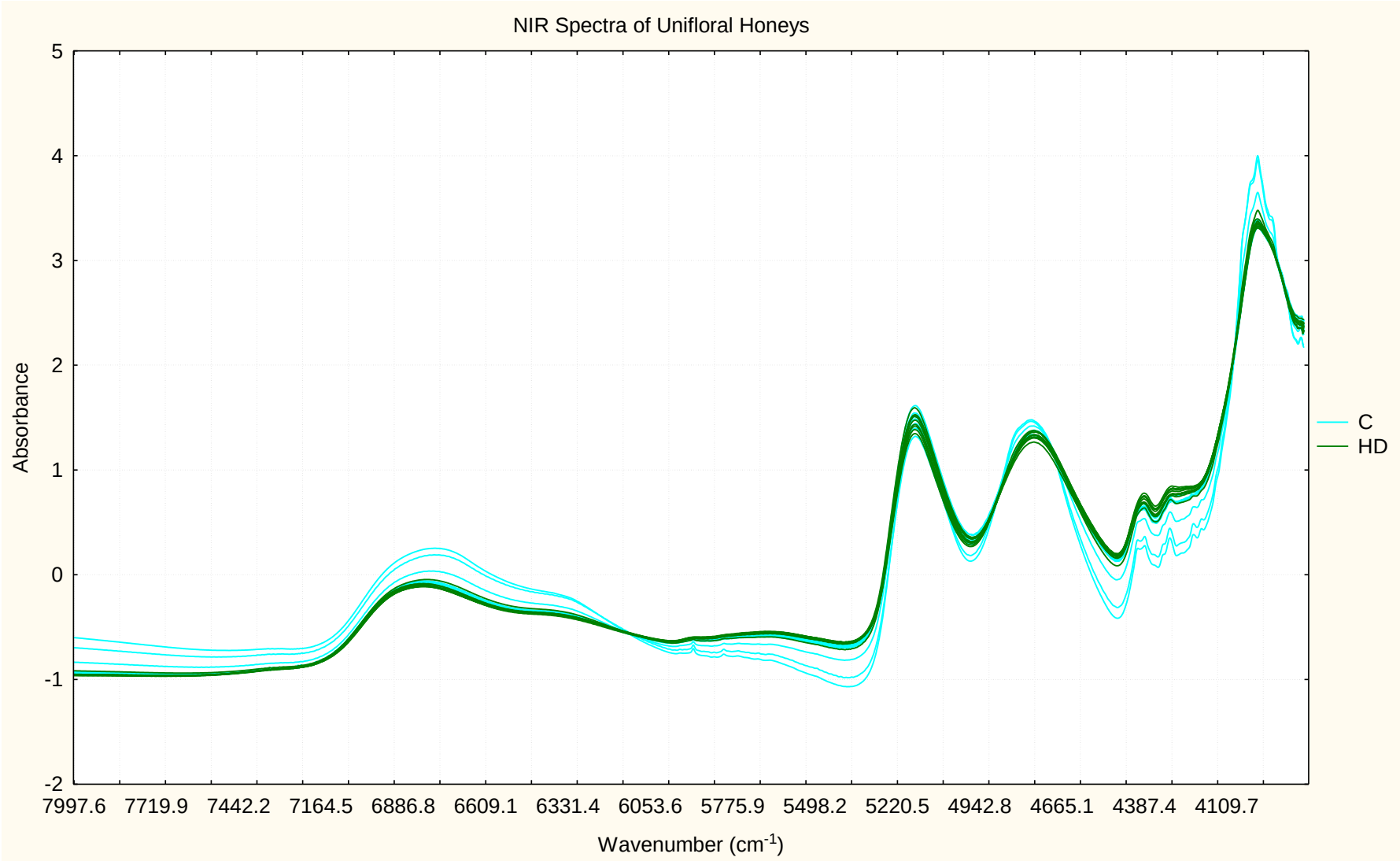


Figure A4.3 NIR spectra of clover (C) and beech honeydew (HD) honey

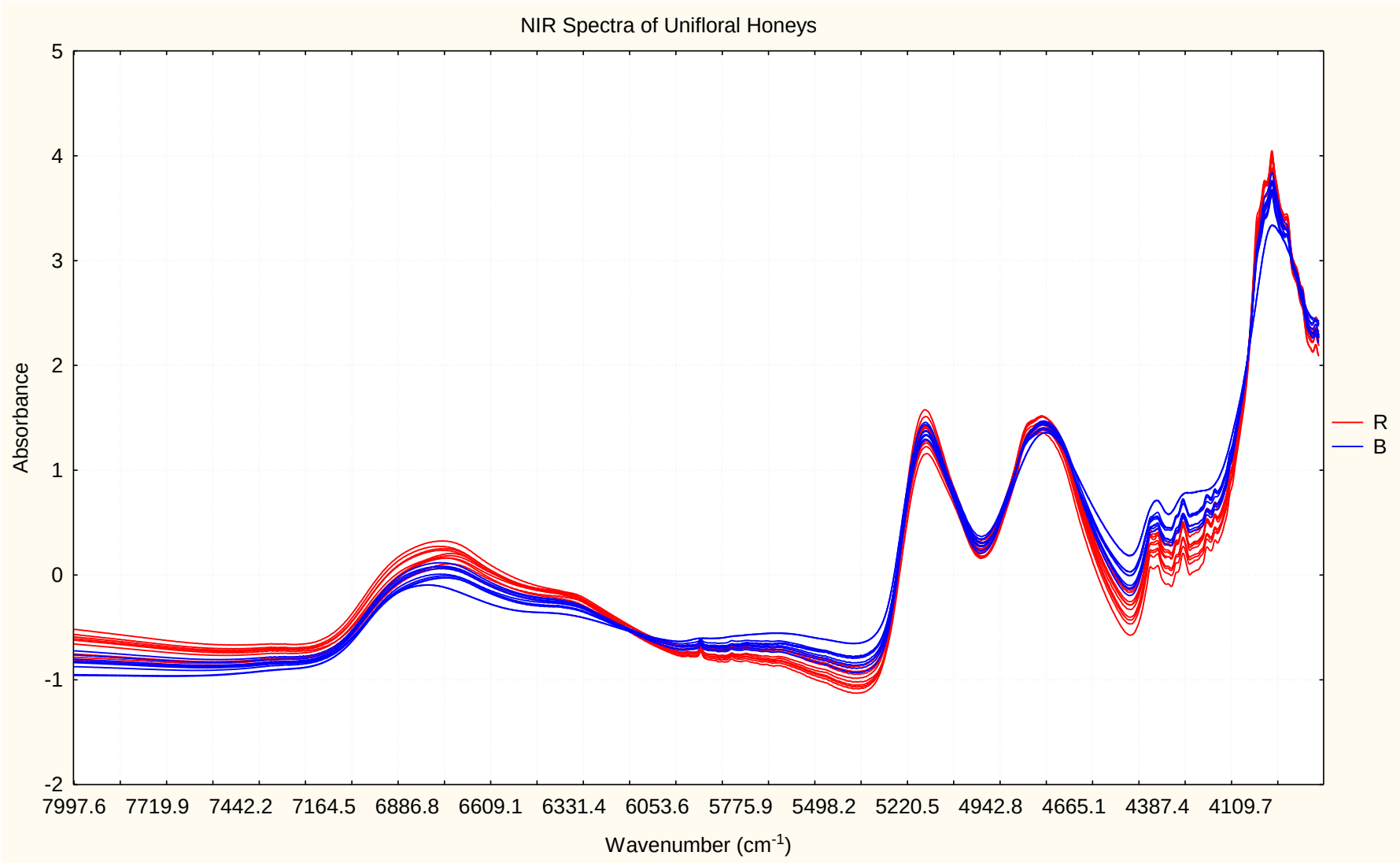


Figure A4.4 NIR spectra of rata (R) and vipers' bugloss (B) honey

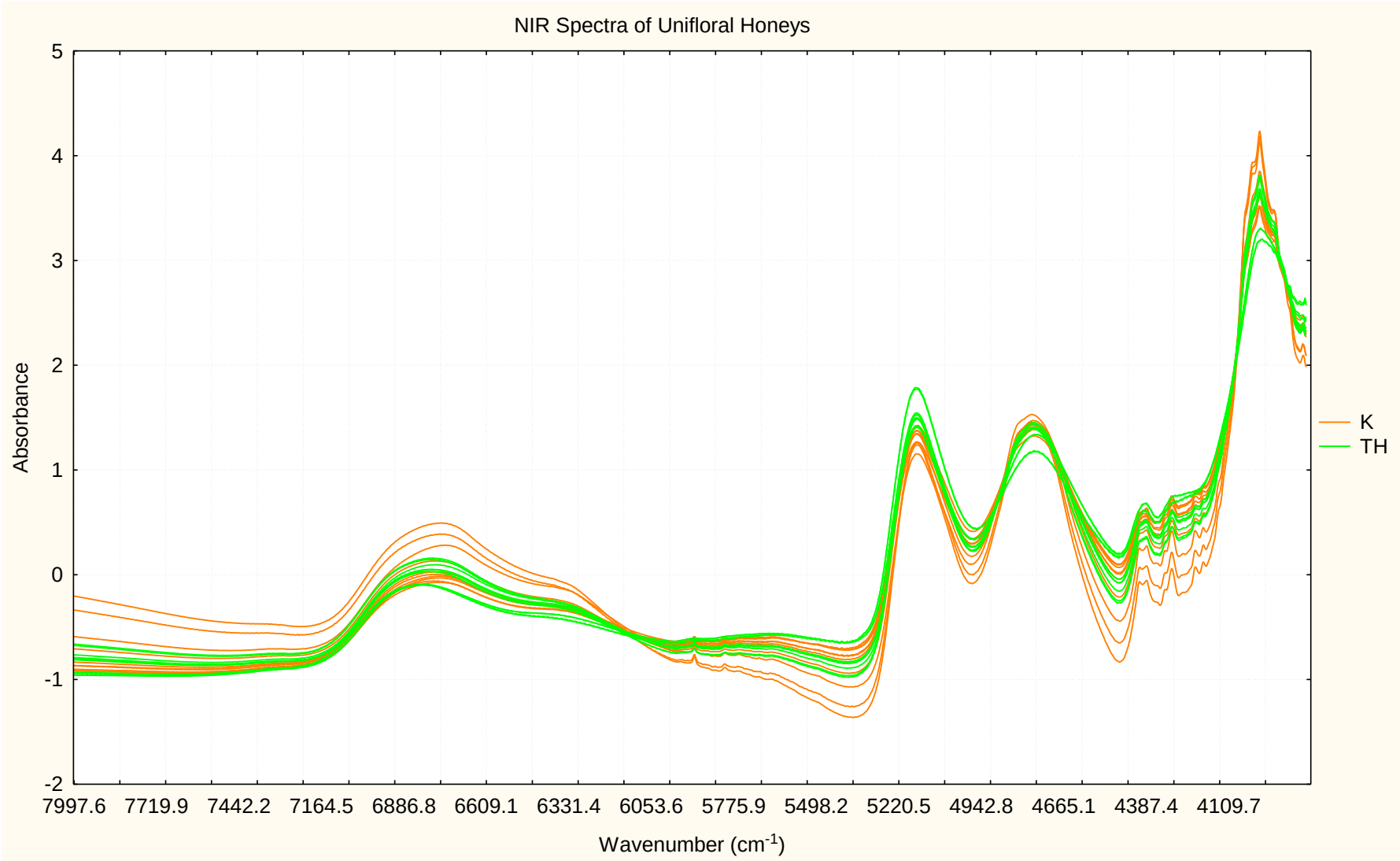


Figure A4.5 NIR spectra of kamahi (K) and thyme (TH) honey

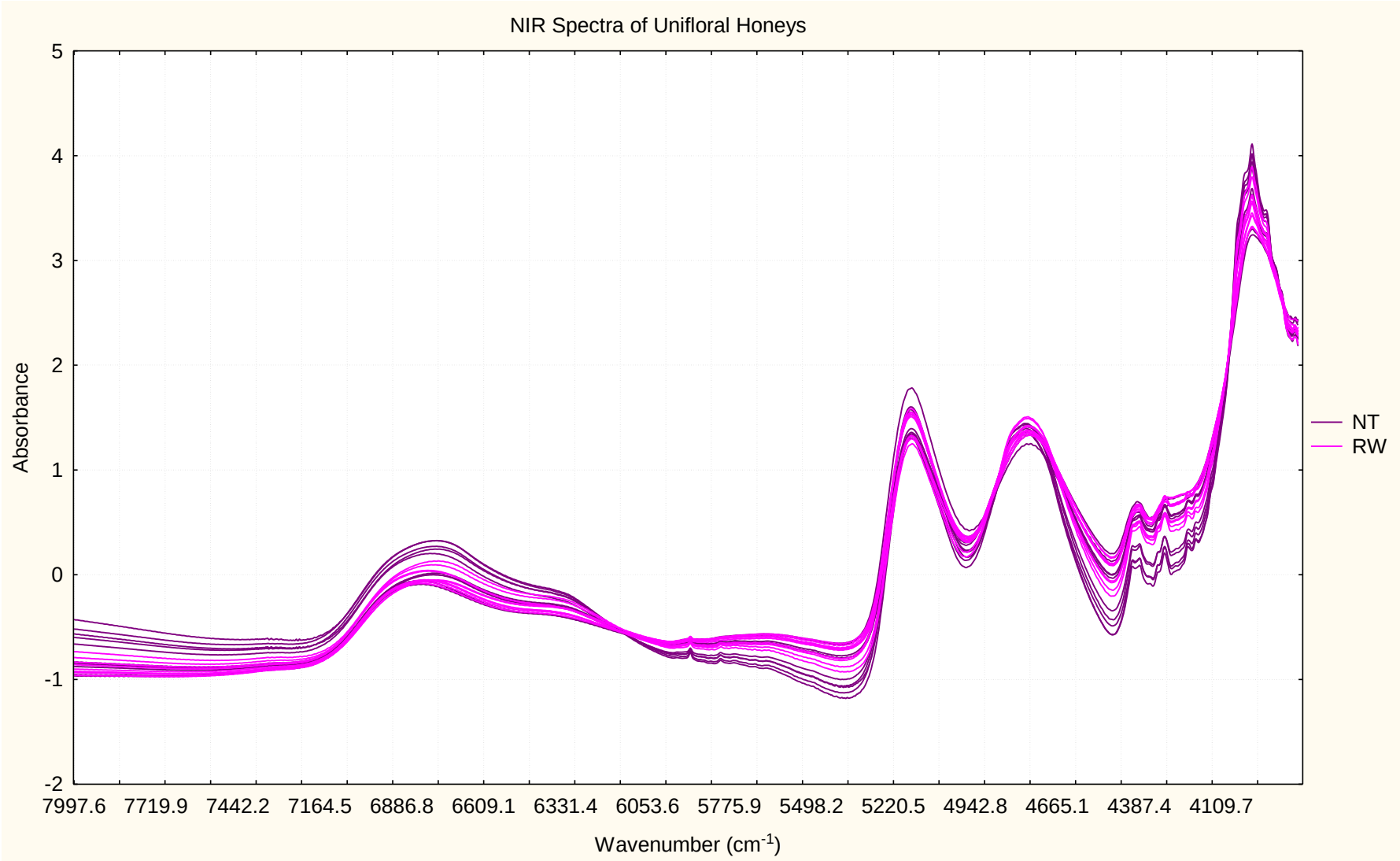


Figure A4.6 NIR spectra of nodding thistle (NT) and rewarewa (RW) honey

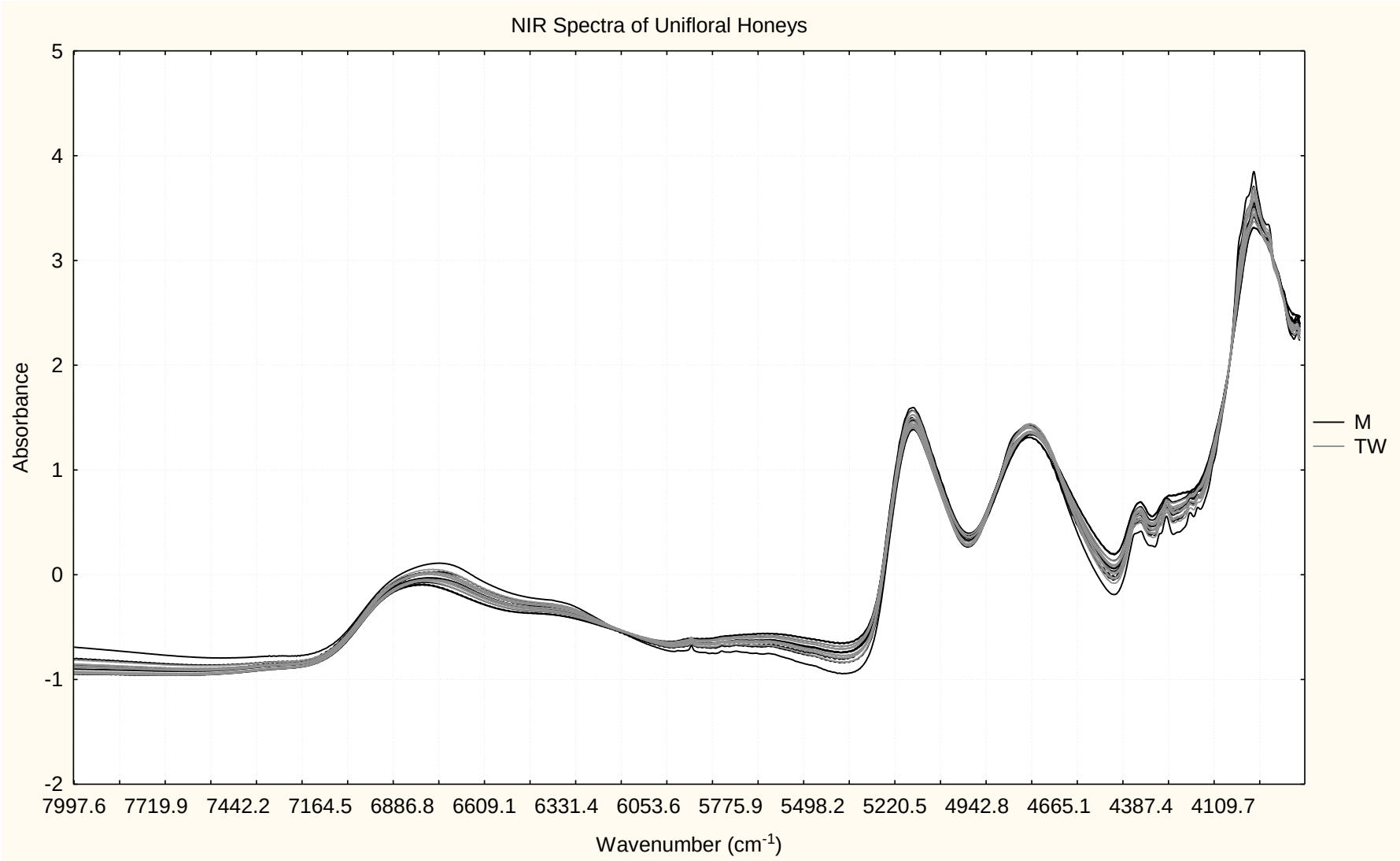


Figure A4.7 NIR spectra of manuka (M) and tawari (TW) honey

A4.3 Scripts and Analysis Procedures used in R

Labels were assigned to the NIR datasets in R using the following script:

```
% labels for 100 floral honey set
honey.type <- factor(c(rep("B",10), rep("C",10), rep("HD",9),
rep("K",10), rep("M",10), rep("NT",10), rep("R",11), rep("RW",10),
rep("TH",10), rep("TW",10)))

% labels for 323 floral honey set
honey.type <- factor(c(rep("R",20), rep("NT",15), rep("C",143),
rep("HD",21), rep("K",19), rep("M",28), rep("RW",15), rep("TW",29),
rep("TH",10), rep("B",23)))

% labels for manuka honey set
honey.type <- factor(c(rep("H",6), rep("M",14), rep("L",18)))
```

The following scripts were used to analyse the carbohydrate, extractives and NIR matrices in R. The symbol <- is an assignment operator, it is used instead of the = sign in some situations to avoid ambiguity. A % symbol is a read only command; therefore any text directly following this symbol is ignored. The "pls" and "MASS" packages[4] were used in the following analysis and was loaded prior to each new session.

Files containing matrices were opened and read by:

```
% choose and read file
df <- file.choose()
honey <- read.csv(df, header = TRUE)
```

[4] Venables, W. N.; Ripley, B. D., *Modern Applied Statistics with S*. 4th ed.; Springer: 2002.

Dendrograms of hierarchical clustering were constructed in R using the following script:

```
% hclust function
x <- hclust(dist(h), method = "ward")
plclust(x)

% Terms "single" "complete" and "average" substituted in place of
"ward" to change the distance method.
```

Principal component analysis was achieved by the following procedure:

```
%conduct PCA and plot loadings
(honey.pca <- princomp((honey), cor = T))
summary(honey.pca)
plot(honey.pca)
loadings(honey.pca)
honey.pc <- predict(honey.pca)

%plot PC1 vs PC2
eqscplot(honey.pc[, 1:2], type = "n", xlab = "PC1", ylab = "PC2")
text(honey.pc[,1:2], labels = as.character(honey.type),
col = 3 + as.numeric(honey.type), cex = 0.8)

%plot PC2 vs PC3
eqscplot(honey.pc[, c(2,3)], type = "n", xlab = "PC2", ylab = "PC3")
text(honey.pc[,c(2,3)], labels = as.character(honey.type),
col = 3 + as.numeric(honey.type), cex = 0.8)

%change scale of plot by including ratio = x after type
%col = vector of colours for plotted symbols (i.e. change symbol
colour)
%cex = character expansion vector (i.e. change text size)
```

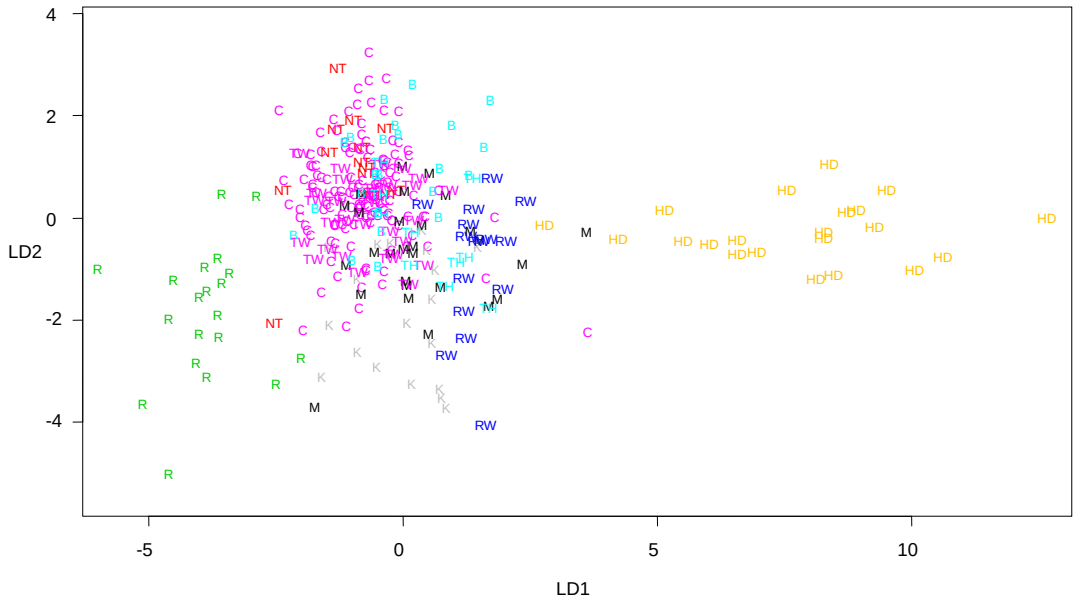
Linear discriminant analysis was conducted using the following method:

```
%LDA on full matrix
(honey.lda <- lda(honey, honey.type))
honey.ld <- predict(honey.lda, dimen = 2)$x

%plot LD1 vs LD2
eqscplot(honey.ld, type = "n", ratio = 0.8, xlab = "LD1", ylab =
"LD2")
text(honey.ld, labels = as.character(honey.type), col = 3 +
as.numeric(honey.type), cex = 0.8)

%plot LD2 vs LD3
eqscplot(honey.ld[,c( 2:3)], type = "n", ratio = 0.8, xlab = "LD2",
ylab = "LD3")
text(honey.ld[,c(2:3)], labels = as.character(honey.type), + col = 3
+ as.numeric(honey.type), cex = 0.8)
```

Linear discriminant analysis was conducted on the PCA scores determined as outlined above using the following method:

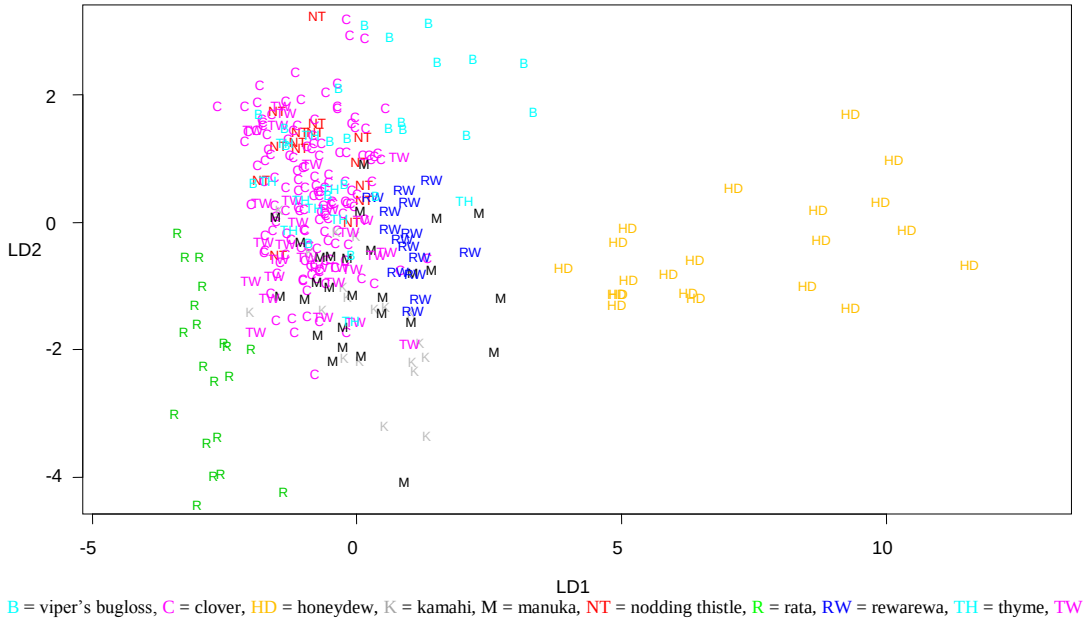
```
%LDA on first 10 PCs
(honey.lda = lda(honey.pc[,1:10], honey.type))
honey.ld = predict(honey.lda, dimen = 2)$x

%plot LD1 vs LD2
eqscplot(honey.ld, type = "n", xlab = "LD1", ylab = "LD2")
text(honey.ld, labels = as.character(honey.type), col = 3 +
as.numeric(honey.type), cex = 0.8)
```

A4.4 Multivariate Statistical Analysis of NIR Spectra



B = viper's bugloss, C = clover, HD = honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure A4.8 Score plot of LD2 vs LD1 conducted on the first 15 PC scores obtained from the analysis of Dataset B



B = viper's bugloss, C = clover, HD = honeydew, K = kamahi, M = manuka, NT = nodding thistle, R = rata, RW = rewarewa, TH = thyme, TW = tawari

Figure 4.9 Score plot of LD2 vs LD1 conducted on the first 15 PC scores obtained from the analysis of Dataset D