A Survey of Dihydroxyacetone in Nectar of *Leptospermum scoparium* from Several Regions of New Zealand

A thesis

submitted in partial fulfilment

of the requirements for the Degree

of

Master of Science in Chemistry

at the

University of Waikato

by

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THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

University of Waikato

2012

Abstract

Mānuka honey has been identified as having non-peroxide antimicrobial activity (NPA). This medical benefit has led to mānuka honey becoming a major export for New Zealand. Hives are currently located in preserved or regenerating mānuka bush. With the growth in the mānuka honey industry, interest has arisen to determine why mānuka trees produce the non-peroxide active honey. Identification of this reason will allow mānuka plantations to be planted that are expected to provide honey with a high level of non-peroxide activity. These plantations will also allow marginal land to become more productive due to mānuka being a resilient plant able to grow under harsh conditions.

It has been shown that the majority of the non-peroxide activity arises from the presence of methylglyoxal (MGO). In fresh honey a minimal amount of MGO was present, but a large amount of dihydroxyacetone (DHA) was found.[1] Previous work has shown that DHA can undergo chemical conversion into MGO under the correct conditions.[2] A similar conversion is postulated to take place in honey, testing suggests that this conversion is a non-enzymatic reaction.[3] Preliminary surveys have been carried out which have identified DHA in the nectar of mānuka flowers.[3,4]

This thesis describes a wider survey of mānuka trees around New Zealand. The trees were sampled in the flowering seasons of 2009 and 2010 between October and January. Flowers were picked and frozen for processing and an aqueous soaking method was developed to extract the DHA and sugar from a pooling of 20 flowers. Analyses of the samples were carried out by gas chromatography with flame ionisation detection. This method was further improved to include the nectar extraction and measurement of DHA levels within a single flower.

To allow the DHA to be related back to honey, it was measured in ratio to the total sugar (Tsugar) in the nectar to give the ratio DHA/Tsugar. It was confirmed that DHA/Tsugar measured in the nectar of the mānuka flower does vary within and between the regions surveyed. Suggested causes of

within region variation are the age of the tree, micro-environments and possibly genetics. Variation between regions is strongly suggested to be genetically linked.[5] Using the work by Adams*et al.*(2008, 2009),[1,3,6] it was possible to predict honey NPA values based upon the DHA/Tsugar found in the nectar and these values were comparable with the measured NPA of the honey as supplied by beekeepers.

Only a poor correlation of DHA/Tsugar was found with the soil components measured by Kiefer(2010);[7] with the leaf oil components measured by Janusch(2010)[8] some correlation was found, when these were correlated across all the sampled regions. When each region was correlated individually, the correlation proved much stronger, suggesting a link, though most likely indirect, to the mānuka oil chemotype. Using these survey results, mānuka trees have been identified for the purpose of breeding and on-going study.

Acknowledgements

I firstly, would like to thank my supervisor, Assoc. Prof. Merilyn Manley-Harris, whose guidance and support over the project has been invaluable. She has time and time again gone above the call of duty whether it be following me up a landslide while sampling or ploughing through my grammar. I am extremely grateful for the opportunities and support you have provided since my first project under you as an undergraduate.

I would also like to thank Steens Honey for their sponsorship of my TechNZ scholarship and the support they have provided throughout the project. A special mention must go to Dr Peter Brooks, of the University of the Sunshine Coast, who was generous in teaching, aid in method development and accommodating me. Also a big thanks to all the Bees N' Trees project members who have provided aid and support for this project.

Special thanks to Annie Barker, Rosslyn Thomas, Jess King, Jessica Macaskill, Cody Wright, Megan Grainger and Beverley Underwood whose help in travelling the country for samples, without which the project could not have been done, is greatly appreciated and an additional thanks to Jessica Macaskill for the help she provided in developing the methodology over a number of summers.

I would also like to thank Jenny Stockdill for her help in the maintenance of the GC-FID, as well as Murphy whose tinkering in the instrument has required me to learn how to service and troubleshoot numerous problems. Also thanks to Wendy Jackson and John Little for their aid in obtaining chemicals and bits of equipment.

A big thanks as well to all the people in the Waikato chemistry labs for their support and warmth no matter how things were going, especially Maria Revell and Megan Grainger who have always been around to bounce ideas off and have put up with me over the years.

Also to my friends who have accepted my workaholic tendencies and especially to Emma who was game enough to read over parts of this thesis.

And of course a big thank you to my family. My Mum and Dad always being just a phone call away and accepting of my sporadic visits home and to my two younger brothers Morgan and Harry. Keeping working hard, science can take you many places.

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Nomenclature

σ	standard deviation
% diff	% difference
% RSD	percent relative standard deviation
AMHA	Active Mānuka Honey Association
ANOVA	ANalysis Of VAriance
AR	analytical reagent
C_3	3 carbon chain
DAK	dihydroxyacetone kinase
DBS	died between sampling
DCM	dichloromethane
DHA	dihydroxyacetone(glycerone)
DHAP	dihydroxyacetone phosphate
F/G	$\frac{Fructose}{Glucose}$ ratio
FID	flame ionisation detector
GC	gas chromatography
GL	glycerol
HA	hydroxyacetone

HPLC	high performance liquid chromatography
IIS	insufficient information to locate for sampling
LC-MS	liquid chromatography with mass spectrometry
LD_{50}	lethal dosage, 50%
LOD	limit of detection
LOQ	limit of quantitation
MGO	methylglyoxal
n	number of
ND	non-detectable
NPA	non-peroxide activity
NS	not sampled
PFBHA	*o*-(2,3,4,5,6-pentafluorobenzyl)hydroxyamine hydrochloride
pptv	parts per trillion by volume
RP18	reversed phase with an 18 carbon chain (HPLC Column)
S	standard error of the regression
SFT	single flower test
Std Dev	standard deviation
subsp.	subspecies
TMSI	1-(trimethylsilyl)imidazole
Tsugar	Total of fructose, glucose and sucrose
UMF™	Unique Mānuka Factor

var	variety

Chapter 1

Introduction

1.1 Overview

In 1989 additional non-peroxide antimicrobial activity was found in mānuka honey. This was a significant find, as such activity was not observed in the other honeys that were part of the survey.[9] As a result of this finding, mānuka honey has grown to be an important export of New Zealand. The main focus of mānuka honey research is into the health properties of the honey with the study of the mānuka tree being restricted.[10] This thesis aims to address some of the gaps in this knowledge.

Honey is typically composed of 7% water and 90% sugar, and generally exhibits antimicrobial activity due to its acidity, osmolality, and generation of hydrogen peroxide.[10] Further work by Allen *et al.*(1991)[11] revealed that some mānuka honeys exhibited non-peroxide antimicrobial activity; and it was generally found that mānuka honey exhibited this activity more often and more strongly than other honeys tested. This discovery has lead to an increase in the demand and therefore price the of mānuka honey. The bioassay used by Allen *et al.*(1991)[11] to measure the non-peroxide activity in honey, has been established as the industrial standard UMF™ (Unique Mānuka Factor) for use by the members of the Active Mānuka Honey Association (AMHA) to define the level of antimicrobial activity in their

mānuka honey. As UMF™ is trademarked by AMHA and not all mānuka honey producers are members of the association, the non-preoxide activity will be referred to as NPA, which is equivalent to UMF™. In 2008, it was shown that the majority of the NPA activity could be attributed to methylglyoxal (MGO)[1,12] and a correlation was demonstrated between NPA and MGO, **Figure 1.1**.[1,6]

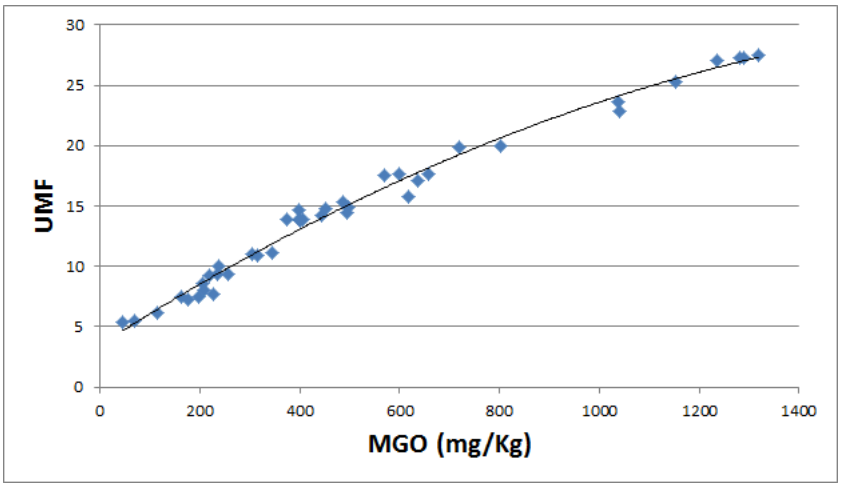


Figure 1.1: The relationship between MGO and UMF™ constructed from data in Adams *et al.*(2008, 2009)[1,6]

Further work by Adams *et al.*(2009) indicated that the source of the MGO was dihydroxyacetone (DHA).[3] DHA was found in high levels in young honey and was shown to decrease as the MGO increased during the ageing of the honey. Testing of mānuka flower nectar revealed the presence of DHA, with MGO being unmeasurable in this testing.[3] This result was supported by Stephens *et al.*(2010) where DHA was reported in mānuka nectar using a different methodology.[4] Since the origin of the antimicrobial activity has been traced to the mānuka tree, the possibility exists to predict the yields and activity levels of the honey derived from an individual tree. It has been strongly suggested that the activity is related to tree genetics.[5] The testing of individual trees, would allow them to be selected and bred to increase the quantity of high activity mānuka honey produced.

1.2 *Leptospermum scoparium*

Figure 1.2: An example of a mānuka tree from the East Cape

Leptosperum scoparium J. R. Forst et G. Forst (also known as tea tree and as mānuka) is a member of the Myrtaceae family. The Myrtaceae family contains over 3800 species broken up into 130 genera naturally distributed in Australia and Southeast Asia as well as Central and South America. The family is characterised by having internal phloem*, a large number of stamens and leaves filled with oil glands. The genus *Leptospermum* which includes mānuka has 83 identified species. *L. scoparium* (mānuka) is found in the *L. myrtifolium* subgroup which is characterised by having persistent woody seed capsules.[5]

L. scoparium is found throughout New Zealand and in the southern reaches of Australia, but the name mānuka is of Māori origin. It is a small tree usually growing from 2 to 8 m tall, although in exposed situations it does not grow beyond much more than a small shrub. The fruit is a woody capsule which ripens during April and May. These capsules can remain viable on the tree for up to 3 years before being shed. This accounts for the mānuka seed

*The phloem is made of living tissue that transports sap (a water solution that contains sugars and other organics from the photosynthetic process).

bank being found on the trees, rather than in or on the ground. Each seed pod contains a large number of seeds which compensates for the low germination rate of individual seeds.[13,14] The bark of the tree is brown and often hangs in strips and in certain areas is often covered in a black fungus (*Capnodium walteri*).[15]

The leaves range in shape from narrow-lanceolate to ovate-coriac,* dependent on the variety and location. Leaf size ranges from 4-12 mm long and 1-4 mm wide with a sharp point and a rigid structure.[16,17]

Mānuka fulfils two roles in the ecology of New Zealand. It has a permanent role in extreme growing environments; such an environment can be characterised by being too wet, too dry or unstable and exposed for climax forests to establish themselves. Mānuka also serves as an early woody species in forest regeneration providing a protecting environment for the growth of larger trees.[18]

Flowers range from mainly white through pink/red tinted to fully pink/red, with a diameter of approximately 12 mm across, although some varieties can have flowers up to 20 mm across.

Flowering in New Zealand occurs normally from September to February. The mānuka flowering season is governed by temperature and daylight hours; an increase in daylight hours, coupled with an increase in temperature, causes the buds, which due to the cold and shorter daylight hours have been dormant over winter, to develop.[19] The advantage of flowering being controlled by daylight hours is that it allows the mānuka tree to fill a niche in the year where few other plants flower.

Mānuka is andromonoecious† with its bisexual flowers appearing commonly

*Lanceolate means spear-shaped; ovate is egg shaped and coriac is a more heart-shaped leaf.

†Andromonoecious plants have both male and bisexual flowers. The male flowers have only stamens while the bisexual flowers contain stamens and ovaries. Even though the plant contains both sexes it is not capable of self-fertilization.[19]

on the upper part of the tree and the terminal of the branch. The ratio of male to bisexual flowers is governed more by environmental effects than plant genetics.[15,18,20]

The mānuka flower's small size means that the tree produces more of them. This creates a large floral display attractive to most insect pollinators. This display is enhanced by trees in a local population, flowering at approximately the same time. Insects were found to primarily visit the flowers for the nectar reward and appeared not to discriminate between male and bisexual flowers.[18]

Limited research on nectar production in the mānuka flower has been carried out. However, it can be assumed that it will share many traits with other trees in its family. This assumption is supported by O'Brien *et al.*(1993), who compared the flowers of *Leptospermum myrsinoides* and *Leptospermum continentale* and found both to be similar. For example: both species have the same dish shape with 5 petals alternating with the sepals* which form a ring around a ring of stamens† with the stigma in the middle of the ring.[21] The mānuka flower shares these same characteristics so should be expected to have similar traits. The nectariferous tissue of the mānuka flower, therefore should be found around the bottom of the dish shape.

This is different to the more common nectar container, which protects the nectar from the rain, but has the advantage of making the nectar more available to a wider variety of pollinators. This is supported by the construction of the mānuka flower being non-specialized. The open dish shape allows a large variety of pollinators to visit with little difficulty. The stamens form a ring around the nectar, thus increasing the chances of insects brushing against the stamen and collecting the pollen.[22]

*In the case of mānuka, sepals alternate with petals around the flower. The sepals act to protect the flower when it is a bud and unfold as the flower blooms

†Stamens are the male part of the plant producing the pollen that is used to fertilise flowers

The flowers of *L. myrsinoides* and *L. continentale* were found to last about 10 to 12 days, dependent on temperature, with the petals falling at the end of this time.[21] Personal observations of mānuka during the current survey demonstrated a similar trend. The nectar was found to be produced throughout the life of the flower in *L. myrsinoides* and *L. continentale* and occasionally was produced even after the petals had fallen.[21] Cases were recorded of some flowers of *L. myrsinoides* and *L. continentale* producing no nectar at all. This result applied to both the male and bisexual flowers, with no difference observed in nectar production between the sexes.[21]

Nor was a significant difference in nectar level seen in *L. myrsinoides* and *L. continentale* between seasons.[21] The *L. myrsinoides* and *L. continentale* trees flowered for 3-4 weeks each during the flowering season. The peak flowering of a population occurred at approximately the middle of the season.[21] The wild species of mānuka, which have predominantly white flowers, follow this 3-4 week flowering period over the months of October to January. The comparison between these two tree species provides an understanding of nectar production in mānuka, until the relevant studies are carried out on the mānuka itself.

1.3 Variations in mānuka

Previous studies support the hypothesis that DHA might vary throughout New Zealand, as the mānuka trees themselves are polymorphic and adapt to environmental conditions, as well as demonstrating a wide variety of genetic variations. A good demonstration of the genetic variation that does occur can be observed in the garden cultivars, as it is often, the genetically rare plants in the wild that are cultivated. The mānuka flower, as mentioned, commonly ranges in colour from white through a pale pink blush to a full pink flower. Strong red-flowered plants do appear, albeit very rarely, in the wild. Another

rarity are double flowered plants that have more than the 5 petals commonly found on mānuka flowers. Although white double flowers are known to appear occasionally, the pink and red double flowers appear to be more rare. Another genetic variation is the prostrate form, where the mānuka exhibits a ground hugging growth habit. In some cases this is an environmental effect.

An example is when the plant grows on ridge tops, which exposes the tree to strong winds encouraging the growth of a smaller plant and/or due to growing on infertile soil, which lack the nutrients allowing the tree to grow tall. In some cases, however, this has been identified as a genetic effect, since trees grown from the seeds of a prostrate form exhibit the same behaviour when grown under more favourable environmental conditions. A dwarf form also exists which is simply a low growing more compact form of the mānuka tree. Some trees have also been identified with variegated leaves. The reasonable number of rare genetic traits has allowed over 150 cultivars to be named, all exhibiting different combinations of these traits.[23]

The garden cultivars exhibit some of the more extreme visual differences observed in the mānuka. A more subtle difference is the shape of the leaves. Yin *et al.*(1984)[16] reported that the leaves of the mānuka changed from a long narrow leaf in northern lower-altitude populations to a broad, short leaf in higher-altitude southern populations. To confirm that this was a genetic difference and not an environmental effect, 17 seedlings were picked to represent the range of sample sites in the study and grown under uniform environmental conditions. It was found that these seedlings maintained their respective leaf shapes even when grown under uniform conditions.[16] This suggests that, although the environment had a limited effect on the seedlings, it is these effects that may have originally shaped the genetics of the trees.

Not all differences reported are visual, numerous studies have been conducted on the oil obtained from mānuka leaves. These studies have found

that the composition of the leaf oil varies throughout New Zealand, illustrated in **Figure 1.3**.

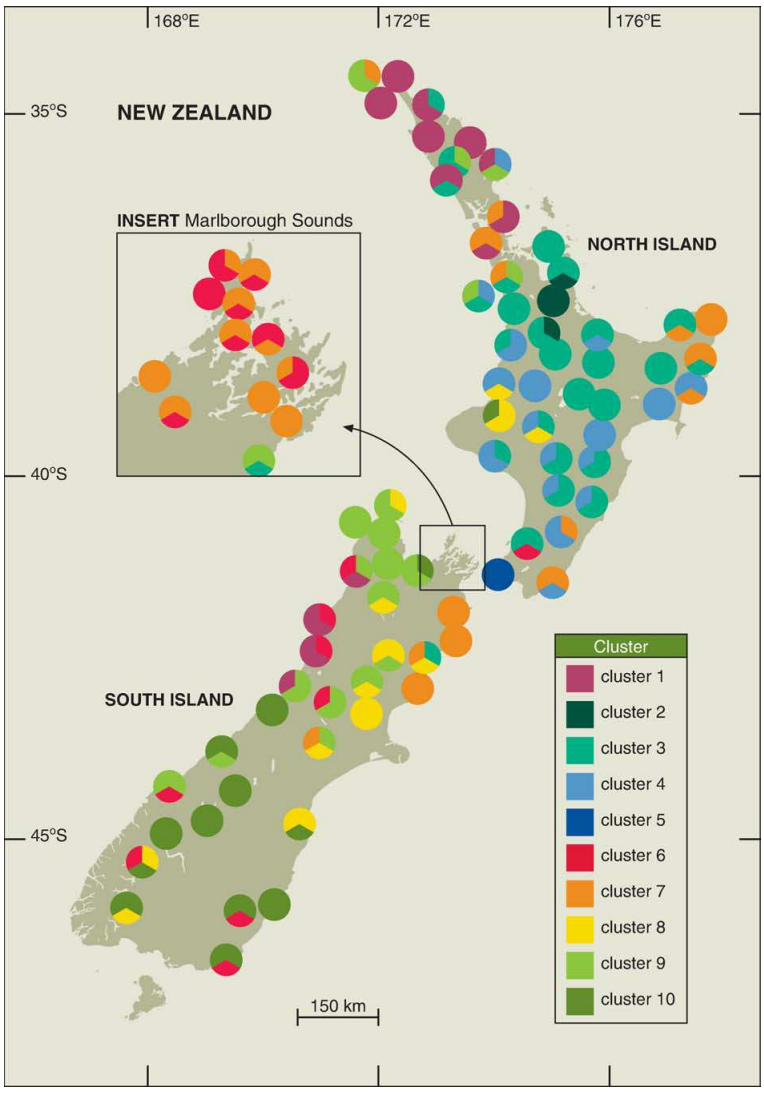


Figure 1.3: The different oil chemotypes observed around New Zealand, each cluster represents a oil chemotype after by Douglas *et al.*(2004).[24]

In the study by Douglas *et al.*(2005)[25] 10 mānuka leaf oil chemotypes were deduced, more than had been previously reported. A larger sample size and a greater area was used in this study compared to previous studies conducted.[8,25,26] To confirm if this was an environmental or genetic effect a test was carried out in which seeds collected from 15 different sites from around the country were grown at one site. This eliminated any major environmental effects on the plants. It was found that these plants still exhibited the same oil chemotypes as the sites from which the seeds were

collected.[25] The composition of the leaf oil chemotypes was found to remain relatively stable throughout the year. However, the oil yields were found to fluctuate during the year due to environmental effects.[25]

An environmental variation that has been investigated is the frost hardiness of the trees. This has been found to have a genetic link and gives a clear demonstration of how changing environments have shaped the genetics of localised tree populations. Sample seedlings were collected from two populations; the first was from a high altitude inland site and the second from a low altitude coastal site. These were grown in four different controlled environments which were cycled between controlled day and night temperatures. It was found that although both populations had a similar threshold for frost temperatures, the higher altitude population seedlings were genetically able to harden to frosts faster, therefore, allowing them to survive more rapid decreases in temperature.[27] A study was carried out comparing mānuka cultivars in New Zealand and France. This study recognised that mānuka cultivars grown in France had difficulties surviving the French inland winters. This difficulty had been attributed to the cultivars in France originating from low altitude plants in New Zealand. To study this, a number of low and high altitude populations were planted at test sites in 3 different climate regions in France and results taken over 2 winters. It was found that the high altitude plants suffered negligible frost damage over those winters, but that damage was significant for the low altitude plants. Furthermore these results show the role of the genetic effect in frost resistance and the possibility to combine frost resistant species with more ornamental cultivars and thus create those that are able to withstand and survive harsh weather conditions.[28]

A minor example of mānuka polymorphism is its water adaptive ability. Samples were taken from two populations of mānuka, one from a semi-arid

region and the other from a moist forest environment. These were planted under the same conditions and grown until the plants were 1 year old. The 1 year old plants were then divided into 4 different groups for testing different water conditions under which the mānuka might grow. These four treatments were defined as "deep running water", "shallow running water", "stagnant water" and a "control treatment". It was found that the two different populations both responded similarly to waterlogging though the samples from the forest environment had a more positive growth demonstrated by an increase in the length of root hairs. Although this indicates the genetic variability that exists in the mānuka plant, the authors suggested that a larger sample size might render the differences observed insignificant.[29] All the foregoing illustrate how well the mānuka is able to adapt to different environmental conditions.

It therefore seems likely that a variation in DHA levels between trees and regions might be expected.

1.4 Possible reasons for DHA to vary in nectar

Only some small sample studies (3-10 trees) have been carried out on mānuka nectar in relation to mānuka honey by Adams *et al.*(2009)[3] and Stephens *et al.*(2010)[4] in which DHA was found in the nectar and in both cases it was noted that there were some differences between the trees sampled. Stephens(2006)[5] looked at some of the factors responsible for the varying levels of NPA observed from mānuka honeys from different parts of New Zealand. The study strongly suggested that mānuka was responsible for the presence of NPA in honey. This conclusion was drawn as no other plant or insect species were found to occur at all the sites that honey containing NPA was collected. Honey bees can also be ruled out as a source since NPA

containing honey is only found where mānuka trees are present for honey bees to visit. Fungal species were unable to be ruled out as there was insufficient data to conclude that they were not present in the same range as the NPA containing honey. It was considered unlikely as honey containing the NPA is collected in parts of New Zealand (such as Otago) that are relatively cool and unfavourable to fungal species.

A similar conclusion can be drawn from honey containing NPA from the Coromandel Peninsula, which could be separated into three regions based on NPA activity. These regions all contain similar environmental conditions, so if fungal species did affect NPA, a uniform distribution would be expected. As this was not observed, it was deduced that fungal species do not have a large effect.[5]

When relating NPA activity to environmental effects it was found that the following factors in decreasing order of effect influenced the NPA of honey: region, drainage, minimum solar radiation, mean annual temperature, minimum winter temperature, slope, mean annual solar radiation and vapour pressure deficit. Region had the most significant effect upon the NPA and this was found to be linked more with mānuka species in the region than any environmental parameter. Rainfall had an effect, but it was dependent upon when the rain fell. Rain falling when the flowers were open resulted in a decrease in NPA due to the open nature of the flowers and hence causing the nectar to be washed out or diluted. Normally an increase in honey is observed when rainfall occurs at night as the additional water in the environment allows more nectar to be produced the next day (although this can dilute some of the contents of the nectar).[5] It was concluded that environmental effects play an insignificant role in the NPA content of honey.

Using tree morphology, leaf oil chemotypes and genetic markers, Stephens(2006)[5] was able to show that NPA content was strongly mānuka variety dependent. Stephens was able to assign identified varieties with the

data and propose the possibility of new varieties in addition to those trees studied in the upper North Island.

L. scoparium var. *incanum* is found in the Northland region. It has lanceolate leaves with flowers that range from white to fully pink. *L. scoparium* var. *linifolium* is found in the Waikato region It has linear leaves and normally has a white flower. *L. scoparium* var. *myrtifolium* is found in the Central North Island with ovate leaves and white flower petal. An undefined variety was found to be present on the most eastern sections of the East Cape with leaves of a linear-lanceolate shape and white flower petals. When relating these to NPA content it was found that the *L. scoparium* var. *incanum* and *L. scoparium* var. *linifolium* varieties had the highest NPA content, on average of 15 NPA while *L. scoparium* var. *myrtifolium* and the undefined variety had lower content of around 9 NPA. Some regions had trees combining characteristics of a number of varieties demonstrating hybridisation in buffer regions.[5] Although not mentioned in the study *L. scoparium* var. *parvum* is also defined and is found around the Wellington region with hairy leathery leaves and small flowers.[17] There is the possibility of several undefined species existing, as the study did not look closely at the trees in the South Island and the variation in the chemotype data suggest there are possibly more varieties. The link between NPA and mānuka varieties demonstrates that NPA is possibly genetically related. To confirm this, the genetic link between the genes and DHA production would need to be identified. This identification would increase the certainty with which trees could be bred.

A difficulty that arose in this testing was the lack of knowledge of the dilution of mānuka honey by other floral sources, either by the activity of the bees, or the beekeeper blending honeys together. This was overcome by the observation that mānuka honey is more viscous than most other honeys. Using this knowledge a standard curve was created that allowed the percentage mānuka of a honey to be calculated based on a honey's viscosity.

Using this curve it was observed that all mānuka honeys contain NPA activity but it was the presence of other honey types that caused the activity to be reduced. Honey containing less than 30% mānuka honey were found to have no measurable NPA activity.[5] A limitation of these results is the collection of the nectar. As bees essentially sample every tree at a site, it is not possible to use the honey results to selectively pick those trees whose nectar contains high levels of DHA. A more specific method is required to allow individual trees to be sampled.

1.5 Nectar

From precedents in the literature it seems likely that that the DHA variation is genetically controlled. With the aim of this research being to sample individual trees, a better understanding is required of how nectar is produced and how the DHA enters the nectar.

1.5.1 How nectar is formed

There is no literature describing the formation of nectar in the mānuka flower. Generally nectar is secreted by glandular trichome cells in the flowers.[30] These cells are supplied with a sugary solution by the phloem to form nectar. The phloem transports a solution primarily composed of sucrose but also containing amino acids, organic acids and other photosynthates from the leaves.[31] Nectar is a sugary solution mainly composed of sucrose, glucose and fructose; other compounds found are organic acids, proteins and mucilages*.[30] Nectar flows down to the base of the stamens which are arranged around the inside edge of the petals. This forces the nectar feeding insect to brush against the stamen and stigma as it collects the nectar. This

*Mucilages are a sticky glue like substance

causes pollen to be collected and also transfers pollen from the bee or the stamen to the stigma which allows fertilisation of the flower **Figure 1.4**.

DHA can then either be formed as part of the nectar-making process or may be a contaminant from the phloem solution. The latter is likely if the DHA is formed in another part of the mānuka tree.

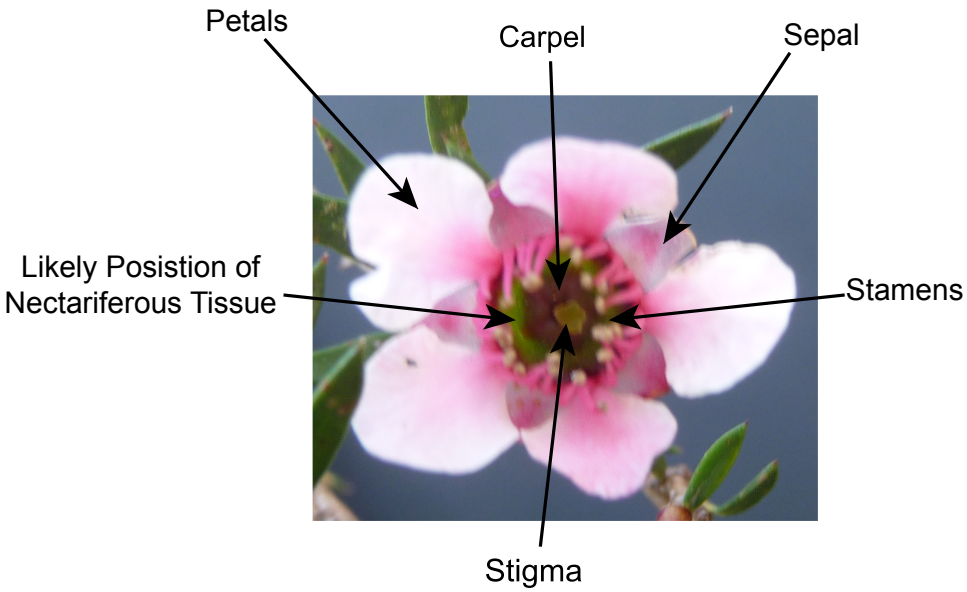


Figure 1.4: An example of a mānuka flower with the likely position of the nectariferous tissue labelled.

1.5.2 Environmental effects on nectar production

In other species, effects have been found to modify floral nectar on a local level. Micro-climates have been demonstrated to have an effect on nectar production in clones of *Asclepias syriaca*, that were planted on the same campus. Due to these being planted on the same site, major environmental effects could be discarded. However, it was found that the amount of sunlight received, combined with small temperature and humidity differences had a larger effect on the nectar production of the flowers than the age of the flowers.[32] The study also showed that nectar production was not uniform, reaching a peak about half way through flowering.[32] The micro-environment

effect was confirmed in a further study on *Asclepias syriaca* in which high humidity and rain was found to cause an increase in nectar volume, while also increasing sugar by a proportional amount so that the sugar concentration remained constant.[33] A simulation of nectar collection with *Pyrostegia venusta* revealed that removal of nectar can cause an increase in the volume of nectar produced, but a reduction in sugar concentrations as it is diluted; this was further reinforced by results observed with *Macaranga tanarius*.[34,35] It was observed in *Echium vulgare* L. and *Sinapsis alba* L. that if nectar is not collected, it may be partially reabsorbed and re-expressed in a more concentrated nectar.[36] A study on *Aquilegia vulgaris* subsp. *vulgaris* and *Aquilegia pyrenaica* subsp. *cazorlensis* comparing plants growing in a glasshouse excluded from pollinators to those in the field with access to pollinators, found that pollinators had a significant affect on the nectar composition and levels in the flowers. It was suggested that this effect was caused by temperature differences, nectar removal by pollinators, addition of enzymes by pollinators and micro-organisms carried by pollinators.[37] The amino acids found in the nectar of *Hibiscus rosa-sinensis* flowers were found to increase when a flower was damaged due to additional asparagine being created in the flower.[38] Fertilizer was found to have little effect on the formation of amino acids in the nectar of *Agrostemma githago*.[39] These effects demonstrate that though genetics may set a base level for nectar production, local conditions can affect nectar levels and composition.

1.6 Nectar collection methods

To allow the DHA to be tested, a method needs to be created to sample the nectar. Most nectar collection and testing methods are based around measuring the sugar content of the nectar. Numerous methods have been used to collect nectar.

The method used by Brink *et al.*(1980) on *Aconitum columbianum* involved waiting for a flower to be visited by a nectar drinking insect. The flower was then bagged with cheese cloth to prevent further insect visits; twenty four hours later the nectar was removed from the flower using a micro-pipette. Sugar levels were measured using a refractometer and, if necessary, the nectars were pooled to give a mean sugar per μL of nectar for a population.[40] A similar technique was applied to *Eucalyptus spathulata* using microcapillary tubes where capillary action was used to withdraw the nectar from the flowers. The height of nectar in the tube was measured to determine the volume collected and then the nectar was rinsed into 2 mL of distilled water for sampling and storage.[41]

Filter paper, from which equilateral triangles with 1 cm sides were created, has also been utilised. The tips of each point of the triangle were dipped into the centre of the flower to absorb the nectar. The triangle was then placed into 2 mL of water, shaken and mixed until the filter paper was reduced to pulp to extract all of the nectar.[41] Ellipsoid shapes with one pointed end and using multiple bits of paper have also been used.[42]

Centrifugal extraction was tested by Swanson *et al.*(1950) using red clover. The flower was removed from the plant, suspended over a capillary tube and centrifuged to pull the nectar out of the flower. This was found to be a suitable technique for small flowers.[43] Specialist equipment was required to be made for this method to allow the flowers to be anchored in the centrifuge.

Rinsing of the flower was found to be effective; a flower on the tree is inverted over a vial and rinsed with a known volume of water multiple times into a collection vial. This sampled water can be reused on further flowers creating a pooled sample if required.[41]

Washing of the flowers is another method used. The flowers were removed from the tree thereby preventing the same flower from being sampled again.

A single flower was agitated in 2 mL of water in a 30 mL vial. This was repeated three times to ensure that the largest quantity of nectar was removed. Further testing of this method showed that a soaking period of sixty minutes gave larger sugar extraction compared to periods of one minute and twenty minutes, though this may be due to sugars leaching out from cut surfaces on the flower.[41]

Tan *et al.*(1988) used a soaking method on mānuka flowers using chloroform as a solvent and a two hour soaking period. The method was aimed at the organic solvent-soluble components within the nectar. No DHA was reported in these extractions.[44] DHA is unlikely to be soluble in chloroform so such a result would be expected.

A similar method was performed by Adams *et al.*(2009) with mānuka flowers. 500 mL of flowers were soaked in 500 mL water for four hours, with the extract concentrated by evaporation under reduced pressure.[3] This proved successful for measuring DHA, although the quantity of flowers required for one sample is large and the method time consuming.

A syringe was used by Stephens *et al.*(2010) to sample the mānuka nectar directly from mānuka flowers; further detail is not provided for this method.[4]

A review of various methods has been carried out by Morrant *et al.*(2009) This found that the rinsing method was the most effective as it allowed the flower to remain on the tree and had the least probability of damaging the flower, an event that can cause additional sugar to be released. The review recognised, however, that this technique was more time consuming. The washing technique was acknowledged as the next acceptable technique though it also showed the possibility of extracting additional sugar from the flowers. Neverless, the advantage still remained that it was quicker and easier to perform in the field.

The review also looked at storage techniques and found that sugar levels remained stable for up to 14 days at refrigerator temperature (4 ℃) and for a minimum of 28 days (time limit of the studies testing) in the freezer (-8 ℃).[41]

Most methods reviewed involved collecting and sampling individual flowers. A study on *Impatiens capensis* looked at the variation of nectar between individual flowers, individual trees and within a local tree population. They found no significant variation between individual flowers on a single tree.[45] This is in contrast to a study carried out on *Aquilegia vulgaris* subsp. *vulgaris* and *Aquilegia pyrenaica* subsp. *cazorlensis* that showed that there was a larger variation in nectar sugar between individual flowers than between trees in the field.[37] This suggests that correct methodology could allow pooling of flowers from a single tree and supports the pooling observed in those methods described by Adam *et al.*(2009)[3] and Morrant *et al.*(2009).[41]

1.7 Dihydroxyacetone

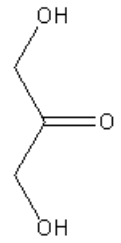


Figure 1.5: Stucture of dihyroxyacetone

Dihydroxyacetone (DHA), **Figure 1.5**, is more well known for its presence in sunless tanning agents then for its presence in plants. DHA is the simplest ketose and the only one without optical activity. It is normally supplied commercially as a white crystalline powder.

DHA is a commonly used chemical in pharmaceutical and food production. It was originally used as a treatment for diabetics, with a chance discovery,

finding that it is able to darken skin.[46] This has given rise to a number of sunless tanning products which use DHA. The focus of most DHA production today is for tanning products and as building blocks in the formation of some chemicals. Industrially, it is primarily formed from a glycerol (GL) to glyceric acid microbial reaction, with DHA being a by-product of this reaction. *Acetobacteria* and *Gluconobacteria* are used industrially in these reactions for the creation of DHA from GL.[46–49]

1.7.1 Chemistry

DHA has been found to be sensitive to pH levels. High pH levels have a great destabilising effect on DHA and very low pH causes it to form dimer compounds. At higher pH levels between 8 and 12 hydroxyacetone is produced together with MGO from DHA degeneration. High pHs also lead to the formation of glycerylaldehyde (an isomer of DHA) together with formic acid. Studies showed that DHA is stable at room temperature for at least six months. Storage at lower temperature helps to increase the storage life of DHA without degradation.[46]

1.7.2 Toxicity

The toxicity of DHA is very low. Tests on rats showed a LD_{50} of over 16 g/kg. In humans, the LD_{50} is very high as it takes part in the human carbohydrate metabolism especially in the phosphorylated form Dihydroxyacetone phosphate (DHAP).[46] The Krebs cycle is one such metabolic cycle. Upon ingestion it has been found that DHA can elevate carbohydrate utilisation in cells and can act as a substitute for glucose as it is an intermediate in the pathway.[50]

1.7.3 DHA in plants

DHA is found in plants as part of their carbohydrate metabolism. DHAP is the form in which DHA is more commonly found in plants; this is a central compound involved in a number of metabolic pathways. DHAP is part of the C_3 carbon photosynthetic cycle, **Figure 1.6**, where it can be converted to hexoses for starch synthesis or for regeneration of ribulose bisphosphate. It is also the transport component of the triose phosphate shuttle out of the chloroplast* and into the cytosol†. In the cytosol it can be converted into hexoses, sucrose and cell wall components or oxidised by glycolysis to pyruvate.[51] The cycle is known as the Benson-Calvin cycle, a 3-carbon, light independent, photosynthetic pathway which is part of carbon fixation in plants. The cycle is also able to form fructose 6-phosphate and glucose 6-phosphate which can be used to form sucrose via enzymatic reactions.[52]

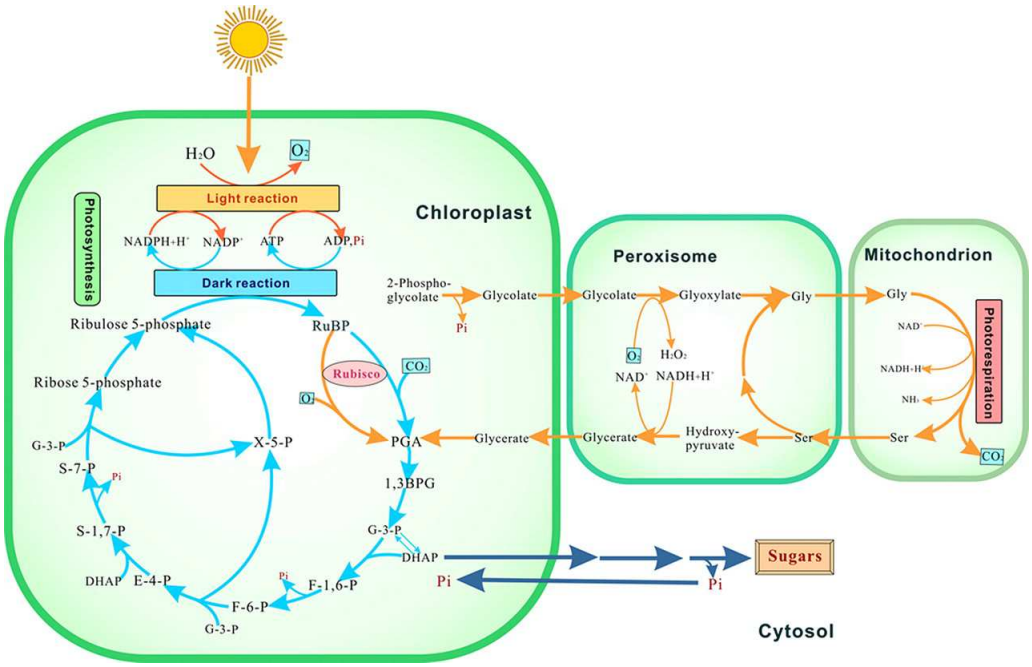


Figure 1.6: Example of how DHAP is involved in the photosynthetic cycle, from Luo *et al.*(2009).[53]

*Chloroplast are responsible for the photosynthetic reactions that take place in a plant cell.

†Cytosol is the liquid found inside plant cells.

Although the role of DHAP is well described in the literature, there is little that explains why DHA is found in mānuka nectar. It has been suggested that DHA may be formed by the Lobry de Bruyn-van Ekenstein rearrangement from D-glyceraldehyde in nature.[54] This work, however, was reviewing the whole of nature and focused primarily on the muscle reactions in animals.[54]

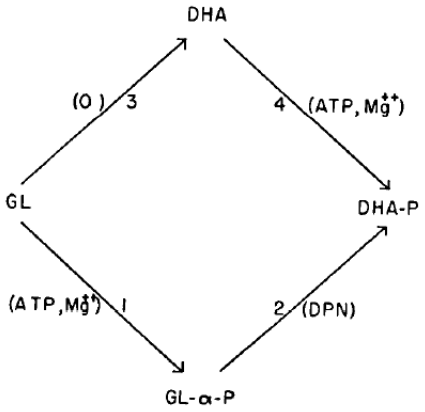


Figure 1.7: An example of how DHA can be an intermediate in the conversion from glycerol (GL) to DHAP in *Acetobacter suboxydans* from Hauge *et al.*(1955).[55]

DHA can be an intermediate in the conversion of GL to DHAP **(Figure 1.7)**.[55] This is part of the glycerolipid metabolism **(Figure 1.8)**. This metabolism has been confirmed in the higher plants, *Populus trichocarpa*, *Ricinus communis* and *Vitis vinifera*.[56] The glycerolipid metabolism is responsible for the formation of lipids in nature. In plants, the lipids are formed in the chloroplasts contained in the leaves.[57] DHA could be explained in mānuka nectar by a possible genetic variation that has arisen that prevents some of the DHA completing the conversion, and therefore causing it to accumulate in the leaves and being transported by the phloem to the flowers.

Dunaliella parva, a green alga, produces GL from DHAP to oppose osmotic pressure in high salt environments.[58] As mānuka is known to grow well in

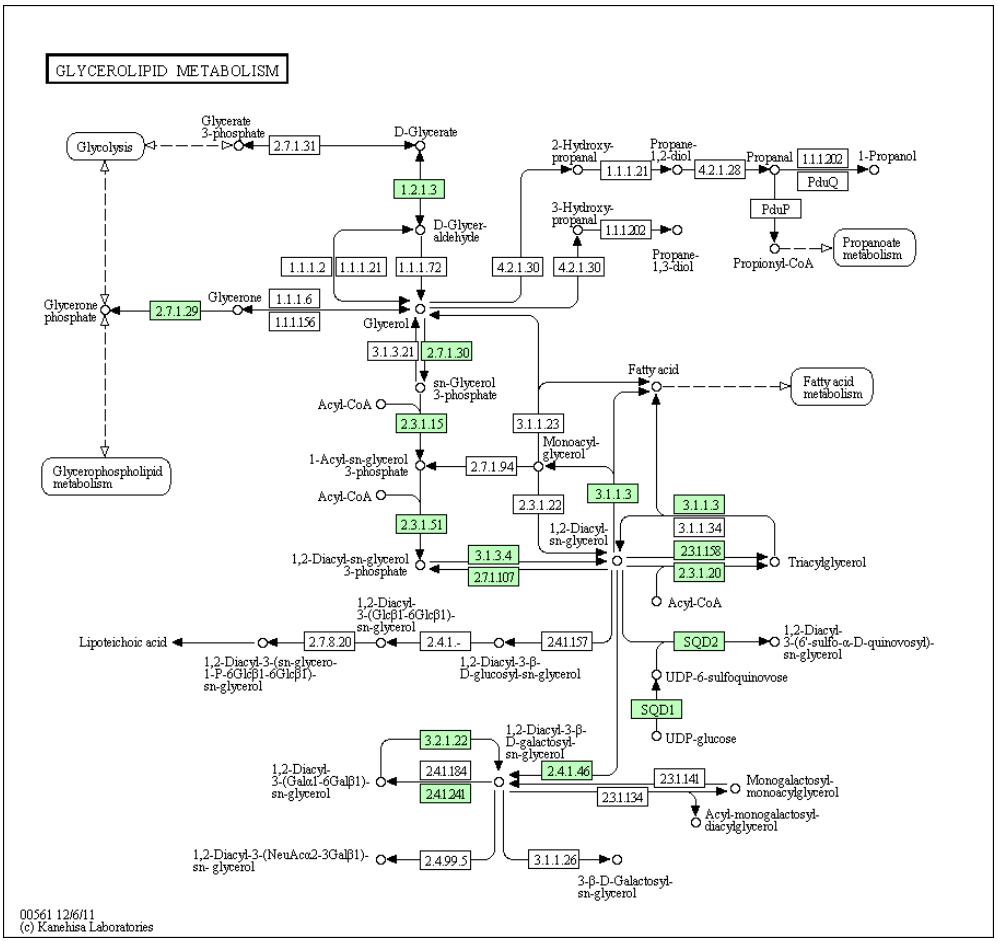


Figure 1.8: How DHA (glycercone) is part of the glycerolipid metabolism.[56]

high salt environments, with a less than 25% reduction in growth,[59] this could be due to a similar pathway as found in *Dunaliella parva*. Thus production of excess GL could lead to excess DHA levels. However, it is unknown if this production of GL due to salt is found in mānuka.

In microbial yeasts, environmental changes such as salinity, heat, osmotic pressure and cadmium can cause stress. The stress can cause excess DHA production resulting in the poisoning of the yeast through an unknown means. The excess DHA is thought to be produced from intracellular GL which is itself promoted by stress such as a high salt environment. Other suggested sources are the dephosphorylation of DHAP and conversion from formaldehyde or some unknown metabolite. The yeast metabolizes the excess DHA through the production of the enzyme dihydroxyacetone kinase (DAK) which reduces the DHA. In the yeast *Saccharomyces cerevisiae* it was found

that when DAK was over produced, the yeast was able to use the DHA as a carbon source.[60]

Mānuka is known to grow in environments that could induce stress, it can also be covered by a black fungus, indicating the presence of scale insects which can stress the tree and possibly kill it.[61] Typical stresses are temperature, drought, irradiation, salts, nutrient deficiency and nutrient toxicity which can affect plants in different ways dependent upon a plant's tolerance. An example is drought stress; the lack of water can cause an increase in concentration of small molecules in sections of the plant as its water levels decrease. Drought stress can also affect the Calvin cycle which is responsible for the formation of carbohydrates in plants.[62] In mānuka, drought stress might possibly cause the DHA to concentrate in the flowers, or impair the Calvin cycle. Impairment of the cycle, may allow the concentration of DHA to increase. A plant's tolerance might also have an effect. For example, a plant that is frost resistant might achieve such a resistance through the production of low molecular weight compounds to increase osmotic pressure. This increases its ability to obtain water from its frozen surroundings and prevent dehydration. Further resistance is possible by production of molecules to protect cells, from the toxins that result from frost damage.[62,63] Thus mānuka may use DHA to resist cold and/or drought. Calcium deficiency is known to affect the bud growth in plants, affecting the resulting flower, which could cause a change in the nectar.[62] Environmental stresses may have a direct effect on the mānuka or an indirect effect, where they have helped shape the plants genetics.

The yeast *Candida boidinii* is able to grow on methanol. DHA is formed as a product of a reaction between xylulose 5-phosphate and methanal (formaldehyde) derived from methanol (**Equation 1.1**). This then reacts to

form DHAP (**Equation 1.2**).[64]

$$\text{Methanol}$$

$$\downarrow$$

$$Xylulose5-phosphate + Formaldehyde \rightarrow DHA + Glyceraldehyde \tag{1.1}$$

$$DHA + ATP \rightarrow DHAP + ADP \tag{1.2}$$

Plants have been found to produce methanol which is known to aid in photosynthesis in the leaves. The pathway for methanol formation in plants is not yet clear, as methanol's small size makes it difficult to detect relative to other organic compounds in the leaves of plants.[65] Thus, the possibility arises that DHA is produced *via* methanol in the leaves and then finally transported to the flower by the phloem. As yeast is known to colonise flowers, it is possible that a yeast has colonised the flower and is changing methanol into DHA in the nectar.[66,67] The regionalism found by Stephens *et al.*(2006) could be explained by the bees carrying yeast between flowers.[5]

Bacteria are also known to colonise flowers. *Acetobacteria* which are used industrially to produce DHA,[46,47] have been found in the flowers of other plants.[68] The strains *A. xylinum* and *A. rancens* were found in Jenias, Marigolds, Cosmos, Kaners and Bougainvilles. Thus, the bacteria (like the yeast) are possibly transported between flowers by pollinators and transform nectar components into DHA.

Another possible source of DHA is decomposition of glucose and fructose in the nectar. It was found that when glucose and fructose were subjected to subcritical (300-350 ℃, 25 MPa) and supercritical (400 ℃, 30-40 MPa) water they broke down into DHA and glyceraldehyde.[69] Such decomposition would be expected to proceed more slowly in the mānuka flower at normal pressure and temperature and leaves the question as to why if this is the source, the process does not appear to occur in other flower species.

A possible reason why mānuka provides DHA is that insects are able to utilize DHA and DHAP as a more readily available energy source than the more common sugars of glucose, fructose and sucrose whilst flying.[70] Support for this idea is the *Dalechampia* plant which provides a precursor for euglossine bees that they require for their sex pheromones;[19] this provides an extra incentive for the bee to visit the plant. Mānuka may provide DHA in its nectar to give bees and other insects an energy boost, thus allowing them to visit more flowers, which increases the chance of pollination and provides an additional incentive for the bees to visit mānuka flowers.

As demonstrated, it is at present difficult to explain the presence of DHA in the nectar of the mānuka flower.It is equally difficult to pin point a source for the production of DHA which might explain why mānuka produces DHA in its nectar when other plants visited by bees do not.

1.8 Detection of DHA

Initial attempts at measuring DHA in nectar were based on the original method for detecting MGO in honey samples. This method uses a mixed mode size-exclusion/ligand exchange HPLC coupled with a refractive index detector. MGO and DHA were detectable without requiring them to be derivatised. The method is limited by poor sensitivity and interference by the large amounts of sugar present.[3] An improvement to this method for MGO detection was derivatization with *o*-phenylenediamine or 1,2-diaminobenzene resulting in a strongly absorbent and stable quinoxaline compound. This allows separation by a RP18 column with detection by a UV detector set at 315 nm. Though this method improves sensitivity for MGO, it is of little use for DHA which lacks the requisite two carbonyl groups to form a quinoxaline compound.[2,3] A compound that has been found effective for both DHA and

MGO is *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxyamine hydrochloride (PFBHA). This reacts *via* the amine on PFBHA which reacts with carbonyl groups on the target compound, thus providing a large benzene ring that improves detection by UV detectors. In addition it improves detection for LC-MS as the attached PFBHA group elevates the target compounds mass making detection easier.[71,72] An alternative to PFBHA is 2,4-dinitrophenylhydrazine, this reacts in the same manner as PFBHA *via* its amine group: it allows detection of more highly polar compounds than PFBHA. A comparison between PFBHA and 2,4-dinitrophenylhydrazine acknowledged that PFBHA still offered better detection over 2,4-dinitrophenylhydrazine for smaller and non-polar compounds.[72] Extraction of the PFBHA derivative compounds into an organic solvent to aid volatility allowed GC to be used; this was found to give more sensitive results than HPLC but with a price increase as an additional solvent extraction has to be carried out increasing the time of the method.[73] A comparison of organic extraction solvents found that dichloromethane (DCM) gave the greatest extraction efficiency for hydroxyacetone (HA) and MGO compared to hexane and methyl-*tert*-butyl ether.[74] When stored in DCM (4 ℃) the PFBHA derivatives were found to be stable for a minimum of 38 days, but as the stability of some derivatives are more variable with longer storage times, the period between derivatisation and analysis should be minimised for samples.[74] An improvement to the PFBHA GC method was the addition of a step which involved reacting the extract with a silylating agent (*bis*(trimethylsilyl)trifluoroacetamide) before addition to the GC. This had the effect of improving sensitivity and volatility of the compounds by replacing hydroxyl groups with trimethylsilyl groups. For the testing of air samples using this method, the method detection limits were achieved to the pptv (parts per trillion by volume) levels for MGO and HA; detection of DHA was also demonstrated.[75]

1.9 Aim of this research

The literature has shown that a link has been found between the NPA content of mānuka honey and DHA levels in the nectar of the mānuka flower. Previous studies support the concept that DHA does vary about the country, but no large scale studies have been carried out. The work by Stephens(2006) indicates that NPA values appear to be linked to different varieties of mānuka. The aim of this study will be to develop a method for testing the DHA and MGO content of nectar from mānuka flowers. With the development of a method, a survey of mānuka trees will be undertaken to look and see whether nectar DHA content varies throughout the sampled areas.

Chapter 2

Methods

2.1 Materials

o-(2,3,4,5,6-pentafluorobenzyl)hydroxyamine hydrochloride (PFBHA) (derivatization grade) and methylglyoxal (MGO)(43.2%) were sourced from Fluka Analytical. Dihydroxyacetone (DHA) (97%), hydroxyacetone (HA) (technical grade 90%), sucrose (99+%), D(-)-fructose (99+%) and D-mannitol (98+%) and citric acid (99.5%) were sourced from Sigma Aldrich. 1-(Trimethylsilyl)imidazole (TMSI) was sourced from Thermoscientific. D(+)-glucose (AR grade) was sourced from BDH Laboratory Supplies. *n*-Heptane (HPLC grade) was sourced from Ajax Fine Chem. Dichloromethane (DCM) was obtained from a Pure Solv Solvent Purification System (Model:PS-SD-5). Liquid Nitrogen was supplied by BOC. MilliQ deionised water, was obtained from a Barnstead Epure water system at 17.9 MΩ.

2.2 General methods

Samples were freeze dried in a freeze dryer (Labconco Freeze Dry systems). Calculations and statistics were carried out using Microsoft Excel Ver: 14.0.4760.1000 (32-bit) and Minitab Ver: 16.1.1.

2.3 Gas chromatography-flame ionisation detector (GC-FID)

GC analysis was carried out using an Agilent Technologies 7890A GC system with a split/splitless inlet and a flame ionisation detector (FID). This was equipped with an Agilent Technologies 7683B series autosampler with a 10 μL syringe and had a Zebron ZB-5, 0.32 mm capillary GC column installed. The instrument was controlled using Agilent Technologies, GC ChemStation, Rev.B.04.03[54] software. 2 μL injections were used for all samples. The autosampler was configured to give 5 washes of heptane and DCM before and after the injection to keep the syringe clean and free running. The FID was held at 300 ℃ with a hydrogen flow of 30 mL/min, air flow 400 mL/min and a make up flow of 10 mL/min.

2.3.1 Oven and inlet gas chromatogram settings

For detection of DHA and MGO the inlet was set to 250 ℃ in splitless mode with a 2 mL/min septum purge flow. The carrier gas (hydrogen) flow was 2 mL/min. The oven program had an initial temperature of 130 ℃ with a 5 min hold, followed by ramping at 10 ℃/min to 250 ℃ with a 6 min hold. For detection of the sugars, the inlet was set to 280 ℃ in splitless mode with a 2 mL/min septum purge flow. The carrier gas (hydrogen) flow was 4 mL/min. The oven program had an initial temperature of 100 ℃ with a 1 min hold, followed by ramping at 30 ℃/min to 200 ℃ with an additional ramp of 10 ℃/min to 250 ℃ and a final ramp 30 ℃/min to 300 ℃.

2.3.2 Organisation of GC-FID sequence

Each GC-FID sequence normally contained two trees with ten extract replicates each. These were tested alternately with the extract replicates of

one tree being tested in reverse order. For the DHA testing sequences, a calibration curve was constructed for each sequence using 6 standards, inter-spaced randomly among the tree replicates. For sugar testing a mannitol standard was tested with each sequence to check against the internal standard.

2.4 Flower collection

The sites for flower collection were picked with the assistance of local bee keepers, Google Earth™ and on the ground reconnaissance. The majority of sites were located near hive sites allowing the DHA levels in the trees to be related to the honey collected from nearby hives. The sites were spread across New Zealand between latitudes 34 °S and 42 °S and longitudes 171 °E and 179 °E. The geography of the sites offered a variety of environments for sampling. As the location of the sites used by beekeepers is commercially sensitive, it was agreed that sites would be only referred to on a regional scale and allocated generic labels. Sampling was carried out over two flowering seasons in 2009 and 2010.

2.4.1 2009 Season

Trees were selected with a bias towards those with a large floral display. This allowed sufficient flowers to be picked for testing. This bias also targeted those trees which would have been more attractive for insects due to the larger number of flowers, thus offering a greater prospective food source.

2.4.2 2010 Season

Where possible, trees sampled in 2009 were sampled again. Some trees had died or finished flowering by the time the sampling took place, so could not

be sampled. In addition trees were sampled around the University of Waikato which has a large variety of mānuka cultivars growing in similar environments.

2.5 Flower collection method

The mānuka trees were sampled, where possible, on a fine day after 2 days of no rain at a time convenient to the sampler. At the time of sampling the date, time, weather, location, elevation, tree details, natural geophysical features and man-made influences, were recorded. The flowers were either picked off individually or scraped off between two fingers, starting from the base of the branch and moving to the tip. These were then deposited into airtight bags and chilled until transfer to a freezer (-20 ℃). A herbarium sample consisting of a branch ($\sim$ 20 cm) was cut containing flowers, seed capsules and leaves. This was deposited at the University of Waikato Herbarium. Refer to **Appendix B** for the full sampling protocol including the form used for records.

2.6 Nectar extraction from flowers

The bulk extraction was carried out by taking flowers (20) from a frozen sample bag and leaving them to stand (20 min) in water (4 mL). The single flower test (SFT) was carried out by taking an individual flower from a frozen sample bag and leaving to stand (20 min) in water (1 mL). Where possible, the flower chosen for the SFT was as complete as possible and fully opened (see **Figure 2.1** for an example). After standing, the flowers were removed from the nectar/water solution and the solution was frozen until analysis.

Figure 2.1: An example of a reasonably complete open flower for testing by the single flower test. Damage to the flowers has been observed as a result of freezing after collection.

2.7 Preparation of nectar for DHA and MGO detection

Table 2.1: Volumes of MGO (1.5 mg/mL) and DHA (1.5 mg/mL) used in standards

Standard	Volume MGO (μL)	Volume DHA (μL)
1	10	0
2	8	2
3	6	4
4	4	6
5	2	8
6	0	10

To a vial containing HA (10 μL, 0.5 mg/mL) was added:

- **Standards**: The required volumes of MGO (1.5 mg/mL) and DHA (1.5 mg/mL) (**Table 2.1**) together with water (190 μL).

- **Bulk Test**: Nectar sample (200 μL).
- **SFT**: Nectar sample (400 μL).

To this solution the derivatising agent PFBHA (50 μL, 20 mg/mL in a pH 4 citric acid buffer) was mixed and let stand (1 h). DCM (2 mL) was added and mixed (SFT used DCM (1 mL)). The vial was placed in a freezer (-20°C) until the aqueous layer froze to aid extraction. An aliquot (1 mL) of the DCM layer was withdrawn into a GC vial. TMSI (50 μL) was added, mixed and let stand (1 h). This was analysed by GC-FID.

2.8 Preparation of nectar for sugar detection

To a vial containing mannitol (5 μL, 5 mg/mL) was added:

- **Blank**: nothing.
- **Bulk Test**: Nectar sample (20 μL).
- **SFT**: Nectar sample (50 μL).

The vial was then freeze dried. To the dry sample TMSI (50 μL) was added, mixed and let stand (1 h) at room temperature. Heptane (1 mL) was added with mixing before analysis by GC-FID.

2.9 Data processing

The GC chromatogram peaks were manually integrated (**Figure 2.2**, **2.3**) using GC Chemstation the peak areas were recorded for processing.

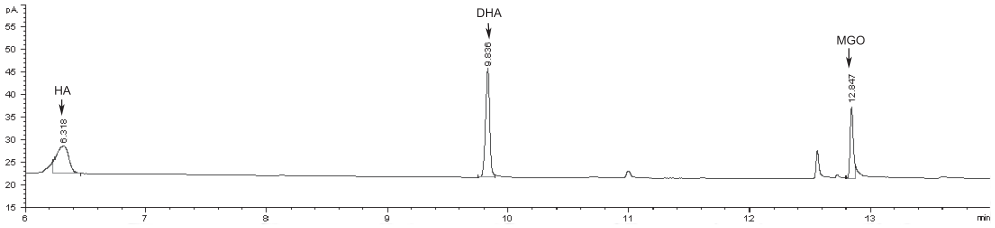


Figure 2.2: An example of a standard integrated for HA (6.318 mins), DHA (9.836 mins) and MGO (12.847 mins). Only a single peak of MGO is integrated, this is the strongest peak observed and in nectar samples it is the one that can be confidently integrated if MGO is observed in the sample.

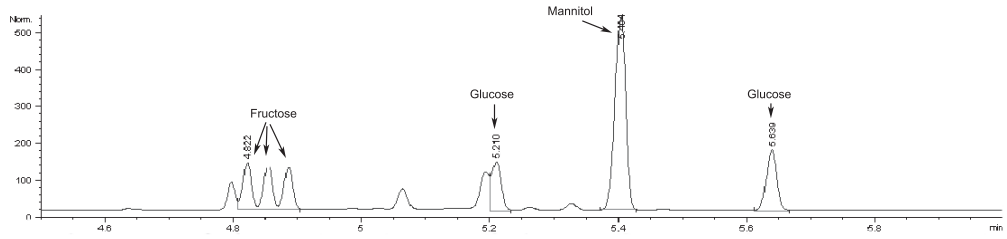


Figure 2.3: An example of a sugar integration from a nectar sample. Fructose (4.822, 4.85 and 4.88 mins), glucose (5.210, 5.6039 mins) and mannitol (5.404 mins). Sucrose (9.8 mins) is not discernible in nectar samples and contributes an insignificant amount to the total sugar value.

2.9.1 Finding DHA

Figure 2.4 provides the calibration curve (DHA peak area/HA peak area vs DHA (mg)) that gives the linear equation to convert DHA/HA to DHA (mg).

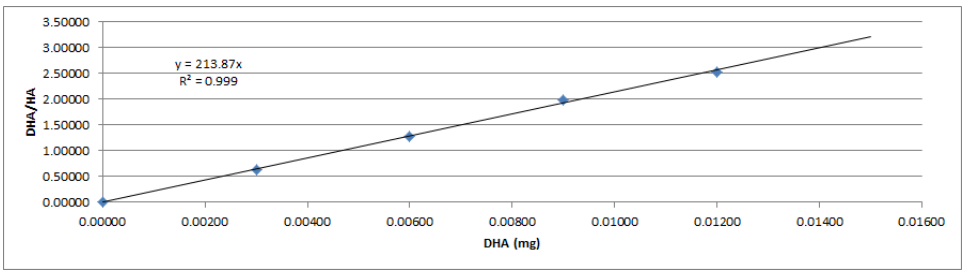


Figure 2.4: An example of a DHA/HA vs DHA(mg) calibration curve. Each point represents an independent preparation.

DHA (mg) for the nectar samples was calculated using **Equation 2.1** and **Equation 2.2**.

$$\frac{DHA(peak\,area)}{HA(peak\,area)} = DHA/HA \tag{2.1}$$

$$\frac{DHA/HA}{calibration\,curve\,gradient\,(mg^{-1})} = DHA(mg) \tag{2.2}$$

This gives the amount of DHA (mg) in the 200 μL (bulk extraction) or 400 μL (SFT).

2.9.2 Finding Tsugar

Tsugar (mg) for the nectar samples, (Tsugar = Total of fructose, glucose and sucrose) was calculated using **Equation 2.3**- **Equation 2.5**.

$$\frac{Sugar(peak\,area)}{Mannitol(peak\,area)} = \frac{Sugar}{Mannitol} \tag{2.3}$$

(sugar = fructose, glucose or sucrose)

$$\frac{\frac{Sugar}{Mannitol} \times Added\,Mannitol\,(mL) \times Mannitol\,Conc(mg/mL))}{(Sugar : Mannitol\,Response\,Factor)} = Sugar(mg) \tag{2.4}$$

- Fructose: Mannitol Response Factor = 0.5335
- Glucose: Mannitol Response Factor = 0.8217
- Sucrose: Mannitol Response Factor = 0.7126

$$Fructose(mg) + Glucose(mg) + Sucrose(mg) \times 10(or\ 8\ for\ SFT) = Tsugar \tag{2.5}$$

This gives the Tsugar amount in 200 μL (bulk) or 400 μL (SFT).

By dividing DHA by Tsugar.

$$DHA(mg)/Tsugar(mg) = DHA/Tsugar(mg/mg) \tag{2.6}$$

The amount of DHA present in relation to the total amount of sugar present could be calculated.

2.9.3 Samples

Each tree was analysed in replicate (n = 10) using either the bulk or SFT methods. The DHA/Tsugar for each replicate was recorded and all were averaged to give the average DHA/Tsugar for a tree.

2.9.4 Outliers

As only a relatively small number of extract replicates are used for each tree, if an abnormal value is produced due to a combination of biological variation and experimental error, it can have a significant effect on the average DHA/Tsugar value. With the small number of extract replicates, it cannot be conclusively proven that the extract replicates are normally distributed. However, it might be expected that a tree would have an average value of DHA/Tsugar with values found around this, so normal distribution can be assumed. If the % RSD was larger than 25% then the sample was tested for the presence of an outlier. To test for outliers the Gibbs' tests for outliers was used. The single outlier test (**Equation 2.7**) was first used to test a suspected outlier:[76]

$$\frac{|\bar{x} - x|}{\sigma} = G \tag{2.7}$$

($\bar{x}$ is the mean of the extract replicates, x is the extract replicate being tested, σ is the standard deviation of the extract replicates)

It can be said with 95% confidence that the value is an outlier if G exceeds 2.176.[76]

If the % RSD was still larger than 25% when a single outlier was removed, it was assumed that it was part of an outlier pairing. The pairing either involved the largest and smallest values or the two largest/smallest values. Having two outliers present causes the data to be skewed and this can disguise the presence of any outlier.

Two tests were then carried out to ascertain the presence of any outlier pairings, depending upon if they were the largest and smallest values or a pairing of the two largest/smallest values.[76] The largest, smallest values test is shown in (**Equation 2.8**):

$$\frac{|x_n - x_1|}{\sigma} = G \tag{2.8}$$

(x_n is the largest value, x_1 is the smallest value)

It can be said with 95% confidence that the two values are outliers if G exceeds 3.68.[76]

The two largest/smallest values tests is given by (**Equation 2.9**):

$$1 - (\frac{(n-3)\sigma^2_{n-2}}{(n-1)\sigma^2}) = G \tag{2.9}$$

(n is the number of extract replicates,σ_{n-2} is the standard deviation of the extract replicates excluding the two possible outliers)

It can be said with 95% confidence that the two values are outliers if G exceeds 0.7695.[76]

If removal of two outliers does not reduce the % RSD below 25, then it was assumed that the variation was due to the whole set of extract replicates and not caused by one or two individual extract replicates.

Chapter 3

Method development

3.1 Gas chromatography methods

Two GC instruments were used for method development. One had a split/splitless inlet and the other an on-column inlet. Both instruments were compared to determine which would be most suitable. DCM and *n*-Heptane were used as wash solutions in both instruments. Five washes with each were required to avoid a jammed syringe on the autosampler.

3.1.1 Split/Splitless inlet GC

The instrument and method used was described in **Section 2.3**.

3.1.2 On-column inlet GC

The instrument used was a Agilent Technologies 6890N Network GC system with an on-column inlet and a FID. This was equipped with an Agilent Technologies 7683 series autosampler and had a Zebron ZB-5, 0.32mm capillary GC column installed. A 2 μL injection was used. The carrier gas (hydrogen) flow was 2.9 mL/min. The oven program had an initial temperature of 50 ℃ with a 2 min hold, followed by ramping at 10 ℃/min to 250 ℃ with a 3 min hold. The FID was held at 325 ℃ with a hydrogen flow of 40 mL/min, air flow 400 mL/min and a make-up flow of 10 mL/min.

3.1.3 Comparison of splitless versus on-column injection

The two instruments were compared to determine which one gave the best reproducibility. One sample was prepared using the initial method (**Section 3.3.2**) with volumes increased to allow more injections to be made. HA (20 μL), DHA (20 μL) and MGO (20 μL) were combined with PFBHA (40 μL) and water (200 μL). DCM (4 mL) was added for extraction. Two aliquots of DCM ($\sim$1 mL) were derivatised with TMSI (120 μL)(1 h). The samples were injected five times (using splitless and on-column injection respectively); heptane injections were used between each replicate to keep the syringe and column clean.

Table 3.1: Comparison of splitless and on-column injections.

On-Column			Splitless		
Replicates	DHA/HA	MGO/HA	Replicates	DHA/HA	MGO/HA
1	3.75	0.46	1	4.98	2.01
2	1.25	0.61	2	4.85	2.08
3	3.32	0.29	3	5.08	2.23
4	2.85	0.65	4	4.79	2.05
5	3.98	0.88	5	5.07	2.37
Average	3.03	0.58	Average	4.95	2.15
Std Dev	1.08	0.22	Std Dev	0.13	0.15
% RSD	35.73	38.26	% RSD	2.64	7.04

The results in **Table 3.1** demonstrate that the splitless injection gave better reproducibility than the on-column. This, combined with greater tolerance to syringe jamming by the splitless auto-sampler and more resolved

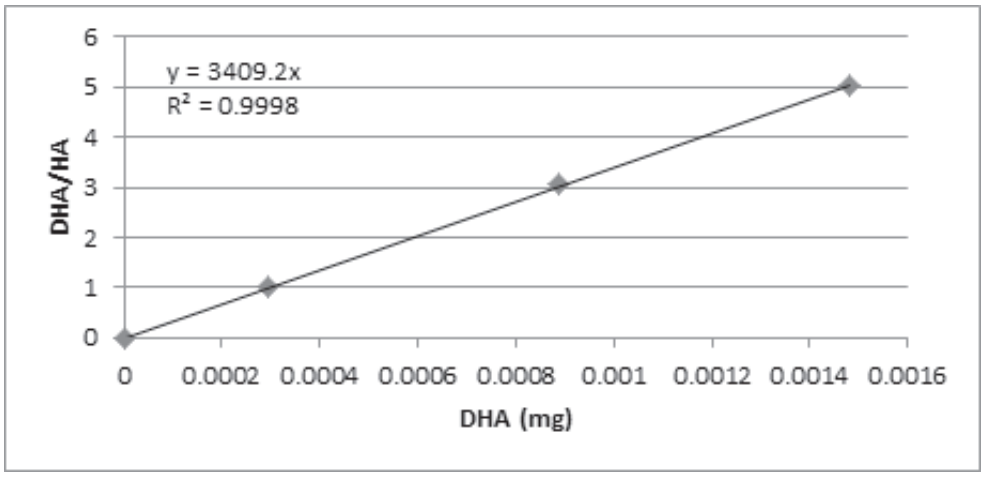
chromatograms, indicated that the splitless injection GC-FID was the most reproducible, and would be used for the quantitative work in this project.

3.2 Detection limits for DHA and MGO for splitless injections

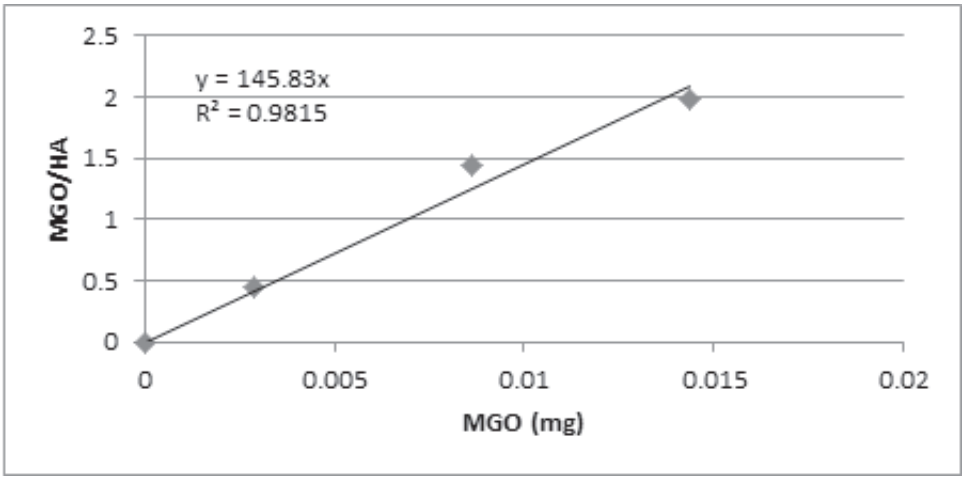
The Limit of detection (LOD) and Limit of quantitation (LOQ) were found for MGO and DHA. The DHA standard (0.15 mg/mL) was diluted by 1/20, 1/40 and 1/200. The MGO (1.5 mg/mL) was diluted by 1/10, 1/20, 1/40 and 1/200. Using a modification of **Section 3.3.2**, an aliquot (5 μL) of the dilution was added to HA (10μL), along with water (200 μL) and PFBHA (20 μL). After standing (1 h), DCM (2 mL) was added. An aliquot of DCM ($\sim$1 mL) was withdrawn into a GC vial (1.5 mL). TMSI (60 μL) was added, mixed and let stand (1 h), followed by analysis.

After analysis on the GC-FID the 1/200th dilution of DHA and the MGO 1/40 dilution were at approximately the LOD. The minimum peak heights that the operator is able to distinguished from the baseline. The 1/200th dilution of DHA and the 1/40 dilution of MGO were remade with HA and re-injected 10 times. Calculation of the standard deviation (σ) for the DHA/HA ratios and MGO/HA ratio in the replicates allowed the LOD and LOQ to be found. The LOD is defined as 3σ and the LOQ is 10σ.[77–79] The ratios of the peak area with HA were converted to DHA (mg) and MGO (mg) using the standard curves **Figure 3.1**.

The LOD and LOQ are given in **Table 3.2**. The % RSD is large due to the difficulty of distinguishing the peaks from the baseline and then manually integrating the peak area. The LOQ translates to a minimum detectable mass of 20.52 ng of DHA and a minimum detectable mass of 312.78 ng of MGO.



(a) DHA standard curve



(c) MGO standard curve

Figure 3.1: Standard curves for DHA and MGO for determination of detection limits.

Though the capability was established to measure MGO using this method, no MGO above the LOD was observed in this survey of mānuka flower nectar. Therefore MGO may be present, but a lower LOD is required to detect it if it is there.

Table 3.2: The LOD and LOQ for DHA and MGO.

Replicates	DHA/HA	Replicates	MGO/HA
1	0.0300	1	0.0169
2	0.0334	2	0.0156
3	0.0208	3	0.0215
4	0.0292	4	0.0238
5	0.0272	5	0.0244
6	0.0134	6	0.0230
7	0.0251	7	0.0292
8	0.0184	8	0.0266
9	0.0193	9	0.0246
10	0.0134	10	0.0292
Average	0.0230	Average	0.0235
Std Dev	0.00700	Std Dev	0.00460
% RSD	30.4	% RSD	19.4

DHA/HA Peak Ratio		MGO/HA Peak Ratio	
LOD	0.0210	LOD	0.0137
LOQ	0.0700	LOQ	0.0456

Mass of DHA in 205 μL		Mass of MGO in 205 μL	
LOD	6.16 ng	LOD	93.8 ng
LOQ	20.5 ng	LOQ	313 ng

3.3 Sample preparation method

3.3.1 Materials

The materials are the same as used in **Section 2.1**

3.3.2 Initial method for preparations of standards

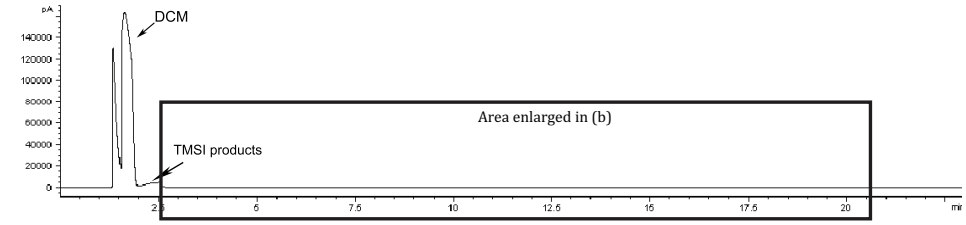
The method was a development of an unpublished method.[80] To the internal standard HA (5 μL, 0.2 mg/mL), the required volumes of MGO (1.5 mg/mL) and DHA (0.15 mg/mL), were added as well as PFBHA (10 μL, 20 mg/mL in a pH 4 citric acid buffer). Water (200 μL) was added, mixed and allowed to stand (1 h). DCM (1 mL) was added mixed and let stand to separate. The vial was placed in a freezer (-20 °C) to allow the water layer to freeze. The DCM ($\sim$250 μL) layer was withdrawn into a GC insert (500 μL), this was inserted into a GC vial (1.5 mL). TMSI (30 μL) was added, mixed and let stand (1 h). The standard/sample was analysed by GC-FID.

Some problems arose while using this method. The GC autosampler syringe was prone to stall, suggesting a build up of TMSI related compounds limiting the autosampler's capability. A noticeable peak, probably TMSI itself, overlapped the HA peak on occasions. Tri-Sil, a HMDS/TMCS in pyridine mixture, was also trialled as a silylating agent. This was unable to be used as it reacted with the amine group on the excess PFBHA that was present in the extract and this gave a peak that overlapped with HA.

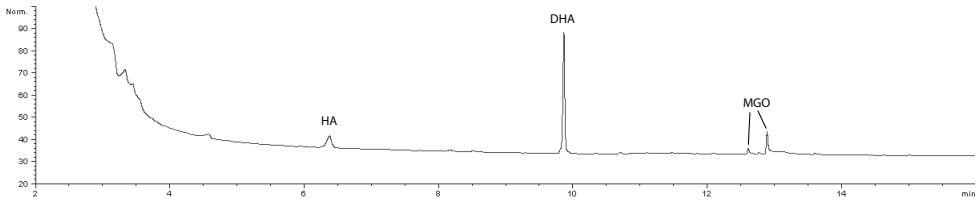
3.3.3 Addition of water to remove TMSI

As TMSI reacts with water to give TMSiOH, which is insoluble in heptane, an experiment using a wash of water after silylation was tried. Three samples

were prepared using the initial method (**Section 3.3.2**) with increased volumes to allow multiple aliquots to be taken. HA (10 μL), DHA (10 μL) and MGO (10 μL) were reacted with PFBHA (20 μL) and water (200 μL). DCM (2 mL) was used for extraction. Two aliquots (∼300 μL and ∼1 mL) were removed. TMSI (50 μL) was reacted with one aliquot (∼300 μL) in a GC insert. The second aliquot (∼1 mL) was reacted with TMSI (120 μL, 1 h). A water wash (100 μL) with centrifugation (1 min, 4000 rpm) was carried out on the second aliquot. Supernatant (∼300 μL) was transferred to a GC insert. 2 μL of each sample was injected with a splitless injection.



(a)



(b)

Figure 3.2: (**3.2a**) Gas chromatogram of a sample of HA, DHA and MGO prepared using the initial method. DCM ∼1.75 min and suspected TMSI products which form a broad peak centred at ∼ 2.75 min. (**3.2b**) HA ∼6.4 min, DHA ∼9.8 min, MGO ∼12.8 min and the TMSI products peak at ∼4 min.

Comparison of **Figure 3.2** and **Figure 3.3** demonstrates that the wash with water reduced the signal strength of HA, DHA and MGO with no apparent effect on the overlapping large peak. This method therefore, was not an improvement on the initial method and hence was not used.

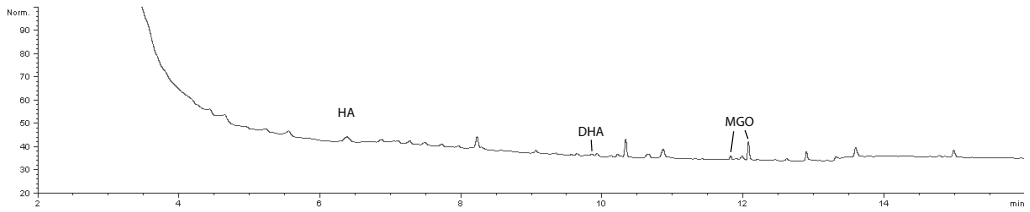


Figure 3.3: Gas chromatogram of a sample of HA, DHA and MGO prepared using the initial method with a water wash. HA ∼6.4 min, DHA ∼9.8 min, MGO ∼12.8 min are reduced in signal and the TMSI products peak is still present at ∼4 min.

3.3.4 Attempt to remove TMSI by evaporation

A possible method to remove unreacted TMSI, was evaporation of the TMSI after the reaction. This was tested by making 8 samples prepared using the initial method (**Section 3.3.2**) with HA (10 μL), DHA (10 μL) and MGO (10 μL). After freezing, two aliquots (∼300 μL and ∼1 mL) were removed. One aliquot (∼300 μL) was reacted with TMSI (50 μL, 1 h) in a GC insert and analysed. The second aliquot (∼1 mL) was reacted with TMSI (50 μL, 1 h). Evaporation of TMSI to dryness in the second aliquot was achieved using a stream of dry nitrogen (∼2 h). The remaining solid was suspended in heptane with sonication, and centrifuged (3 min, 4000 rpm) to remove any undissolved material. Supernatant (∼300 μL) was transferred to a GC insert; the sample was then analysed.

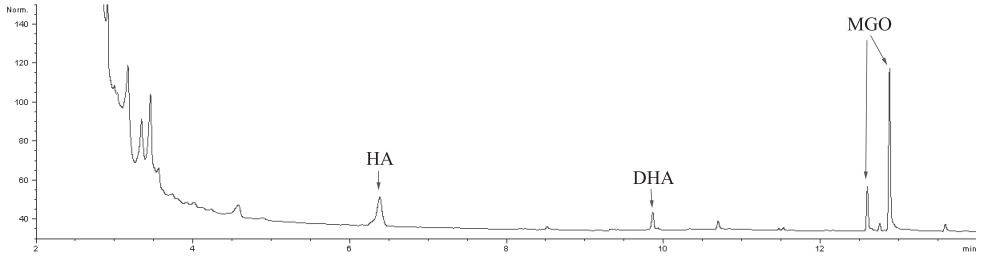


Figure 3.4: Gas chromatogram of a sample of HA, DHA and MGO prepared using the initial method without TMSI evaporation. HA ∼6.5 min, DHA ∼9.8 min and MGO ∼12.9 min and large interfering peak ∼4 min.

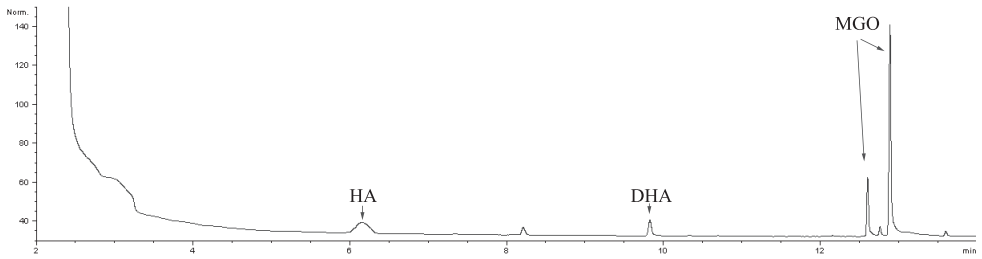


Figure 3.5: Gas chromatogram of a sample of HA, DHA and MGO prepared using the initial method but with TMSI evaporation. HA ∼6.5 min, DHA ∼9.8 min and MGO ∼12.9 min and large interfering peak at ∼4 min.

Figure 3.4 shows the HA, DHA and MGO peaks. The edge of the interfering large peak can be seen at ∼4 min, this can shift the baseline sufficiently to reduce the HA signal. **Figure 3.5** shows that the offending peak has been reduced by evaporation. Coincidentally, the problems with the syringe have disappeared. It also shows that the HA peak has become broader, increasing the possibility of overlapping peaks in nectar samples.

Table 3.3 demonstrates that there is a slight increase in DHA/HA and MGO/HA ratios with the evaporation method. This is because HA is lost preferentially, during evaporation. Also the increase is accompanied by an increase in % RSD from 6.75% to 18.7% for the DHA/HA ratio which is not an acceptable % RSD for a standard sample.

A student z test on the DHA/HA ratio (**Table 3.4**) confirms with 95% confidence that the two methods do give different results rather than a cleaner chromatogram as desired. This however was not observed visually, except in the broadening of the HA peak.

Spaulding *et al.*(2002) have also observed low molecular weight molecules like HA being lost in those PFBHA derivatising methods which use evaporation to remove TMSI.[74]

Evaporation to remove TMSI was shown to be effective at reducing the overlapping peak; however, the increase in % RSD was not acceptable for a

Table 3.3: Comparison of no-evaporation and evaporation method

No-Evaporation Method						Evaporation Method					
Sample (Aliquot 1)	HA	DHA	MGO	$\frac{DHA}{HA}$	$\frac{MGO}{HA}$	Sample (Aliquot 2)	HA	DHA	MGO	$\frac{DHA}{HA}$	$\frac{MGO}{HA}$
1	65.76	15.34	117.30	0.233	1.784	1	62.78	23.21	160.90	0.370	2.563
2	66.59	15.40	122.75	0.231	1.843	2	59.34	12.13	152.57	0.204	2.571
3	64.70	15.40	131.07	0.238	2.026	3	64.95	19.12	187.88	0.294	2.893
4	66.97	15.63	138.69	0.233	2.071	4	65.67	23.27	216.45	0.354	3.296
5	68.75	15.46	150.06	0.225	2.183	5	65.73	21.85	178.56	0.332	2.717
6	71.82	16.20	156.11	0.226	2.174	6	71.67	26.03	252.28	0.363	3.520
7	77.10	20.94	196.43	0.272	2.548	7	76.53	29.03	297.59	0.379	3.889
Average	68.81	16.34	144.63	0.24	2.09	Average	66.667	22.091	206.604	0.328	3.064
Std Dev	4.33	2.05	26.74	0.026	0.253	Std Dev	5.71	5.39	52.61	0.062	0.514
% RSD	6.29	12.55	18.49	6.75	12.11	% RSD	8.57	24.41	25.46	18.75	16.78

Table 3.4: z-Test: no-evaporation method compared to evaporation method

	No-Evaporation	Evaporation
Mean	0.2369	0.3283
Known Variance	0.0003	0.0038
Observations	7	7
Hypothesized Mean Difference	0	
z	-3.8027	
z critical two-tail	1.9600	
As z > (z critical two-tail) there is 95% confidence that the two methods are different		

quantitative method, nor was the change in the ratio. The initial method was therefore retained.

3.3.5 Volume of dichloromethane used

To test if increasing DCM volume affects the result the following samples were prepared using a modification of **Section 3.3.2**. HA (20 μL), DHA (20 μL) and MGO (20 μL) were mixed with PFBHA (40 μL) and water (200 μL). The samples were extracted with DCM (4 mL and 2 mL respectively). Once the water had frozen, aliquots (1 mL) were silylated with TMSI (120 μL) before analysis.

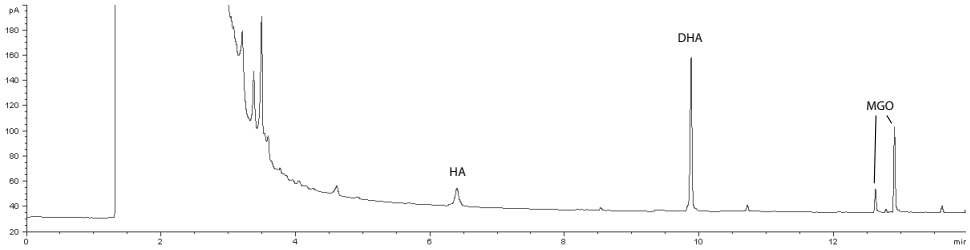


Figure 3.6: Gas chromatogram of extraction using 2 mL of DCM. HA ∼6.5 min, DHA ∼9.8 min and MGO ∼12.9 min.

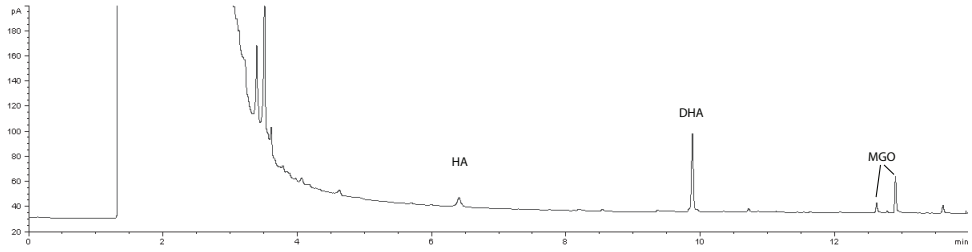


Figure 3.7: Gas chromatogram of extraction using 4 mL of DCM. HA ∼6.5 min, DHA ∼9.8 min and MGO ∼12.9 min.

Table 3.5: Comparison of peak areas and ratios of HA, DHA and MGO using 2 mL and 4 mL DCM for extractions

Sample	HA	DHA	MGO	DHA/HA	MGO/HA
2 mL of DCM	43.0	224.3	106.3	5.2	2.5
4 mL of DCM	20.9	109.1	46.9	5.2	2.2

Figure 3.6 and **Figure 3.7** show that the chromatograms differ in intensity.

But the DHA:HA ratio remains consistent and the MGO:HA ratio is within 13% (**Table 3.5**). From this it can be seen that 2 mL of DCM is as efficient as 4 mL of DCM at extracting the sample; so the lesser volume was retained.

This survey used DCM (2 mL). The use of DCM (1 mL) for the single flower test however, demonstrated that DCM (1 mL) can be used to possibly increase the sensitivity of future iterations of this method.

3.3.6 Volume of TMSI used

TMSI is added in excess to silylate the PFBHA derivatised HA, DHA and MGO. To ensure that excess is added a number of volumes of TMSI (50 μL, 100 μL, 150 μL and 200 μL) were tested. Each volume of TMSI was tested in triplicate using the following initial preparation based off the method in **Section 3.3.2**:

HA (10 μL), DHA (10 μL) and water (170 μL) were mixed with PFBHA (50 μL, 1 h). The samples were extracted with DCM (2 mL). Once the water had frozen, aliquots of DCM (1 mL) were silylated with TMSI. The samples were analysed on the GC-FID.

The amount of DHA used was well in excess of that expected to be found in a flower extract.

From **Table 3.6**, TMSI (50 μL) is shown to be in sufficient excess to silylate the test samples; addition of larger volumes of TMSI did not have a significant effect since the standard deviation range of all volumes overlapped. Therefore TMSI (50 μL) was used for subsequent experiments.

Table 3.6: Comparing different volumes of TMSI used for silylation to the DHA/HA recorded.

TMSI μL added	Average DHA/HA	Std Dev
50	0.086	0.005
100	0.090	0.001
150	0.091	0.004
200	0.094	0.005

3.4 Sugar analyses

The sugars in honey are derived from the nectar collected by the bees. The amount of DHA that is expected to be found in honey could be predicted by using the relationship between DHA and the sugars in nectar. The literature[41,45] demonstrates that the extraction method used for the DHA in flowers is also effective for the sugars, so further extraction testing was not required.

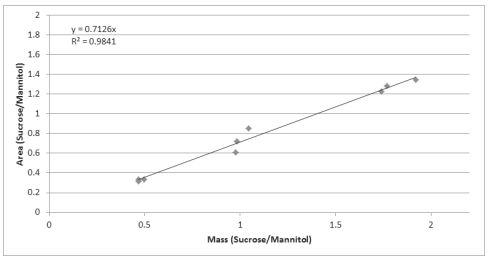
3.4.1 Initial method for preparation of nectar sugars' standards

The method is a development of an unpublished method.[80]

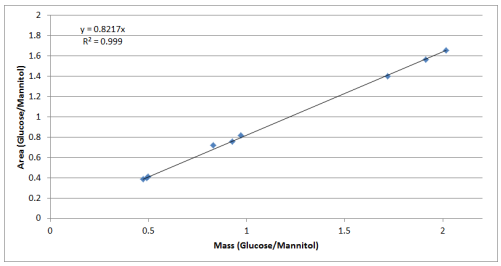
Equal masses of fructose, glucose, sucrose and mannitol (∼100 mg) were dissolved in water (100 mL). An aliquot (20 μL) of this was freeze dried. TMSI (50 μL) was added and left to react (1 h) at room temperature. Heptane (1 mL) was added mixed and centrifuged (1 min , 4000 rpm) before analysis.

3.4.2 Response factors of sugars

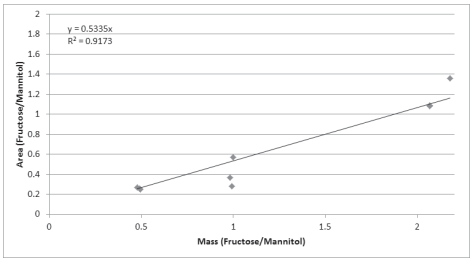
Mannitol is used as the internal standard for the sugars as it is not found in the nectar of mānuka flowers. The response factor of each sugar to mannitol is required, allowing the mannitol peak area to enable the amount of sugars present to be calculated. This was achieved using a modified version of the initial method (**Section 3.4.1**) with only one sugar (S) added together with mannitol (M) and with weight ratios of S (∼50 mg): M (∼50 mg), 2S (∼50 mg): M (∼25 mg) and S (∼25 mg): 2M (∼50 mg). The rest of the method remained the same.



(a) Response factor curve for sucrose, giving the response factor as 0.7126



(b) Response factor curve for glucose, giving the response factor as 0.8217



(c) Response factor curve for fructose, giving the response factor as 0.5335

Figure 3.8: Response factor curves for the three sugars, each point represents a separate preparation.

These response factors were used in **Equation 2.4**.

3.4.3 Amount of TMSI added

It is suggested that, although fructose, glucose and sucrose are the major constituents of nectar, other organic compounds such as carboxylic acids and amino acids are also present. These may also react with TMSI, leaving insufficient TMSI to react with the fructose, glucose and sucrose. To test this, nectar from L3995-2009 was used with varying amounts of TMSI (50 μL, 100 μL and 200 μL), and tested in duplicate. The samples were prepared as in **Section 2.8** until the TMSI addition and different volumes of TMSI were added to each.

Table 3.7: Addition of different volumes of TMSI to nectar sugar to determine if TMSI is in excess.

TMSI (μL) added	Replicate	Fructose (mg) in 20 μL	Glucose (mg) in 20 μL	$\frac{Fructose}{Glucose}$	Tsugar (mg) in 200 μL
50	a	0.031	0.0189	1.67	0.50
	b	0.033	0.0187	1.75	0.51
100	a	0.033	0.0192	1.73	0.53
	b	0.034	0.0188	1.81	0.53
200	a	0.033	0.0176	1.88	0.51
	b	0.035	0.0186	1.87	0.53
Average		0.033	0.0186	1.78	0.52
Std Dev		0.001	0.0006	0.08	0.01
% RSD		3.43	3.03	4.58	2.46

The sugars did not appear to increase, as the volumes of TMSI were increased. TMSI (50 μL), is therefore in excess of that which is required to

silylate the nectar sugars (**Table 3.7**). Subsequent work used TMSI (50 μL) for silylation.

3.5 Nectar sampling methods

This section describes the development of the method to allow sufficient DHA to be extracted from mānuka flowers for quantitative analysis. The development work in this section was carried out on flowers from mānuka trees that were not part of the survey group unless stated otherwise. The flowers from these trees were collected at the same time and frozen (-20 ℃). One tree was used in each test.

3.5.1 Quantity of flowers required for extraction

Due to the variation in the number of flowers collected for each tree, it was important to establish how many flowers were required to give a measurable signal. The test was carried out using a variation of the method in ref.[80]

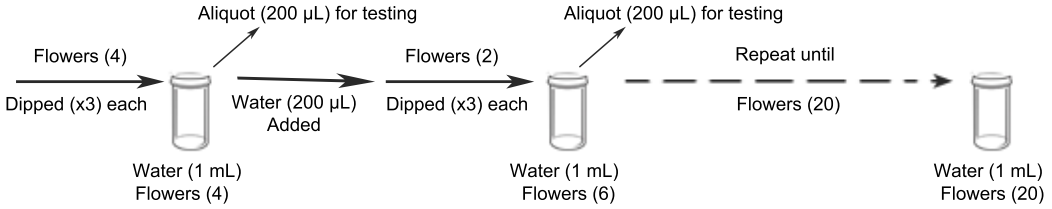


Figure 3.9: Flow chart demonstrating how the flower dipping was performed and aliquots taken for sampling.

Flowers (4) were dipped (x3) into water (1 mL). Aliquots (200 μL) were removed to another vial and replaced by water (200 μL). Two further flowers were dipped into the solution and an aliquot taken and replaced by water. This was repeated until a total of 20 flowers had been dipped (**Figure 3.9**). HA (5 μL) was added to the aliquots, these were derivatised with PFBHA

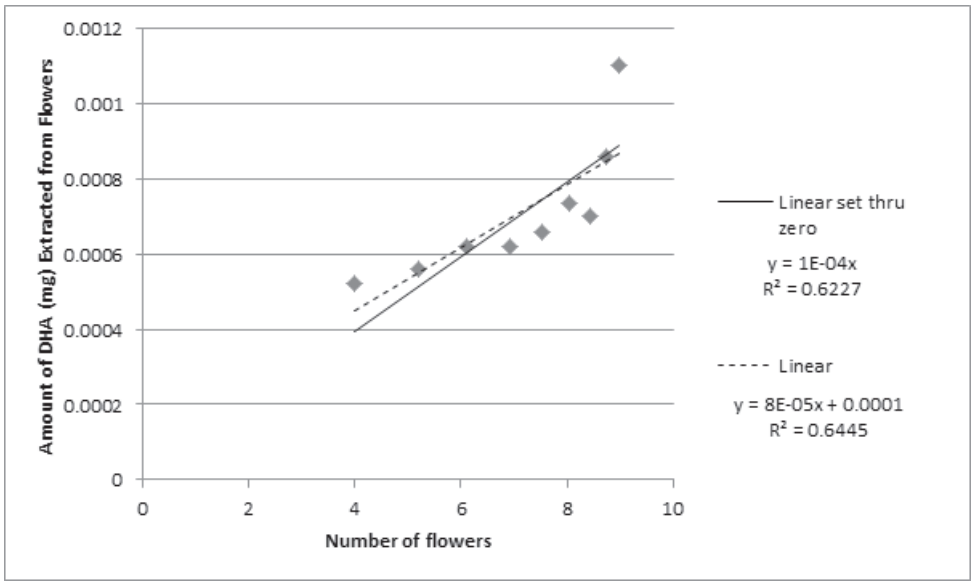


Figure 3.10: Comparison of the number of flowers dipped with the amount of DHA extracted

(20 μL) and extracted with DCM (1 mL). The extract (250 μL) was silylated with TMSI (30 μL) for analysis by GC-FID.

As expected the amount of DHA extracted, increases with the number of flowers used (dotted line) (**Figure 3.10**). As DHA varies between trees, 20 flowers were used where possible to ensure detectability and to compensate for inter-flower variability. The biological variation between flowers can be seen by the deviation of the flowers from the trend (filled line) going through zero, as well as the moderate correlation with the trend, $R^2 = 0.64$. This could possibly be attributed to the flowers being picked from the same tree at different stages of their flowering cycle.

3.5.2 Extraction method

Alternative methods to dipping have been reported that might have been used to sample flowers. As the flowers had already been picked, the methods were restricted. The work by Lanza *et al.*(1995) confirmed that flowers from the same tree could be combined to form one sample.[45] Three methods were

chosen to determine the best method for the extraction of the mānuka flowers sampled.

These were:

Method 1: Flowers (20) were taken from a frozen testing bag, added to water (4 mL) and mixed. After 20 mins the flowers were removed with shaking from the nectar/water solution. The solution was then placed in the freezer.

Method 2: Flowers (20) were taken from a frozen testing bag. The flowers were individually dipped (x3) into the same water (4 mL) and shaken to remove excess water. After the flowers were dipped the vial was placed in the freezer.

Method 3: Flowers (20) were taken from a frozen testing bag. Each flower had water (10 μL) from a stock solution, (4 mL) added via an autopipette to its center. This was pumped and swirled around the flower. The water was transferred, with the nectar, back into the stock solution. This was repeated twice for each flower. Once all the 20 flowers were extracted, the stock solution was placed in the freezer.

Each method was repeated 8 times using flowers from the same bag. Nectar solution (200 μL) was combined with HA (5 μL ,0.2 mg/mL) and derivatised using PFBHA (10 μL, 20 mg/mL). After the reaction (1 h), DCM (1 mL) was added. These were stored (-20 ℃) to freeze the water layer. DCM extract ($\sim$250 μL) was transferred to a GC insert and silylated with TMSI (30 μL). These were injected using an on-column injector *.

*This work was carried out before the work in **Section 3.1.3** showed the splitless was better than on-column injection, it was not repeated, as the difference between the methods, was judged to be greater than that between the instruments

Table 3.8: Comparison of the three extraction methods; **method 1:** Soaking the flowers (20) for 20 mins, **method 2:** individually dipping (x3) flowers (20) and **method 3:** individually rinsing (x2) the flowers (20) with stock solution (10 μL). In some samples non-detectable (ND) levels of DHA were found.

Method	1	2	3
Sample	Mass DHA (mg)	Mass DHA (mg)	Mass DHA (mg)
1	0.00031	0.00010	0.000080
2	0.00054	ND	0.00033
3	0.00044	0.00028	ND
4	0.00065	0.00022	ND
5	0.00057	0.00049	0.00038
6	0.00064	0.00029	ND
7	0.00076	ND	ND
Average	0.00056	0.00020	0.00011
Std Dev	0.00015	0.00018	0.00017
% RSD	26	90	150

The results, **Table 3.8**, demonstrate that **method 1** gave the best % RSD. The relatively poor % RSD overall was expected due to biological variations between the flowers on the same tree and due to the on-column GC instrument. **Method 1** also had the highest mass of DHA extracted and therefore extracts the largest amount of DHA. Method 3 was discontinued as it failed to extract enough DHA to allow quantitation. **Method 1** was used throughout this survey, although **method 2** was continued in **Section 3.5.4** to confirm that **method 1** continued to give good results when different trees were sampled.

3.5.3 Nectar residue in sample bags

The possibility exists that nectar may be lost or transferred between flowers while in the sample bag. To test if this occurs, the sample bag was rinsed with water after all the flowers were removed and analysed. Using a modified preparation method based upon **Section 3.3.2**; water (4 mL) was added to the sample bag and shaken; an aliquot (200 μL) was combined with HA (5 μL) and derivatised using PFBHA (10 μL). DCM (1 mL) was added and an aliquot of DCM ($\sim$250 μL) was transferred to a GC insert and silylated with TMSI (30 μL, 1 h).

Table 3.9: DHA extracted from an empty sample bag.

DHA extracted from bag		
Sample	DHA/HA	DHA (mg)
1	0.036	0.000205
2	0.044	0.000249
3	0.082	0.000465
Average	0.054	306.38
Std Dev	0.025	139.06
% RSD	45.39	45.39

Table 3.9 reveals that DHA was found in the bag confirming that the nectar is transferred between flowers and the sample bag and likely also between individual flowers. Comparing **Table 3.9** and **method 1** (**Table 3.8**) *, it is demonstrated for this work that the bag had a DHA content approximately half that of 20 flowers †. This transfer should not be significant as the transfer of nectar around the bag would be a form of averaging the nectar,

*The bag used for testing was from the testing in **Section 3.5.2.**

†A bag can typically contains $\sim$ 500 flowers dependent on the flowers' size and shape.

complementing the average found by using 20 flowers in the extraction. This work only looked at the DHA of the nectar remaining in the bag, as it was the limiting factor for this project. Future work may also wish to look at the sugar content to determine the DHA/Tsugar remaining in the bag.

3.5.4 Differentiating mānuka trees

To test that the method is able to differentiate between mānuka trees, one sample bag from each of two different mānuka trees were tested. These trees were assumed to be different due to their environmental and physical differences. Tree VB014 is located in a well maintained urban garden and has dark pink/red flowers. Tree VB190 is located on semi-managed farmland and has white flowers. Both **method 1** and **method 2** were used for the comparison. **Method 2** was used to confirm that **method 1** continued to give the best results for an inter-tree comparison.

Table 3.10: Comparing the mānuka trees VB014 and VB190 using soaking (**Method 1**) and dipping (**Method 2**)

Method 1 (DHA\HA)						
Sample	1	2	3	Average	Std Dev	% RSD
VB014	2.65	2.85	2.37	2.61	0.34	12.83
VB190	0.0914	0.0819	0.0893	0.0856	0.0052	6.11

Method 2 (DHA\HA)						
Sample	1	2	3	Average	Std Dev	% RSD
VB014	0.248	0.299	0.440	0.37	0.10	26.92
VB190	0.0487	0.0443	0.0517	0.0480	0.0052	10.93

Both methods were able to distinguish a difference between VB014 and VB190 but **method 1** consistently extracts more DHA than **method 2** and has a smaller % RSD. The partial origin of the variation responsible for the large % RSD can be seen in **Figure 3.11**, which demonstrates the range of states the mānuka flowers are in after being stored and frozen (20 ℃) in the sample bag.

Figure 3.11: An example of the range of states of the sampled flowers after storage (VB014)

3.6 Single flower test (SFT)

During sample collection it was found that not all trees sampled provided sufficient flowers for the standard method, (**Section 2.6**) which requires 20 flowers per replicate. This method was modified to allow a single flower to be extracted for a replicate, as described in **Section 2.7 (DHA method)** and **Section 2.8 (Tsugar method)**.

3.6.1 Does the appearance of the flower affect DHA/Tsugar?

The age of the flower and state of the flower after freezing might possibly have an effect on the DHA/Tsugar levels. To test this, replicates using the

SFT were carried out.

Table 3.11: Comparing DHA/Tsugar values with the state of the flowers.

SFT Replicate	$\frac{DHA}{Tsugar}$	Image	SFT Replicate	$\frac{DHA}{Tsugar}$	Image
SFT-1	0.00072		SFT-11	0.00127	
SFT-2	0.00037		SFT-12	0.00035	
SFT-3	0.00068		SFT-13	0.00041	
SFT-4	0.00054		SFT-14	ND	
SFT-5	0.00040		SFT-15	0.00130	
SFT-6	0.00061		SFT-16	0.00064	
SFT-7	0.00160		SFT-17	0.00048	
SFT-8	0.00092		SFT-18	0.0011	
SFT-9	ND		SFT-19	0.00026	
SFT-10	ND		SFT-20	ND	

Table 3.11 shows that there is a large variation between flowers from a single tree. Flowers in bud provided no detectable (ND) DHA/Tsugar, therefore these should not be used for testing. This is reinforced by the fact that bees do not collect nectar from flowers in bud. An observation could be made that those flowers with more flung back petals (suggesting older flowers) have a higher DHA/Tsugar. However, due to the manner in which the flowers were packed and frozen in the bags, this is difficult to say with confidence. More testing with age identified flowers would help clarify this.

The results confirm the use of 20 flowers in the standard method for testing. Pooling the flowers reduces the variation by averaging the flowers, thus providing a more uniform result than the SFT. This also shows that the SFT

is able to detect DHA across a range of flower states. Further more it shows that where possible more than one SFT per tree should be performed to reduce variation, when the quantity of flowers collected for a tree is insufficient for the standard method. Work on flowers of *L. myrsinoides* and *L. continentale* found that some flowers may not produce any nectar.[21] Hence using more than one SFT is recommend to give a sample that is as uniform as possible.

3.6.2 Effect of the colour of flower petals

Personal observation during this survey, showed that it was possible for flower petals to range in colour from white through to pink on a single tree. Using the SFT, flowers were picked on a continuum from white to pink petals to discover if there was a correlation with DHA/Tsugar levels.

Table 3.12 shows that for these results, there is no correlation between the colour of the flower petals and the DHA/Tsugar content measured. Future work may use a larger sample size to confirm that indeed no correlation is exhibited and also measure the age of the flower, as this may affect the petal colour or the DHA/Tsugar levels measured.

3.6.3 Does the colour of the flower centre have an effect on the DHA/Tsugar level?

During this survey it was noticed that the centre of the mānuka flower is often green or a dark red colour in some trees. To test the effect this has on the DHA/Tsugar levels, replicates (10) were carried out using the SFT from two trees. Half the replicates were selected with red centres and the other half had green centres.

Table 3.13 shows no apparent trend, between the colour of the flower centre and DHA/Tsugar. It was found that the centre of *Chamelaucium uncinatum* (also of the Myrtaceae family) changes from green to red as the flower ages, this was thought to provide a visual signal that nectar production had stopped.[81] As DHA/Tsugar was still detected in the red centred mānuka flowers, this may not apply to mānuka. To confirm that this applies to mānuka, and if the age or possibly the fertilisation of the flower affects the centre colour and DHA/Tsugar levels, a future investigation using a larger sample size with age identified flowers would be required.

Table 3.12: Comparison of the colour of the mānuka flower petals to the DHA/Tsugar measured using the SFT.

	VB013		VB072		VB014	
Colour Continuum	$\frac{DHA}{Tsugar}$	Image	$\frac{DHA}{Tsugar}$	Image	$\frac{DHA}{Tsugar}$	Image
White/Pink	0.0003		0.0015		0.0008	
	0.0017		0.0005		0.0009	
|	0.0018		0.0004		0.0021	
|	0.0026		0.0013		0.0022	
|	0.0011		0.0015		0.0009	
|	0.0030		0.0033		0.0009	
|	0.0009		0.0025		0.0008	
V	0.0003		0.0023		0.0021	
	0.0036		0.0031		0.0010	
Pink/Red	0.0005		0.0025		0.0018	

Table 3.13: Comparison of the colour of the mānuka flower centre to the DHA/Tsugar measured using the SFT.

	VB013		VB072	
Centre Colour	$\frac{DHA}{Tsugar}$	Image	$\frac{DHA}{Tsugar}$	Image
Green	0.0013		0.0015	
Green	0.0034		0.0027	
Green	0.0022		ND	
Green	0.0034		0.0005	
Green	0.0009		0.0004	
Red	0.0023		ND	
Red	0.0015		0.0028	
Red	0.0011		0.0020	
Red	0.0015		0.0013	
Red	0.0009		0.0017	

3.6.4 Relating SFT to standard method

Only a small number of trees sampled require testing using the SFT. To test how the SFT relates to the standard method, only trees with flowers in excess of the requirement for the standard method were used. The standard method was used to prepare replicates (n= 10) to compare with replicates (n= 10) of the SFT.

Table 3.14: Comparing the standard and SFT methods and the difference between the methods.

Sample	Method	Average $\frac{DHA}{Tsugar}$	% RSD	% diff	Average $\frac{Fructose}{Glucose}$	% diff
VB019-2010(2)	Standard	0.00106	27.9	11.60	2.20	17.44
	SFT	0.00118	50.1		1.82	
VB169-2009	Standard	0.00047	24.8	20.21	2.43	16.71
	SFT	0.00037	65.3		2.02	
L3980-2010(3)	Standard	0.00182	12.5	5.52	1.87	1.83
	SFT	0.00192	41.0		1.90	

Comparing the methods in **Table 3.14**, it can be concluded that the SFT gives a DHA/Tsugar level approximately the same as the standard method. As the average DHA/Tsugar and F/G ratio is reasonably consistent between the standard and SFT methods, it can be seen that the SFT maintains the sugar ratio comparable to the standard method. As the % difference (% diff) to the standard methods is smaller than the % RSD of the SFT, a large amount of this difference is most likely linked to the variability between individual flowers on a tree. A better understanding of effects such as flower age, nectar production, flower position on tree, time of day, if the flower had being visited by a pollinator and weather conditions could allow some of this

variability to be reduced. Reducing the variability would be expected to reduce the % RSD, improving the accuracy of the SFT to match that of the standard method.

3.7 Testing the robustness of the method for nectar samples

3.7.1 Volume of PFBHA used

To ensure that all the HA, DHA and MGO has reacted, excess PFBHA must be used. Testing this with a standard solution was not ideal as it does not take into account the possibility of PFBHA reacting with other carbonyl containing compounds in the nectar. Nectar from L3995-2009 was used instead for this study.

PFBHA volumes (50 μL, 100 μL and 200 μL) were prepared in duplicate. An aliquot of nectar (200 μL) was combined with HA (10 μL) and derivatised using PFBHA (50 μL or 100 μL or 200 μL). DCM (2 mL) was added and the aqueous layer allowed to freeze. DCM extract ($\sim$1 mL) was transferred to a GC vial and silylated with TMSI (50 μL, 1 h) followed by GC-FID analysis.

Table 3.15 shows that 50 μL is sufficient excess to ensure that all of the HA and DHA reacts in the 1 hr reaction time (MGO was not detected in the sample). The additional PFBHA added in the 100 μL and 200 μL does not have any noticeable effect on the DHA measured. This confirms that the 50 μL of PFBHA of concentration 20 mg/mL is sufficient for this study.

Table 3.15: Effect of different amounts of PFBHA upon derivatisation

PFBHA (20 mg/mL)	Replicate	Mass (mg) of DHA in 200 μL
50 μL	a	0.0040
	b	0.0039
100 μL	a	0.0045
	b	0.0041
200 μL	a	0.0041
	b	0.0039
Average		0.0041
Std Dev		0.0002
% RSD		5.31

3.7.2 Period of flower extraction

Due to issues arising from laboratory work, occasions arose when the flowers were left to soak for greater than the suggested 20 minute period. Morrant *et al.*(2009) found no significant difference between a 1 min and a 20 min soaking duration when testing *Eucalyptus leucoxylon*. Although a significant different was observed between a 20 min and 1 h soak.[41] To investigate the effect of this with mānuka, 3 nectar extractions of a tree sample were left to soak for an excessive period ($\sim$ 240 mins) in order to observe the effect in comparison with that of the nectar extractions which had been left for the required 20 mins. To ensure that the effect was not tree specific, the experiment was repeated with two different trees. The trees used were VB202-2009 and VB192-2009(1). The extracts were treated as in **Section 2.6** with 3 of the 13 replicates left to soak for an excessive time. They were processed as in **Section 2.7**.

Table 3.16: Comparison of a soaking period of 20 minutes to that of a period in excess of 230 minutes.

Tree	Soak period (min)	DHA (mg) in 200 μL	Tsugar (mg) in 200 μL	Average DHA/Tsugar
VB202-2009	20	0.00014	0.33	0.00045
	249	0.00023	0.69	0.00034
VB192-2009(1)	20	0.00013	0.42	0.00031
	237	0.00024	1.00	0.00023

Table 3.16 shows that in all cases more DHA and Tsugar were extracted using the longer soaking. However, the excessive extraction favoured sugar and thus brought about a decrease in DHA/Tsugar level. Interpreting this, it is suggested that when the flowers are added to water, the free nectar is extracted relativity quickly. Using a 20 min soak ensures that all the free nectar is extracted. A longer period can result in sugar leaching from other parts of the flower.[41] The z-test in **Table 3.17** shows that the longer soaking period does significantly affect the results, the effect of a small time increase is probably minimal, but longer soaking should be avoided. This study should be repeated with more replicates and plotting DHA/Tsugar versus soak period to determine the optimal extraction length.

Table 3.17: Using the z-test for two sample means to determine if the extended soak (230 mins) results are the same as the standard soak (20 mins).

VB202-2009	Standard soak	Extended soak
Mean	0.00045	0.00034
Known Variance	0.0000000138	0.0000000045
Observations	10	3
Hypothesized Mean Difference	0	
z	2.15	
z Critical two-tail	1.96	
As z > (z critical two-tail) there is 95% confidence that the two methods are different		
VB192-2009(1)	Standard soak	Extended soak
Mean	0.00031	0.00023
Known Variance	0.00000000691	0.00000000058
Observations	10	3
Hypothesized Mean Difference	0	
z	2.63	
z critical two-tail	1.96	
As z > (z critical two-tail) there is 95% confidence that the two methods are different		

3.7.3 Using a second extraction

A decision was made to see if there was any DHA remaining in the flowers after the initial wash. To test this, a second wash was performed after the first wash. This was achieved by transferring the flowers, (20) from the initial vial to a second vial containing water (4 ml), after the initial 20 minutes had passed. These were then left for a further 20 minutes to extract DHA and sugar from the nectar. After the second wash the flower extracts were processed as in **Section 2.7**. Three different tree samples were tested to eliminate tree specific effects.

Table 3.18: A second extraction compared to a single extraction.

Sample	Extraction	DHA (mg) in 200 μL	Tsugar (mg) in 200 μL	$\frac{DHA}{Tsugar}$	$\frac{DHA}{Tsugar}$ % reduced
L3962-2009	First	0.00128	0.56	0.00233	17.23
	Second	0.00054	0.28	0.00193	
L3961-2010	First	0.00152	0.54	0.00282	12.84
	Second	0.00064	0.26	0.00246	
L3998-2009	First	0.00131	0.38	0.00346	11.57
	Second	0.00062	0.20	0.00306	

In **Table 3.18** it is observed that DHA and sugar is still found although at reduced levels. The second extraction results are approximately half that of the first extraction. In each case DHA/Tsugar decreased by 12-17%. The amount extracted in the second wash is more than might have been transferred between washes accidentally. This suggests that a second extraction may be advantageous to increase yield. However, there is an

advantage to not depleting the flower. It ensures that a uniform sample is obtained for all trees in the survey. If a sample was depleted of DHA, additional sugar could still be extracted, by leaching of the sugar from other parts of the flower. This would result in the measured DHA/Tsugar ratio to be skewed towards the Tsugar, giving a lower reading. By ensuring that DHA remains, the DHA/Tsugar ratio will remain consistent for the extraction period, due to the contribution from the leached sugar being minimal compared to the sugar extracted from the nectar.

3.7.4 Nectar stability

Once the nectar has been extracted into the water and the flowers removed, the extract often sits at room temperature during the period of sample preparation. As these samples are likely to contain biological activity, the samples themselves may become degraded throughout the period of sample preparation and thus affect the results. To test this possibility, an extreme example was taken, with the extracts left overnight before processing. 10 replicates from three trees samples were prepared. 10 replicates followed the procedure outlined in **Section 2.6** and **Section 2.7**. 5 of the replicates were left overnight after the procedures described in **Section 2.6**. After being left overnight, the procedure in **Section 2.7** was again performed on these replicates.

A z-test, **(Table 3.20)** shows that there is no significant difference between those extracts left at room temperature overnight and those processed on the same day. Therefore the samples are stable during the sample preparation period. It was observed that the nectar extract did darken overnight, possibly due to the Maillard reaction, but this had no observed effect on the DHA/Tsugar value measured. Future projects might investigate the changes that occur in the nectar when stored, and how this compares to the changes in the formation of honey.

Table 3.19: Comparison of samples processed on one day compared to those allowed to stand overnight.

Sample	Test	DHA (mg) in 200 μL	Tsugar (mg) in 200 μ	$\frac{DHA}{Tsugar}$
VB203-2009	Normal	0.00017	0.30	0.00058
	Overnight	0.00015	0.30	0.00051
L3969-2009	Normal	0.00044	0.54	0.00081
	Overnight	0.00047	0.55	0.00087
VB072-2011(3)	Normal	0.00096	0.28	0.00349
	Overnight	0.00104	0.27	0.00381

Table 3.20: Determining if the extraction left overnight is the same as the standard extraction method by using the z-test for two sample means, with a hypothesized mean difference of 0.

VB203-2009	Standard Extract	Extract Left Overnight
Mean	0.00058	0.00051
Known Variance	0.000000007	0.000000004
Observations	10	5
z	1.81	
z critical two-tail	1.96	
As z < (z critical two-tail) there is 95% confidence that the two methods are the same		

L3969-2009	Standard Extract	Extract Left Overnight
Mean	0.00081	0.00087
Known Variance	0.000000024	0.000000008
Observations	10	5
z	-1.06	
z critical two-tail	1.96	
As z < (z critical two-tail) there is 95% confidence that the two methods are the same		

VB072-2011(3)	Standard Extract	Extract Left Overnight
Mean	0.00349	0.00381
Known Variance	0.000000479	0.000000598
Observations	10	5
z	-0.80	
z critical two-tail	1.96	
As z < (z critical two-tail) there is 95% confidence that the two methods are the same		

3.7.5 Stability of samples over GC-FID sequence

The auto-sampler on the GC-FID allows samples to be processed more quickly and with better reproducibility then manual injection methods. A problem is possible sample degradation while the derivatised samples wait at room temperature (22.5 ℃) to be injected. To check for sample degradation, 2 vials from the tree L3986-2010 were duplicated for both DHA and sugar testing. One set of duplicates were tested at the beginning of the sequence with the second set tested at the end to look for any change over the sequence length. For the sugar test there were ∼315 minutes between duplicates and for the DHA test ∼702 minutes.

Table 3.21: Comparison of samples injected at the beginning and end of the GC sequence

Sample	Sequence Posistion	DHA (mg) in 200 μL	Tsugar (mg) 200 μL	$\frac{DHA}{Tsugar}$
L3986-2010-1	Beginning	0.00082	0.59	0.0014
	End	0.00083	0.61	0.0014
L3986-2010-2	Beginning	0.00068	0.59	0.0011
	End	0.00070	0.61	0.0011

Table 3.21 shows no significant difference is found between the vials as DHA, Tsugar and DHA/Tsugar is within the standard deviation determined for L3986-2010 tree sample; 0.0002 for DHA, 0.05 for Tsugar and 0.0003 for DHA/Tsugar. Observing this, there is no concern that the samples may degrade during a vial sequence.

Further testing showed that both sugar and nectar samples gave reproducible results after 4 days sitting in the auto-sampler rack with pierced septa at room temperature. This however, is not recommended, as the DHA samples

had an additional non-interfering peak appear in the chromatogram, and the sugar samples showed signs of crystallisation. No significant changes were observed over the 4 days, longer periods may prove to have a more significant effect. It is still recommended therefore that the samples are silylated and analysed in the same 12 h period.

3.8 How does the biological variation compare to the experimental variation?

To test the variation that the method causes in the extract replicates, three trees were analysed for DHA and sugar using five replicates each from a single extraction of 20 flowers. This was then compared to the variation found between 10 nectar extracts of 20 flowers used in the testing of the trees. The extraction was carried out as in **Section 2.6**, 5 aliquots (200 μL) were treated as in **Section 2.7(DHA)**, an additional 5 aliquots (20 μL) were treated as in **Section 2.8(sugar)**. The testing used 3 trees L3980-2009, L3998-2009(1) and VB210-2010.

Table 3.22: A single nectar extract repeated 5 times compared to the variation within the standard method of 10 nectar extracts, each repeated once.

	% RSD	
Tree	Single nectar extract	Standard Method
L3980-2009	3.13	25.80
L3998-2009(1)	2.54	9.06
VB210-2010	1.20	14.36

Table 3.22 shows that variation between extracts is much greater than within each individual extract. The former originates in biological variation but the latter is experimental error. It can then be concluded that the biological effects have the most significant effect on the variation between extracts.

3.9 Day to day reproducibility within a single tree

It is important to ensure that tree samples, if repeated, are reproducible. To test this, some trees were chosen and were prepared again on different days. Each tree had 10 nectar extracts of 20 flowers prepared on the day it was tested.

Table 3.23 shows the variation that can occur between samples from the same tree prepared on different days. However, as the range given by the standard deviations of the individual repeats overlap with that of the combined value, there it not a significant difference between such repeats.

Table 3.23: Comparing tree samples (10 nectar extracts each) that have been repeated on different days to observe any changes in the tree, and if this change is significant when compared to all nectar extracts combined.

	Repeat of standard method (10 replicate extractions of 20 flowers each)									
	1		2		3		4		All combined	
Tree	$\frac{DHA}{Tsugar}$	Std dev	$\frac{DHA}{Tsugar}$	Std dev	$\frac{DHA}{Tsugar}$	Std dev	$\frac{DHA}{Tsugar}$	Std dev	$\frac{DHA}{Tsugar}$	Std dev
VB163-2010	0.0019	0.00037	0.0020	0.00030	0.0033	0.00044	0.00056	27.21	0.0023	0.00066
VB197-2009	0.00034	0.000073	0.00059	0.00012	0.00054	0.00012			0.00049	0.00013
VB218-2009	0.00036	0.000076	0.00032	0.00004					0.00034	0.000030
L3998-2009	0.0035	0.00049	0.0027	0.00024					0.0031	0.00056
VB192-2009	0.00019	0.000048	0.00031	0.000083					0.00025	0.000085
VB065-2010	0.0010	0.000084	0.00077	0.00010					0.00088	0.00016
L3980-2009	0.0013	0.000350	0.0023	0.000031					0.0018	0.00077
VB168-2009	0.00070	0.000140	0.0010	0.00022					0.00085	0.00022

3.10 Elimination of the possibility that DHAP is present

Dihydroxyacetone phosphate (DHAP) is part of the Calvin Cycle in plants. This is a light independent reaction that is responsible for creating carbohydrates from carbon dioxide and water.[82,83] This raised the possibility that the method was actually detecting DHAP as a derivative of DHA, or possibly both chemicals together. The phosphate group is able to be silylated by TSMI.[84] There is a possibility that the phosphate ester might be hydrolysed during the derivatisation process as well.[85]

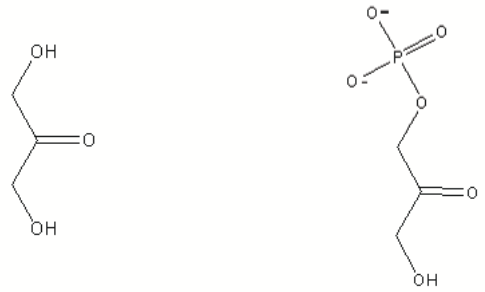


Figure 3.12: Structures of DHA and DHAP.

Two samples were prepared using the initial method (**Section 3.3.2**) with volumes increased to allow for more injections. One sample (DHAP) had DHAP (20 μL, $\sim$0.87 mg/mL, dihydroxyacetone phosphate dilithium salt 97% Sigma-Aldrich) added and the other DHA (20 μL). To both of these HA (20 μL) and PFBHA (30 μL) was added with water (200 μL). Extraction was with DCM (2 mL) and silylation with TMSI (120 μL).

Figure 3.13 has a clear HA and DHA peak and **Figure 3.14** has only an HA peak. Therefore there is no interference from DHAP and only DHA is detected using this method. The DHAP, which is anionic, would be expected to favour the water during the extraction step even with the PFBHA group attached. So if unhydrolysed DHAP was present, it would be removed, in this step. If it was not removed, silylation of the phosphate group by TMSI would

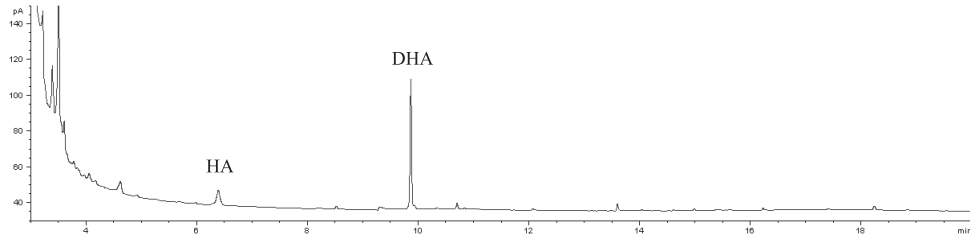


Figure 3.13: Gas chromatogram of a sample of DHA + HA prepared using the initial method. HA ∼6.5 min, DHA ∼9.8 min.

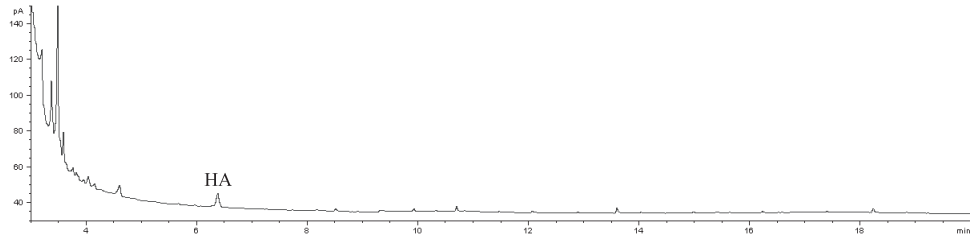


Figure 3.14: Gas chromatogram of a sample of DHAP + HA prepared using the initial method. HA ∼6.5 min and no DHAP is detected.

be expected to give a retention time different to that of DHA. Since DHA alone is detected, it can be concluded that any DHA detected in a nectar sample is due only to free DHA and not DHAP. DHAP may be present in the flower nectar, but it is outside the aim of this research to confirm its presence.

3.11 Investigating the possibility of DHA in other tree parts

Literature suggests the possibility of DHA in other parts of the tree, a small investigation was mounted to discover if this was correct.

3.11.1 Bark

A length of mānuka branch (10 cm) was stripped of its bark. The bark was frozen in liquid N_2 and ground to a powder. The powder was added to water

(1 mL), mixed, and centrifuged (1 min, 4000 rpm). An aliquot (200 μL) was taken for testing as in **Section 2.7**.

3.11.2 Stem

A length of mānuka branch (10 cm) was stripped of its bark (the bark was used in **Section 3.11.1**). The remaining stem was frozen in liquid N_2, and ground to a powder. The powder was added to water (1 mL,) mixed, and centrifuged (1 min, 4000 rpm). An aliquot (200 μL) was taken for testing as in **Section 2.7**.

3.11.3 Twigs

Twigs are small thin terminal branches of a woody plant. Twigs (10) were picked, frozen intact in liquid N_2, and ground to a powder. The powder was added to water (1 mL), mixed, and centrifuged (1 min, 4000 rpm). An aliquot (200 μL) was taken for testing as in **Section 2.7**.

3.11.4 Leaves

Leaves (40) were picked, frozen in liquid N_2 and ground to a powder. The powder was added to water (1 mL) mixed and centrifuged (1 min, 4000 rpm). An aliquot (200 μL) was taken for testing as in **Section 2.7**.

3.11.5 Flowers

To confirm DHA was present in the mānuka tree and could be detected using this sample preparation. Flowers (7) were frozen in liquid N_2 and ground to a powder. The powder was added to water (1 mL), mixed, and centrifuged (1 min, 4000 rpm). An aliquot (200 μL) was taken for testing as in **Section 2.7**.

The chromatograms showed the possibility of DHA in the twig and leaf samples, and the presence of DHA in the flowers; thus confirming that the tree did have DHA that is detectable by this method.

A limitation of the method was the size of the sample. Since the leaves proved most promising, the leaves were repeated using a larger sample size. Fragments of leaves and twigs are often included in the flower washing process in smaller amounts then that tested. These tests show that any contribution of DHA from the bits of branch and leaves would be insignificant compared to that of the flowers.

3.11.6 Analysis of a large leaf sample

Leaves (1.8 g, ∼600 leaves) were picked, frozen in liquid N_2 and ground to a powder. The powder was added to water (4 mL), mixed, and centrifuged (1 min, 4000 rpm). An aliquot (200 μL) was taken for testing as in **Section 2.7**.

An increase in DHA was possibly observed, but this increase did not correspond to the increase in number of leaves used.

3.11.7 Summary of DHA in other parts of the mānuka tree

This testing does not disprove the possibility of DHA in other parts of the tree. A larger sample size for the different parts and a modified method may give better results. This was outside the scope of this project so was not pursued.

Chapter 4

Results

4.1 Collection outcomes

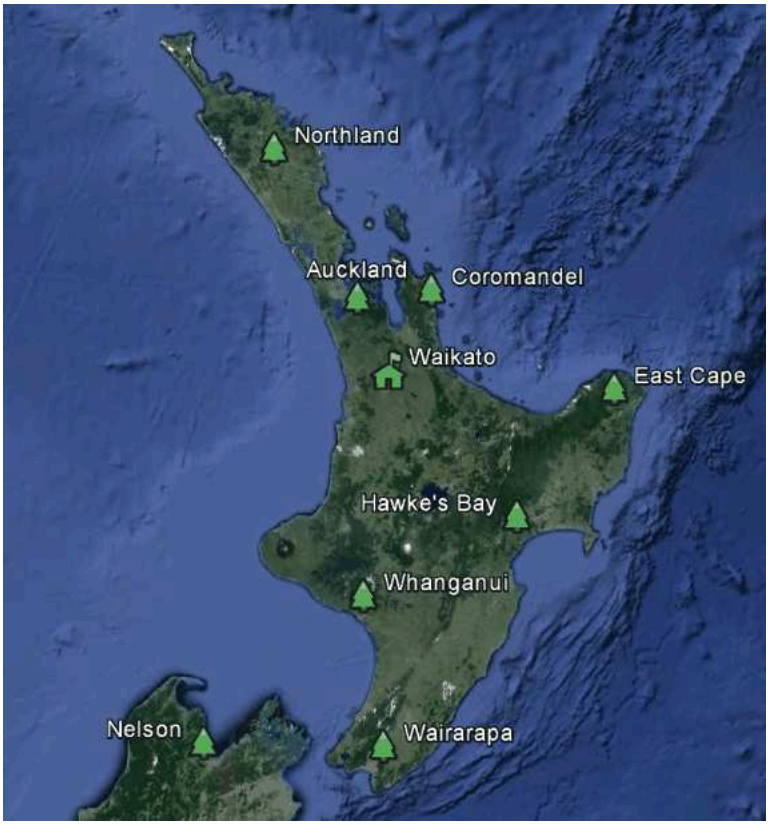


Figure 4.1: Areas from which trees were sampled.[86]

In 2009 and 2010, samples were collected from the areas shown in **Figure 4.1**. For all sites the collecting was carried out during the flowering period from November to December. More detailed information on the flowering period cannot be identified due to commercial interests.

10 replicates were initially analysed for each tree. For those trees that had a % RSD in excess of 25%, Grubbs' test for outliers was used to remove outlier values with 95% confidence (**Section 2.9.4** describes this in more detail). The 95% confidence interval used in the following graphs was calculated using the replicates for each tree.

Table 4.1: List of trees collected. Terms: Not Sampled (NS), Insufficient information to locate (IIS) , Died between sampling (DBS) .

Sample ID	2009	2010	Sample region	Sample ID	2009	2010	Sample region
VB151	NS	Sampled	Auckland 1	VB177	NS	Sampled	Auckland 1
VB187	NS	Sampled	Auckland 1	GCP022	Sampled	NS	Auckland 2
GCP130	Sampled	NS	Auckland 3	GCP128	Sampled	NS	Auckland 4
L3961	NS	Sampled	Coromandel 1	L3969	Sampled	Sampled	Coromandel 1
L3978	Sampled	Sampled	Coromandel 1	L3980	Sampled	Sampled	Coromandel 1
L3986	Sampled	Sampled	Coromandel 1	L3989	Sampled	Sampled	Coromandel 1
L3990	Sampled	Sampled	Coromandel 1	VB213	Sampled	NS	East Cape 1
VB181	Sampled	Sampled	East Cape 1	VB183	Sampled	NS	East Cape 1
VB200	Sampled	NS	East Cape 1	VB216	Sampled	DBS	East Cape 1
VB184	Sampled	Sampled	East Cape 2	VB186	Sampled	Sampled	East Cape 2
VB188	Sampled	Sampled	East Cape 2	VB192	Sampled	Sampled	East Cape 2
VB198	Sampled	IIS	East Cape 2	VB207	Sampled	NS	East Cape 2
VB190	Sampled	Sampled	East Cape 3	VB191	Sampled	NS	East Cape 3
VB193	Sampled	Sampled	East Cape 3	VB195	Sampled	Sampled	East Cape 3
VB197	Sampled	Sampled	East Cape 3	VB201	Sampled	DBS	East Cape 3
VB203	Sampled	DBS	East Cape 3	VB204	Sampled	DBS	East Cape 3
VB206	Sampled	Sampled	East Cape 3	VB208	Sampled	Sampled	East Cape 3
VB199	Sampled	Sampled	East Cape 4	VB202	Sampled	Sampled	East Cape 4
VB209	Sampled	DBS	East Cape 4	VB210	Sampled	Sampled	East Cape 4
VB212	Sampled	Sampled	East Cape 4	VB214	Sampled	Sampled	East Cape 4
VB275	Sampled	DBS	East Cape 5	VB218	Sampled	NS	Hawks Bay 1
L2253	Sampled	NS	Nelson 1	L3962	Sampled	NS	Nelson 1
L3995	Sampled	NS	Nelson 1	GCP119	Sampled	NS	Northland 1
GCP124	Sampled	NS	Northland 1	VB014	Sampled	Sampled	Waikato 1
VB065	NS	Sampled	Waikato 2	VB112	NS	Sampled	Waikato 2
VB084	NS	Sampled	Waikato 3	VB015	NS	Sampled	Waikato 4
VB018	NS	Sampled	Waikato 4	VB042	NS	Sampled	Waikato 5
VB077	NS	Sampled	Waikato 5	VB091	NS	Sampled	Waikato 6
VB101	NS	Sampled	Waikato 6	VB036	NS	Sampled	Waikato 7
L3998	Sampled	NS	Wairarapa 1	VB162	Sampled	NS	Wairarapa 1
VB165	Sampled	NS	Wairarapa 1	VB170	Sampled	NS	Wairarapa 1
VB189	Sampled	NS	Wairarapa 1	VB164	Sampled	IIS	Wairarapa 2
VB169	Sampled	NS	Wairarapa 2	VB172	Sampled	NS	Wairarapa 2
VB194	Sampled	NS	Wairarapa 2	VB168	Sampled	IIS	Wairarapa 3
VB173	Sampled	Sampled	Wairarapa 3	VB175	Sampled	NS	Wairarapa 3
VB217	Sampled	Sampled	Wairarapa 3	L3999	Sampled	NS	Whanganui 1
VB167	Sampled	NS	Whanganui 1	VB185	Sampled	NS	Whanganui 1
VB205	Sampled	Sampled	Whanganui 1				

Table 4.1 shows that 57 samples were collected in 2009 and 45 new or re-sampled trees in 2010. Eight of the 2009 samples could not be collected again in 2010, due to insufficient location information from the 2009 survey (IIB), or the tree having died in the intervening year (DBA). Furthermore the flowering periods of a few 2009 trees did not coincide with collection trips in 2010. Eight new samples were collected from the Waikato region, to look at intentionally planted cultivars growing under similar environmental conditions. An additional two trees were sampled from the Coromandel region to investigate the effect of close proximity of trees at this site.

4.2 Overview of samples for 2009 and 2010

Figures 4.2 and **4.3a** give an overview of the DHA/Tsugar for all trees sampled in both years grouped by region. A variation in DHA/Tsugar is seen both within and between regions.

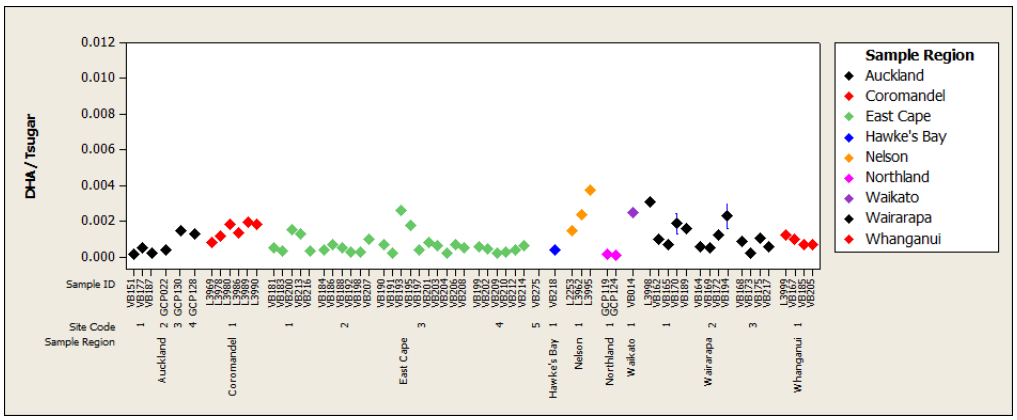
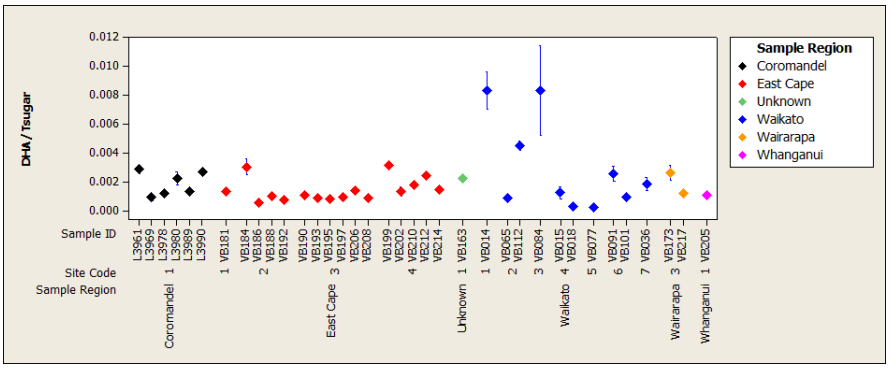
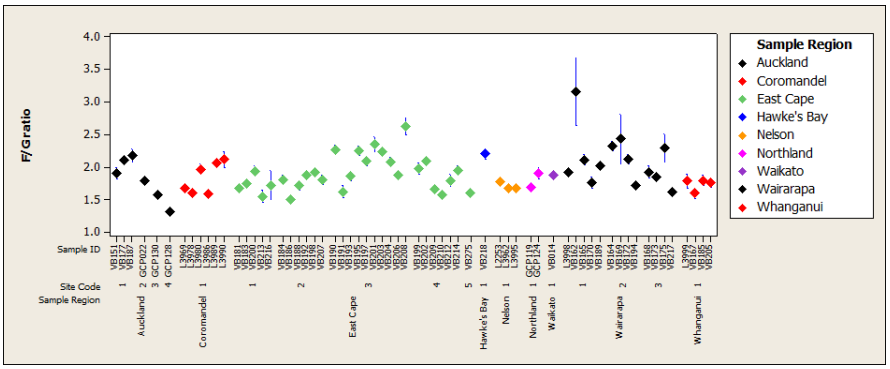


Figure 4.2: Interval plot of DHA/Tsugar ratios for trees sampled in 2009, grouped by regions and with error bars defined by the 95% confidence interval, see enlarged in **Appendix C**.

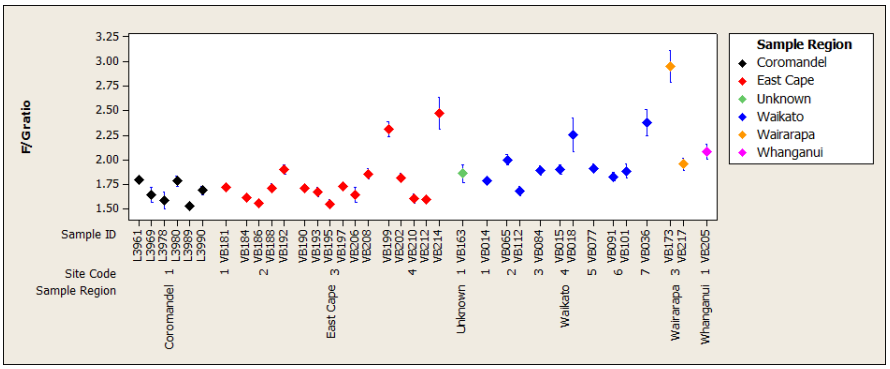
Figures 4.3b and **4.3c** gave an overview of the F/G ratio for all trees sampled in both years grouped by regions. These show that the F/G ratio also varies within and between regions.



(a) Interval plot of DHA/Tsugar ratios for trees sampled in 2010, grouped by regions and with error bars defined by the 95% confidence interval, see enlarged in **Appendix C**.



(b) Interval plot of F/G ratios for trees sampled in 2009, grouped by regions and with error bars defined by the 95% confidence interval, see enlarged in **Appendix C**.



(c) Interval plot of F/G ratios for trees sampled in 2010, grouped by regions and with error bars defined by the 95% confidence interval, see enlarged in **Appendix C**.

4.3 Variation in DHA/Tsugar and F/G ratios between 2009 and 2010

For those trees that were able to be sampled in 2009 and 2010, a comparison was carried out to see if there was any variation between the samples collected in 2009 and 2010.

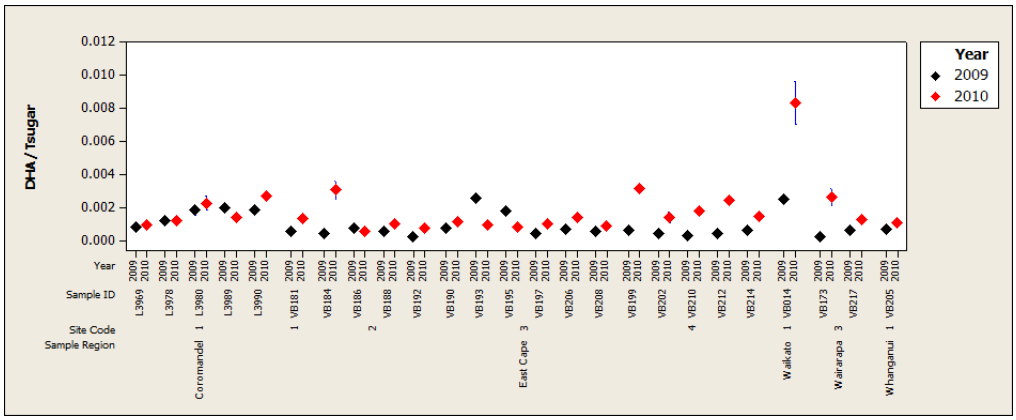


Figure 4.3: Interval plot of the mean DHA/Tsugar values for trees sampled in 2009 and 2010 with error bars showing the 95% confidence interval, see enlarged in **Appendix C**.

Figure 4.3 demonstrates that the DHA/Tsugar levels in flowers may vary annually. That is, the 2010 samples generally appear higher than the 2009 samples for the East Cape samples. A balanced ANOVA, hypothesising that the regional DHA/Tsugar means for 2009 and 2010 are equal, (when $p > 0.05$) was 95% confident that the Coromandel means for 2009 and 2010 were the same ($p = 0.5$). For the East Cape the ANOVA test was 95% confident that the means for 2009 and 2010 were not the same ($p = 0.02$). Other regions were not tested due to lack of samples that sufficiently covered both time periods.

Figure 4.4 demonstrates that F/G ratios in flowers may also vary annually which is shown by the 2009 samples being generally higher then the 2010 samples. Conditions in 2010 could possibly have increased the amount of

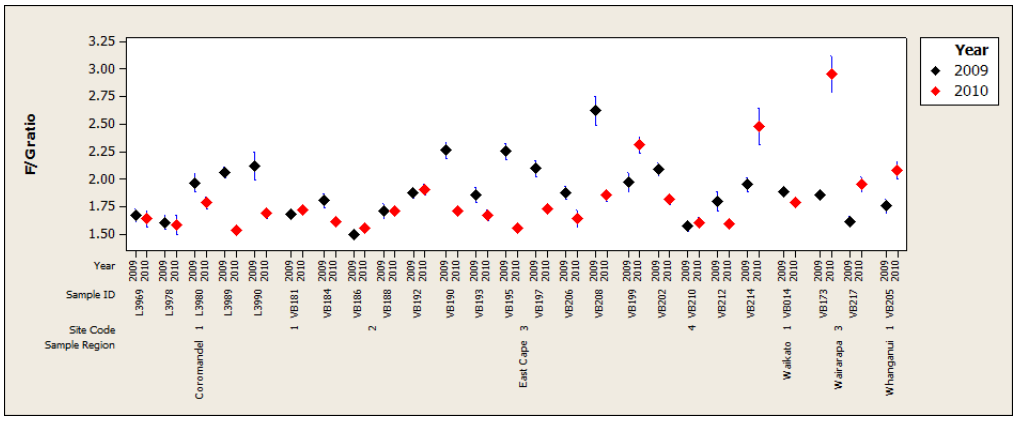


Figure 4.4: Interval plot of the mean F/G ratos for trees sampled in 2009 and 2010 with error bars showing the 95% confidence interval, see enlarged in **Appendix C**.

glucose in nectar reducing the F/G ratio. A balanced ANOVA, hypothesising that the regional F/G ratio means for 2009 and 2010 are equal, (when $p > 0.05$) was 95% confident that the Coromandel regional means for 2009 and 2010 were the same ($p = 0.08$) as were the East Cape regional means ($p = 0.097$). Other regions were not tested due to lack of samples that covered both time periods sufficiently.

The variation in the mean DHA/Tsugar between the years was expected. The NPA values of honey are known to vary each year[87] and local environmental conditions are equally known to affect the nectar in other plants.[32–35] The age of the trees theoretically could have an effect as younger trees may produce more DHA/Tsugar than older trees, that upon reaching a certain age, produce a consistent amount of DHA/Tsugar. The consistency in the mean F/G ratio for both regions over 2009 and 2010, shows that the F/G ratio might remain the same even if the amount of nectar produced changes between the years. This similarity may be weak as the p values (Coromandel, $p = 0.08$ and East Cape, $p = 0.097$) are only slightly higher than the $p = 0.05$ cut off. A larger number of regions with sampling over multiple flowering seasons will be required to improve our understanding of this variation.

4.4 Variation of DHA/Tsugar between regions

The Coromandel, East Cape and Wairarapa region samples were tested for similarity. The other regions were excluded due to lack of samples . VB214, VB199 and VB209 are excluded from the East Cape as these are deliberately planted cultivars.

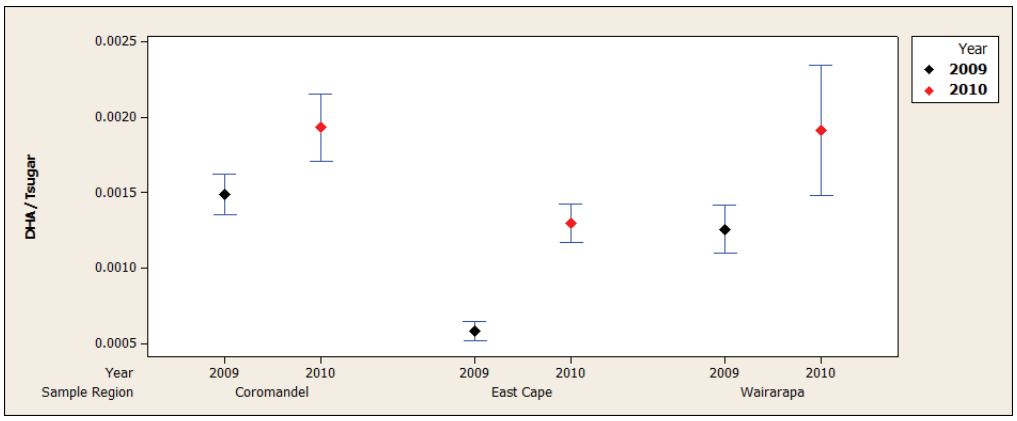


Figure 4.5: Interval plot of the mean DHA/Tsugar values across all trees in the Coromandel, East Cape and Wairarapa regions sampled in 2009 and 2010 with error bars showing the 95% confidence interval.

The Coromandel and Wairarapa samples appear to be similar in **Figure 4.5** and the East Cape samples different. A one-way ANOVA test with grouping (provided by the Tukey method) found with 95% confidence that the Coromandel and Wairarapa samples were grouped together and that the East Cape samples were grouped separately for both the 2009 and 2010 samples. Based on these groupings, it can be said that there was a significant difference between the East Cape and the Coromandel and Wairarapa samples in both 2009 and 2010, but not between the Coromandel and Wairarapa samples themselves. As these groups are maintained in both 2009 and 2010, DHA/Tsugar does vary between regions.

Figure 4.6 expands the data to all regions, and despite the fact that there is insufficient data for a one-way ANOVA. In other regions it appears that in

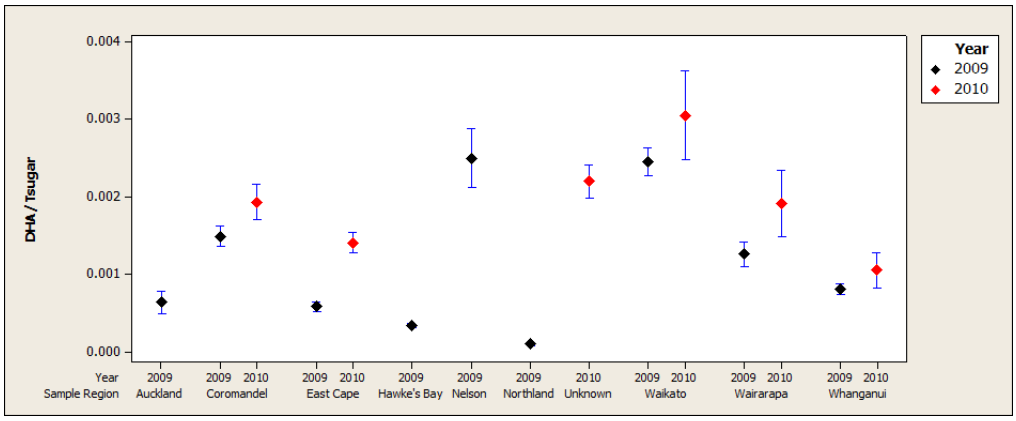


Figure 4.6: Interval plot of the mean DHA/Tsugar values across all regions sampled in 2009 and 2010 with error bars showing the 95% confidence interval.

some cases they differ from the East Cape, Coromandel and Wairarapa regions by more than the significant difference found between the East Cape and Coromandel/Wairarapa groupings.

4.5 Intra-region differences

The Auckland, Hawkes Bay, Nelson and Northland regions are not examined in depth due to low number of samples per site.

4.5.1 Coromandel samples

The Coromandel samples all fell within a circle of 100 m radius (**Figure 4.7**). Previous studies had endeavoured to ensure that trees were at least 100 m apart when sampling. This limits the possibility of sampling genetically similar trees.

As the trees are within 100 m radius circle, it could be hypothesised that the trees may be genetically related, therefore giving similar DHA/Tsugar values.

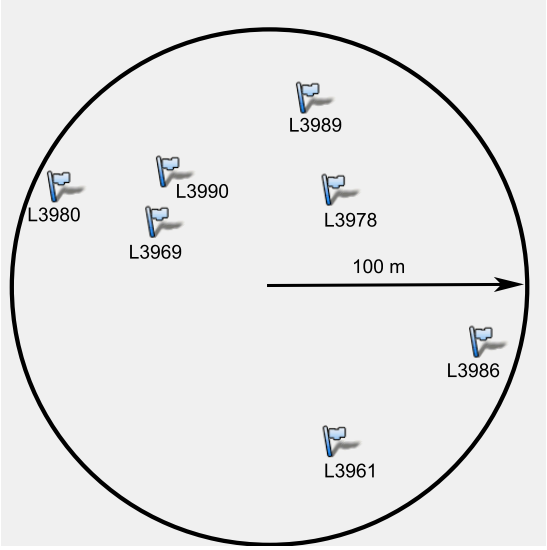


Figure 4.7: An illustration of the position of the Coromandel sampled trees and their relationship to each other.

If similarities in DHA/Tsugar do indicate a genetic link based on proximity, then L3969 and L3990 would be expected to be similar, as would L3978 and L3989. This is not observed (**Figure 4.8**).

Tree age may have an effect. In this survey, tree age was estimated based on height, diameter of trunk, location and appearance of the tree. L3969, L3978 and L3986 were identified as mature trees and these have lower DHA/Tsugar values compared to that of L3961, L3980, L3989 and L3990. Confirmation of these results would require the use of a increment borer. This would allow the tree rings to be counted, and thus an estimation of the trees age. Unfortunately mānuka trunks are normally narrow so careful treatment is necessary so not to harm or kill the tree. To do a genetic comparison it may be necessary to have trees of the same age, in order to remove any possible effects of age on the DHA/Tsugar levels. The soil order (**Section 4.9.1**) shows that the sampled Coromandel trees are spread across 3 soil orders. These do not correspond with the DHA/Tsugar, as L3980, L3990 and L3969 have all the same soil order, yet L3969 is lower than both L3980 and L3990.

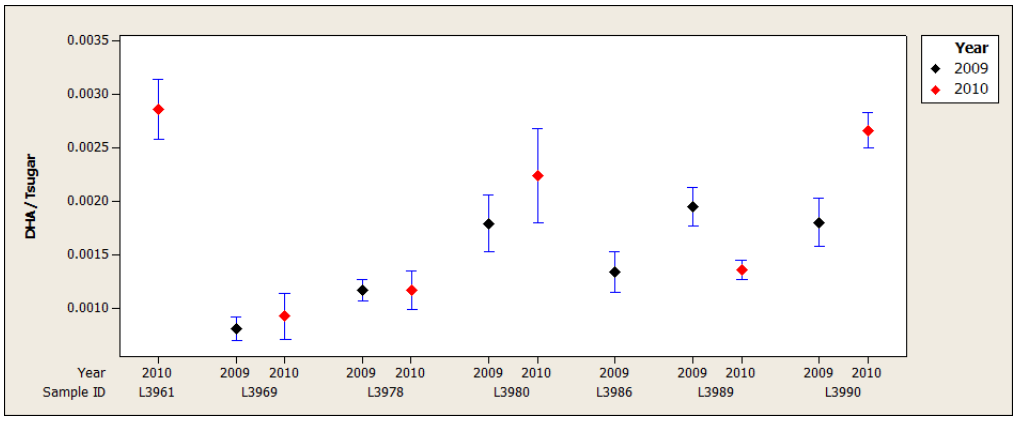


Figure 4.8: Interval plot of the mean DHA/Tsugar values for the Coromandel trees sampled in 2009 and 2010 with error bars showing the 95% confidence interval.

4.5.2 East Cape samples

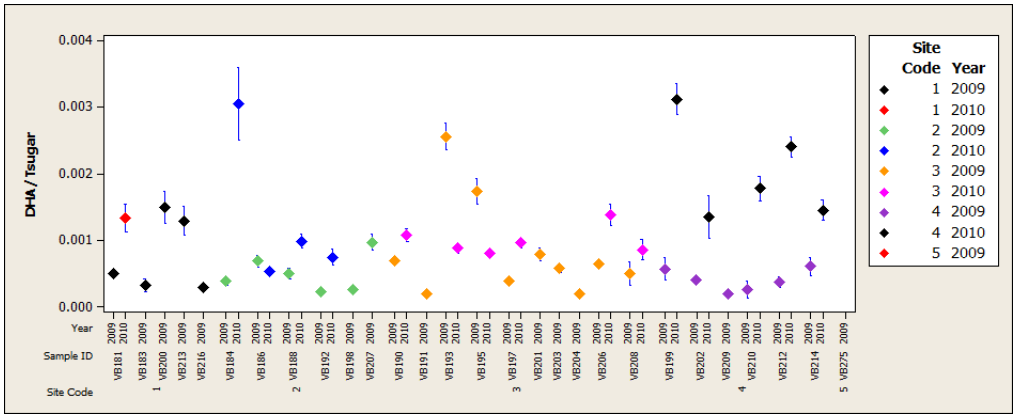


Figure 4.9: Interval plot of the mean DHA/Tsugar values for the East Cape trees sampled in 2009 and 2010 with error bars showing the 95% confidence interval, see enlarged in **Appendix C**.

For the East Cape samples, **Figure 4.9** shows that the majority of the 2009 samples fall within the same DHA/Tsugar ratio range of 0.00012-0.001. VB200 and VB213 at site 1 and VB193 and VB195 at site 3 are outside this range. No obvious reasons can be found for these high values as they grow under similar conditions and are judged to be of a similar age to other sampled trees at their respective sites. VB193 and VB195 at site 3 are within 50 m of each other so could be related possibly explaining their similar

DHA/Tsugar values. VB200 and VB213 at site 1 are 600 m apart so there is a greatly reduced chance of being related. However, there is a possibility, due to VB200 and VB213 being in close proximity to a river, that the seed could have been transported by river allowing related plants to grow further apart. Sampling trees within a 50 m radius of these trees would determine if proximity between trees relates to DHA/Tsugar. VB275 was not found to have any detectable DHA, which suggest that it may not have been mānuka. This was a possibility as its physical appearance had cast doubt on its species. Site 2 and site 3 have 2010 samples that are similar to their 2009 samples. For VB195 and VB193 the 2009 samples are elevated compared to 2010, where as for VB184 the situation is reversed. The 2010 samples from the trees at site 4 are larger and have a greater variation than that observed for the 2009 samples. Again there are no obvious reasons for this.

More years of sampling are required to understand the change in DHA/Tsugar over a number of years. A complication with the East Cape sites is the range of environments presence. Trees grow from river banks to ridge tops, creating a number of micro-environments; these micro-environments have been shown to affect nectar in plants.[32–35] Sampling at a site took place over a wide area encompassing a number of micro-environments and a broad range of tree ages. Although this gave a broad view of a site, it did not allow any single or group effects to be properly understood.

A future study may wish to plant a single mānuka culitvar over a uniform site and have the trees tested each year. This would increase our understanding of tree variation as the effects of micro-environments would be minimal. Such a study would also permit the study of changes in DHA/Tsugar as the mānuka matures.

4.5.3 Wairarapa samples.

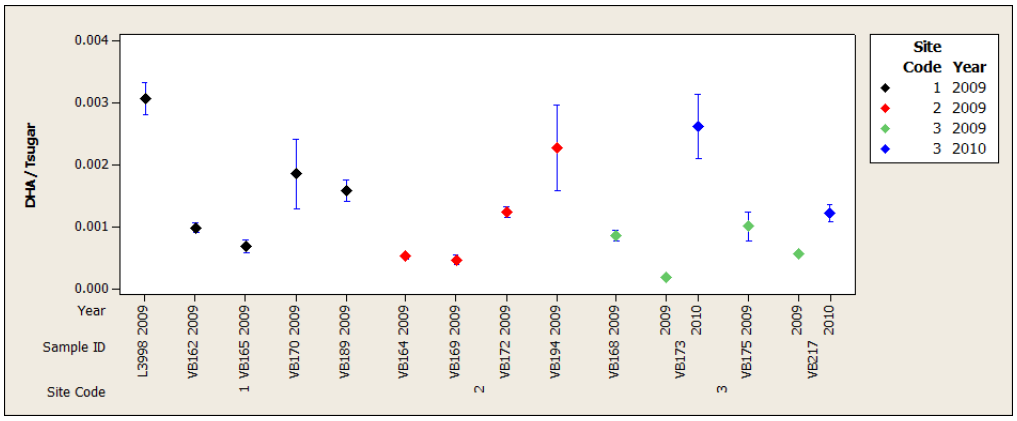


Figure 4.10: Interval plot of the mean DHA/Tsugar values for the Wairarapa trees sampled in 2009 and 2010 with error bars showing the 95% confidence interval, see enlarged in **Appendix C**

Figure 4.10 of the Wairarapa samples show a greater variation intra site than the East Cape. A one-way ANOVA test with grouping provided by the Tukey method was 95% confident that sites 2 and 3 were grouped together and were significantly different to site 1. However, this is most likely due to the presence of L3998 which skews the site 1 mean higher. Removal of L3998 from the test, changes the result so that sites 1 and site 2 are grouped and site 3 becomes significantly different. This suggests that more samples are needed from each site to provide a large enough pool of data to separate out abnormal samples, and to determine if each site is significantly different to one another.

4.5.4 Whanganui samples

The Whanganui samples can be divided into two sets based on their location. L3999 and VB205 are within 50 m of each other on a river bank, VB185 and VB167 are also within 50 m of each other on a cliff edge and the two sets are 430 m apart horizontally and 79 m vertically. The trees have been judged to

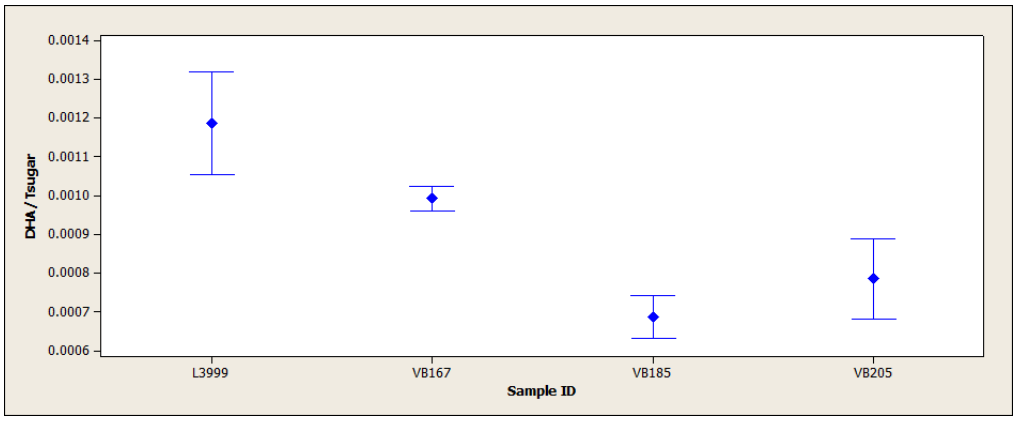


Figure 4.11: Interval plot of the mean DHA/Tsugar values for the Whanganui trees sampled in 2009 with error bars showing the 95% confidence interval.

be about the same age. Based on age and proximity the pairs would be expected to be similar. **Figure 4.11**, however, shows that this is not the case with L399 and VB167 forming a pair as well as VB185 and VB205. From their proximity to each other, the location pairs would be expected to have similar micro-environments and to be related. It would appear that the genetic relationships based on proximity is not a factor in controlling DHA/Tsugar. With only a few samples from this site, confirmation would require a larger sample of trees to be collected from each of the two collection locations at this site.

4.5.5 Waikato samples

The Waikato trees sampled were deliberately planted cultivars, that exhibit a number of physiological differences. **Figure 4.12** shows the range of DHA/Tsugar ratios recorded. Only a few of each cultivar type was tested, and although physiological differences were observed, the cultivars were not clearly identified. The Waikato samples show the possibility that cultivar variety may play a part in determining DHA/Tsugar with the range of values observed. To confirm this, testing a larger number of formally identified cultivars would be required.

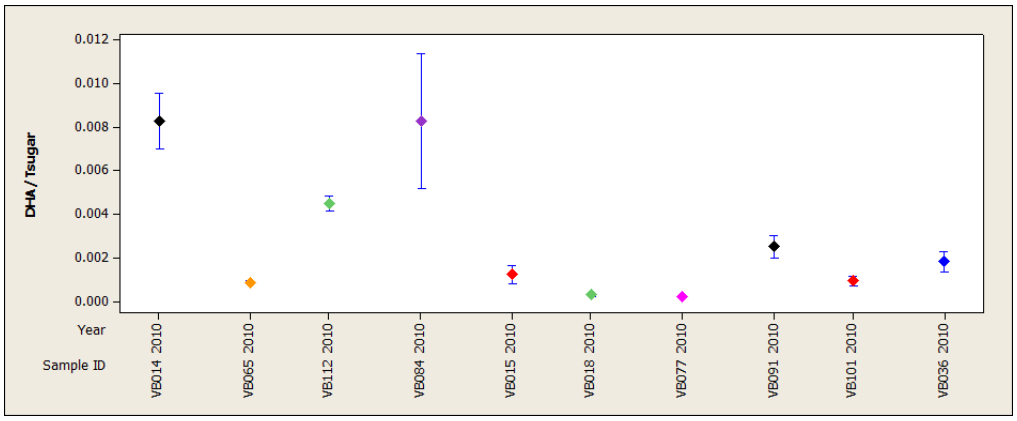


Figure 4.12: Interval plot of the mean DHA/Tsugar values for the Waikato trees sampled in 2010 with error bars showing the 95% confidence interval, see enlarged in **Appendix C**.

4.5.6 Intra-region summary

By looking at the differences within a region, it has become apparent that a larger number of samples are required from each site. This will allow a better understanding of how micro-environments, age, proximity and variety will affect the trees and provide enough data to allow abnormal values to be clearly identified.

4.6 Variation in DHA/Tsugar based on tree height

The intra-region comparisons suggested that the age of the mānuka might have an effect on the DHA/Tsugar. However the trees ages are difficult to estimate. Heights of the trees were thus used instead as a rough estimate of age.

Figure 4.13 does not show a strong trend correlation between DHA/Tsugar and tree height. This does not disprove age as a factor since tree height is known to change based on environments For example, trees on a hill top are

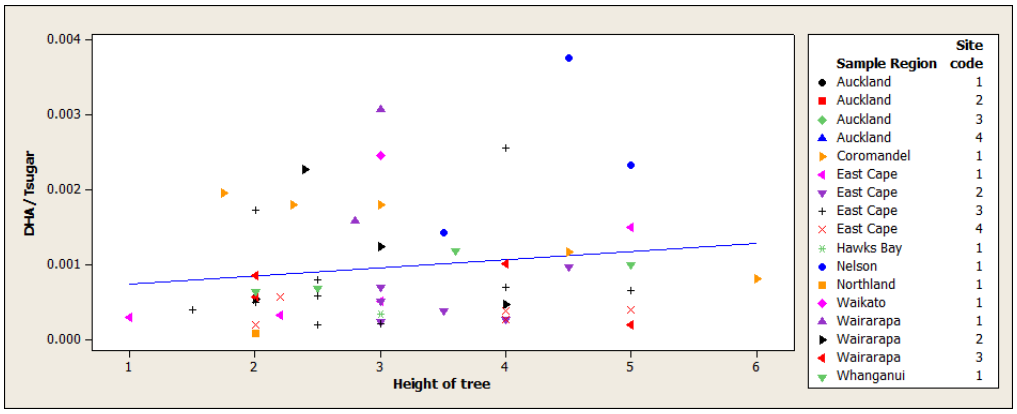


Figure 4.13: Scatter plot of the mean DHA/Tsugar values vs tree height for trees sampled in 2009.

more likely to be short and stocky to resist the wind. To confirm the effect of age, a better method will be required.

4.7 Waikato Gold

Through testing, VB014 was found to have the highest DHA/Tsugar. Having identified the cultivar it was from (due to commercial reasons this will be known as Waikato Gold), samples were collected from the tree in 2009, 2010 and 2011 to see how this tree varied over the three year period.

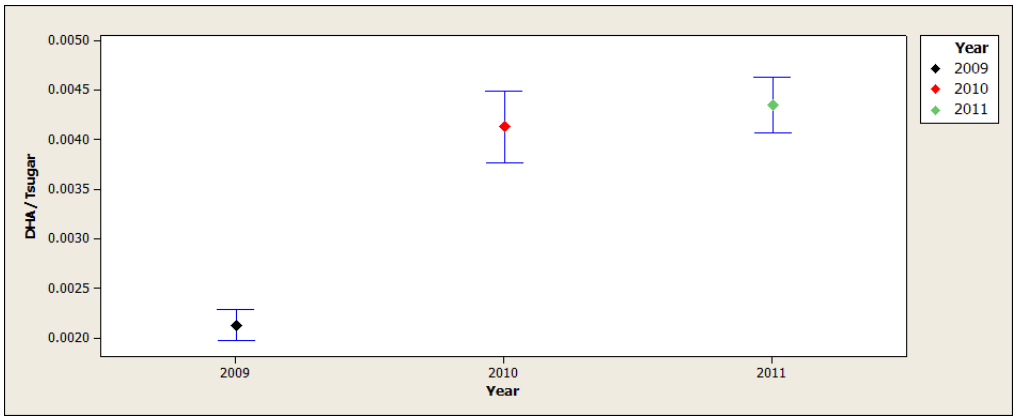


Figure 4.14: Interval plot of the mean DHA/Tsugar values for the VB014 tree sampled in 2009, 2010 and 2011 with error bars showing the 95% confidence interval.

Figure 4.14 shows that the 2010 and 2011 years gave the same results, while the 2009 year was lower. As the tree is considered mature, this difference cannot be explained by age alone. The difference could be explained by an environmental effect on the tree. For example, the Waikato was considered in drought for the flowering period in late 2009.[88] Another possibility is with the experimental method. However, as the number of flowers collected in 2009 were limited the 2009 sample could not be repeated to confirm the result. Further collection of samples from VB014 would allow this annual change, if indeed it is real to be better understood.

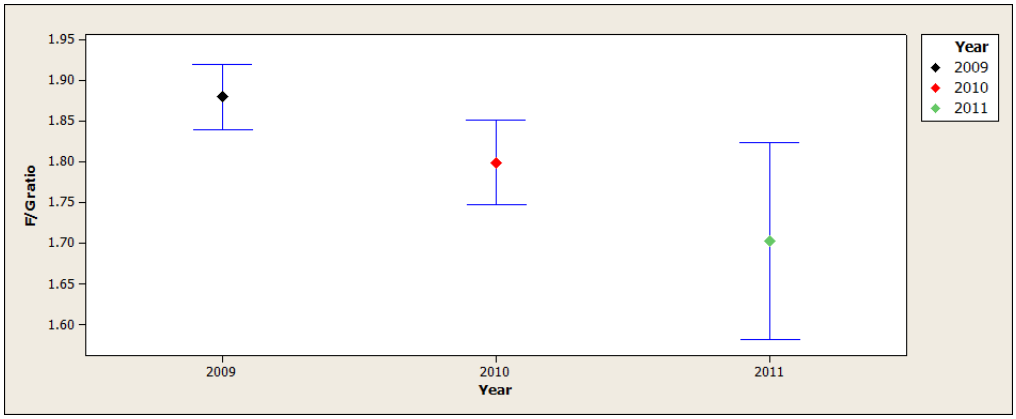


Figure 4.15: Interval plot of the mean F/G ratio values for the VB014 tree sampled in 2009, 2010 and 2011 with error bars showing the 95% confidence interval.

Figure 4.15 show that the F/G ratio is relatively consistent over the 3 year period with the 95 % confidence bars overlapping.

More work is required on the Waikato Gold variety to confirm it as a good candidate for breeding, but it does provide a starting point for future work.

4.8 Predicting honey NPA using the nectar DHA/Tsugar

The results of this research are presented in the format of DHA/Tsugar to describe the DHA content of a mānuka tree. To relate this back to the NPA of honey a conversion was required.

4.8.1 Prediction of DHA in immature honey based on DHA in nectar

Honey is approximately 90% glucose, fructose and sucrose. Tsugar is composed of glucose, fructose and sucrose found in the nectar. Assuming all the sugar in honey is from the nectar, then **Equation 4.1** gives the amount of DHA in a kg of sugar.

$$DHA/Tsugar(mg/mg) \times 1\,000\,000 = DHA/Tsugar(mg/kg) \tag{4.1}$$

As only ∼90% of honey is sugar **Equation 4.2** gives the amount of DHA assumed to be found in fresh honey from a beehive. This assumes that all the honey in the beehive is from the trees that have been assayed.

$$DHA/Tsugar(mg/kg) \times 0.90 = DHA/Honey(mg/kg) \tag{4.2}$$

4.8.2 Prediction of final MGO value in mature honey based on DHA in immature honey

Adams *et al.*(2009) showed that DHA is converted to MGO over time in honey. Using this conversion it is possible to change DHA to MGO in honey.[3]

$$(\frac{DHA}{Honey}(mg/kg) \times 0.2125) + 26.378 = \frac{MGO}{Honey}(mg/kg) \tag{4.3}$$

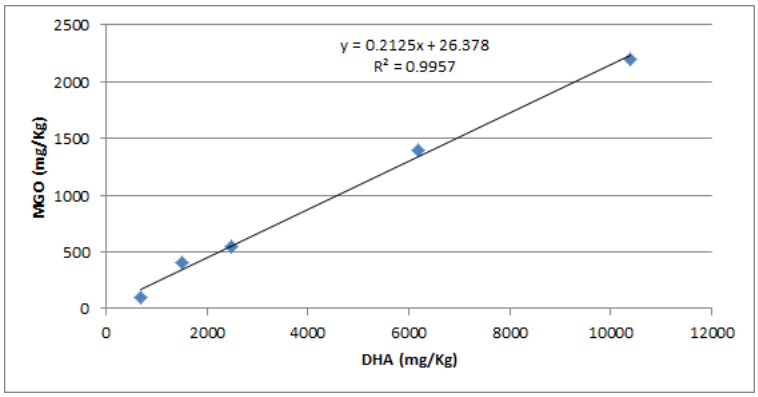


Figure 4.16: Graph demonstrating the relationship between initial DHA added and the MGO measured after 175 days constructed from the data in Adams *et al.*(2009).[3] This also provides the linear equation for the conversion in **Equation 4.3**.

The data for **Figure 4.16** came from doping clover honey with known amounts of DHA and storing it for 175 days at 37 ℃ and then measuring the MGO content.[3] It is assumed that this imitates the reaction which takes place in mānuka honey to give a correct conversion for DHA to MGO.

4.8.3 Prediction of NPA from the MGO content of mature honey

Adams *et al.*(2008, 2009) demonstrated that there was a relationship between MGO content and NPA in mānuka honey.[1,6]

Figure 4.17 provides the quadratic equation (**Equation 4.4**) for the conversion of MGO to NPA. The equation was shown to fit with a higher R^2 than a linear equation and provide a better y-intercept.

$$-6E^{-06}(\frac{MGO}{Honey}(mg/kg))^2 + 0.0266(\frac{MGO}{Honey}(mg/kg)) + 3.4677 = NPA \tag{4.4}$$

The data for **Figure 4.17** came from testing the NPA and MGO content of a range of mature mānuka honeys.

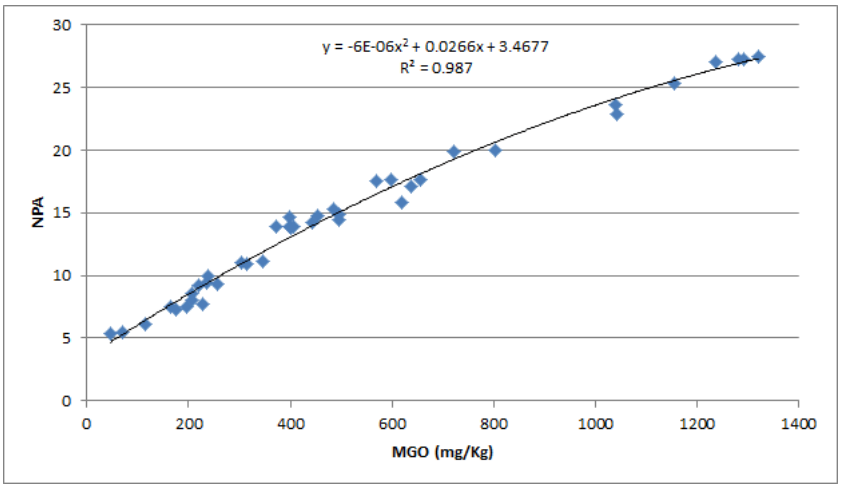


Figure 4.17: Graph demonstrating the relationship between MGO and NPA constructed from the data in Adams *et al.*(2008, 2009).[1,6]

4.8.4 Relating predicted NPA values to those obtained from hive sites

To confirm that the conversion worked, the calculated NPA of the sampled trees was compared with the NPA obtained from the honey collected from hive sites near the trees. As it can be assumed that the bees sample many trees from a site for their honey, the trees at each site were averaged to provide a representative sample, similar to what might be expected from the bees sampling many trees for their honey. The number of trees sampled in this survey was very small relative to the number that bees visit, so a representative sample of a site is not guaranteed, limiting the accuarcy of this prediction.

Figure 4.18 demonstrates a rough agreement between the recorded and predicted values. Taking into account the assumptions and limitations of this prediction, the results show that a predicted honey NPA value can be obtained for a hive site, based on sampling the trees at that site. This also demonstrates that it could be possible to predict the NPA of the honey which might be produced from a plantation of trees. A better understanding of the

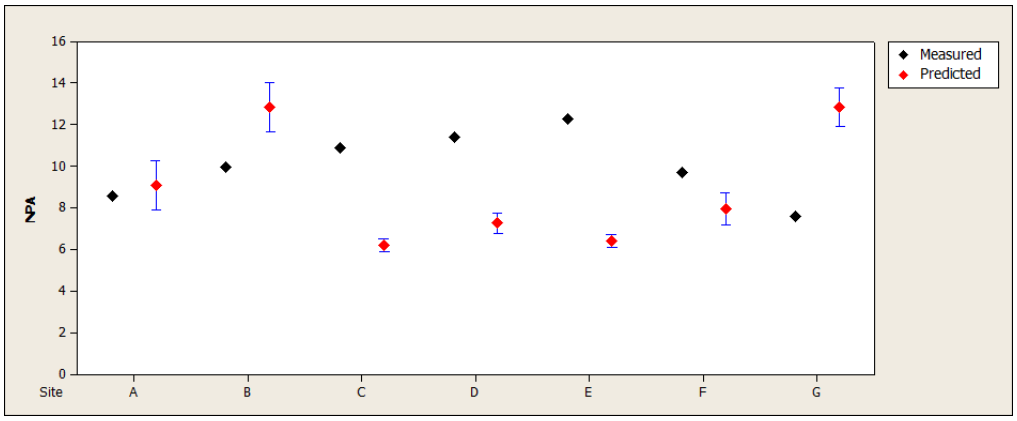


Figure 4.18: Comparing the predicted NPA to the actual NPA values. The predicted values have error bars showing the 95% confidence interval.

conversation of DHA to MGO in honey would aid in improving this prediction.

4.9 Relationship between mānuka flower nectar and soil

As part of the 2009 survey, soil from around the base of the mānuka trees sampled was collected. Analysis of the soil, is described by Kiefer(2010).[7] The soils were described by their colour (Munsell notation) as well as their order and texture. The pH, total soluble salts (TSS), Olsen P, total exchangeable base (TSB), cation exchange capacity (CEC), base saturation (BS), total carbon (TC), Total nitrogen (TN), carbon:nitrogen ratio(C/N) and trace elements, were measured.

It was observed in the studies by Kiefer(2010) and Janusch(2010) that there was no correlation observed between the leaf oils and soil samples taken from the same trees.[7,8]

To see if this observation is also true for the DHA/Tsugar, the 2009 samples will be compared to the soil analysis carried out on the soil from the sample sites.

4.9.1 DHA/Tsugar related to soil order

The most basic soil analysis is the soil order, that describes the type of soil.

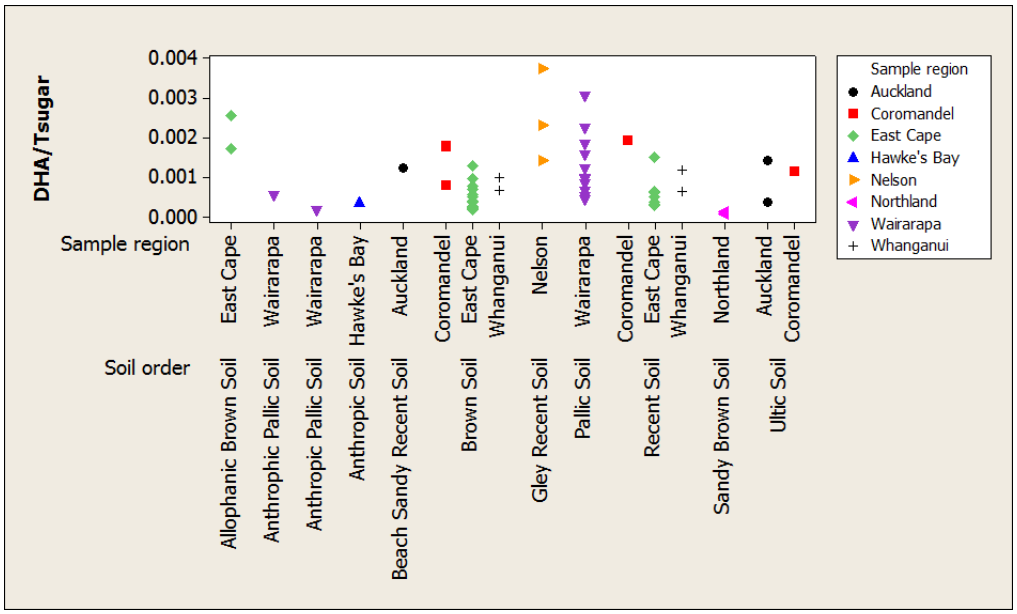


Figure 4.19: Interval plot of DHA/Tsugars for trees, grouped according to soil order and sample region, see enlarged in **Appendix C**.

Figure 4.19 shows the range of soil orders in which the sampled trees grow. The soil order is seen to have little effect, demonstrated by the similarities in DHA/Tsugar values located on different soils in the same region, for example, the Coromandel and East Cape samples. The Wairarapa samples also demonstrate the lack of effect with the large range of values for the pallic soil order. Therefore the soil order is not responsible for the variation seen in the DHA/Tsugar values.

4.9.2 DHA/Tsugar relative to soil components

To look at the quantifiable soil components, a linear regression test was used to see if the components, either individually or working together correlated, with the DHA/Tsugar from the flowers. The linear regression test was used to give an indication of any trends that are possibly present. Other

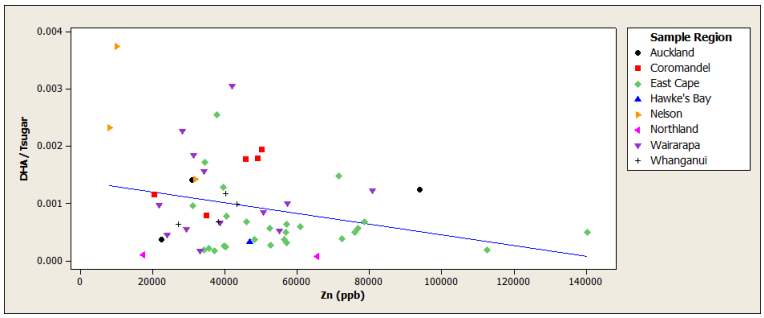
regressions were not fitted as the linear relationships demonstrated that the spread of data was large.

The soil components were first tested individually to find those with the greatest linear correlation to DHA/Tsugar **Table 4.2**. Some are illustrated in **Figure 4.20**.

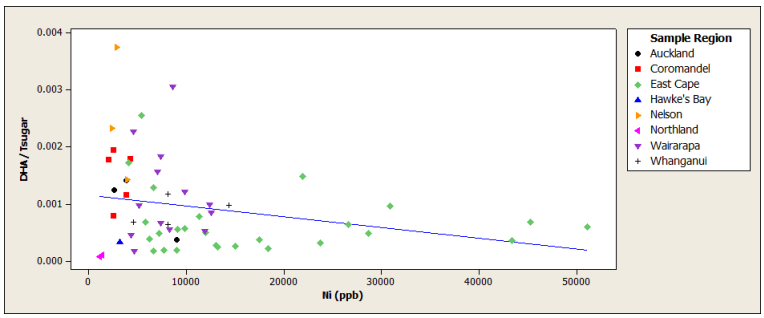
Table 4.2: Correlation of individual soil components to DHA/Tsugar.

Soil Component	R^2	Soil Component	R^2
Zn (ppb)	8.2	U (ppb)	1.8
Ni (ppb)	7.0	pH	1.7
C/N ratio	6.9	Sr (ppb)	1.7
TEB [cmol/100g]	5.4	TN[%]	1.5
Cu (ppb)	5.0	K (ppb)	1.3
B (ppb)	4.5	Tl (ppb)	0.9
In (ppb)	3.9	P (ppb)	0.8
Mg (ppb)	3.5	Na (ppb)	0.7
CEC [cmol/100g]	3.4	Se (ppb)	0.5
TSS [%]	3.3	Cd (ppb)	0.4
Ba (ppb)	3.2	Fe (ppb)	0.4
As (ppb)	3.0	Mn (ppb)	0.3
Olsen P[ppm]	3.0	Al (ppb)	0.1
Ca (ppb)	2.3	BS [%]	0.1
Cr (ppb)	2.3	TC [%]	0.0
Co (ppb)	2.0	Hg (ppb)	0.0
Pb (ppb)	1.9	V (ppb)	0.0
Li (ppb)	1.9		

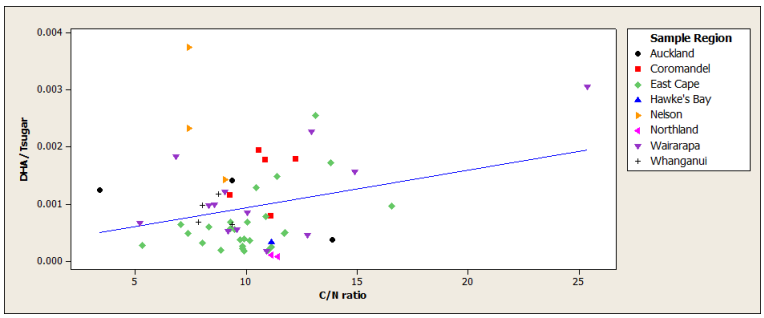
Table 4.2 shows that no single soil component is responsible for the level of DHA/Tsugar observed in the nectar of the flowers. This is shown by the lack of a strong trend in the scatter plots and the low R^2 values observed for each



(a) Zn, R^2 = 8.2 %, S = 0.00075



(b) Ni, R^2 = 7.0 %, S = 0.00075



(c) C/N ratio, R^2 = 6.9 %, S = 0.00076

Figure 4.20: Scatter plots of the three individual soil components that have the highest R^2 with DHA/Tsugar, to demonstrate the low linear correlation.

plot. More than one environmental effect is known to work on a plant, thus this result was anticipated.

Combinations of more than one soil component might have an effect, and hence the regression test was carried out using multiple soil components as variables to determine which ones had the strongest correlation with DHA/Tsugar.

The test gave **Equation 4.5**, which predicts DHA/Tsugar based on the soil components.

$$0.000899 + 0.000001\,U\,(ppb) - 0.0000001\,Zn\,(ppb) - 0.0000001\,P\,(ppb)$$
$$- 0.00377\,TN\,(\%) + 0.000307\,TC\,(\%) + 0.000007\,BS\,(\%)$$
$$+ 0.000058\,OlsenP\,(ppm) - 0.000040\,TEB\,(cmol/100g) = DHA/Tsugar \tag{4.5}$$

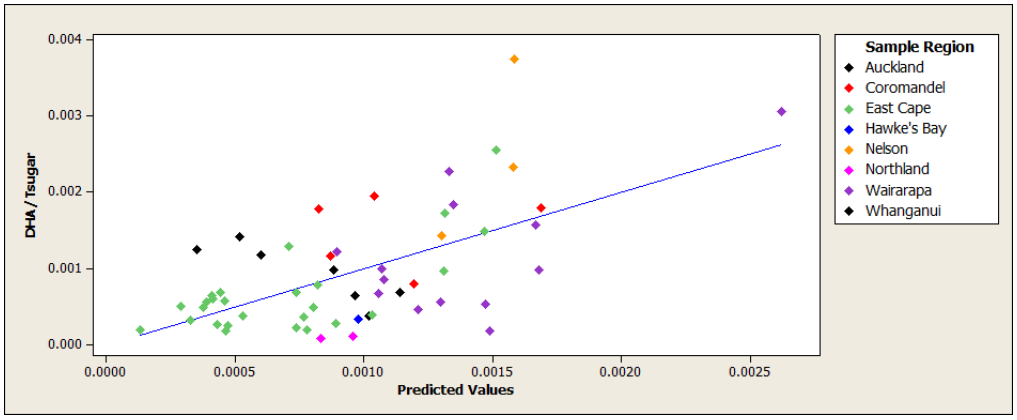


Figure 4.21: Scatter plot of the measured DHA/Tsugar vs the values predicted by **Equation 4.5**, R^2 (adj) = 26.5 %, S = 0.00067 using soil components across all regions.

Figure 4.21 compares the measured DHA/Tsugar values to those that **Equation 4.5** predicts. A trend is suggested, but with a R^2 (adj) = 26.5%, S = 0.00067 *†, there is poor linear correlation to confirm it. As the linear correlation is very weak, it can be concluded that the soil in which the mānuka tree grows has an insignificant effect on the DHA/Tsugar values found in the nectar. This is reinforced by the scatter of data observed in **Figure 4.20** and is similar to the observations that soil components, had little effect on the leaf oils[25] and NPA of honey[5] from the mānuka tree.

*R^2 (adj) is a modification of R^2 that adjusts for the number of variables used in the model

†S is the standard error of the regression, and represents the standard distance the data values fall from the regression line

4.9.3 F/G ratio relative to soil components

Although it has been shown that the soil components do not have a significant effect on the DHA/Tsugar directly, there may be an indirect effect through other components in the nectar. Fructose and glucose are two major components of the nectar, so if a relationship were found between the F/G ratio and the soil components, it would suggest that the soil may have an effect on the nectar and therefore an indirect effect on the DHA/Tsugar.

Table 4.3 lists the individual soil components and their correlation to the F/G ratio.

Table 4.3 shows that no single soil component is responsible for the level of F/G ratio, as observed in the nectar of the flowers. This is shown by the lack of a strong linear trend in the scatter plots **Figure 4.22** and the low R^2 values observed for each plot.

It is possible that combinations of more than one soil component might have an effect. The linear regression test was carried out using multiple soil components to determine which, if any combinations, provide the strongest correlation with the F/G ratio.

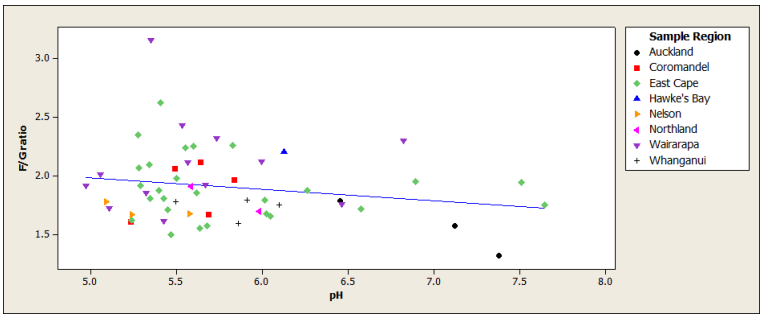
The test gave **Equation 4.6** which predicts F/G ratio based on the soil components.

$$\begin{aligned} 2.84 - 0.167\,pH + 0.000019\,Li\,(ppb) + 0.000244\,B\,(ppb) \\ + 0.0000001\,Al\,(ppb) - 0.0000001\,K\,(ppb) - 0.000012\,Cu\,(ppb) \\ - 0.000012\,Ni\,(ppb) - 0.000305\,U\,(ppb) = F/G\,ratio \end{aligned} \quad (4.6)$$

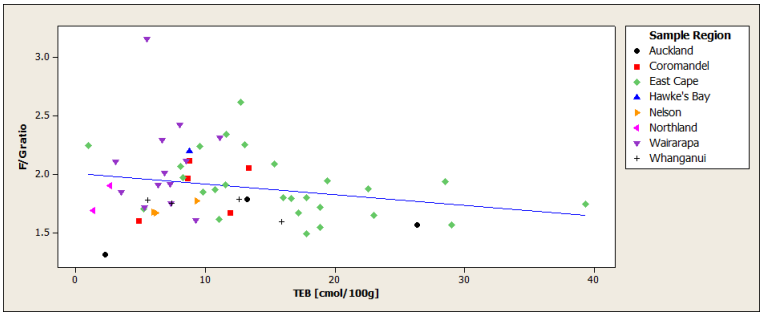
Figure 4.23 compares the measured F/G ratio values to those that **Equation 4.6** predicts. A trend is suggested, but with a R^2(adj) of 37.3% and a S of 0.24, there is poor correlation to confirm it. A larger sample size

Table 4.3: Correlation of individual soil components to F/G ratio for all regions.

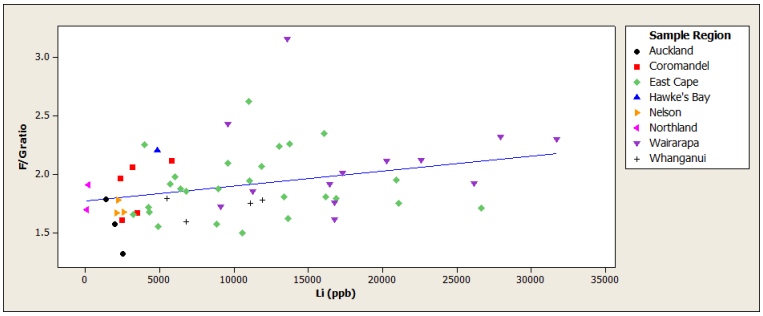
Soil Component	R^2	Soil Component	R^2
Li (ppb)	9.8	Co (ppb)	1.0
TEB [cmol/100g]	5.0	Ba (ppb)	0.9
pH	3.7	Cr (ppb)	0.8
Al (ppb)	3.7	Hg (ppb)	0.8
Se (ppb)	3.5	P (ppb)	0.8
Cu (ppb)	2.6	BS [%]	0.7
Pb (ppb)	2.3	Sr (ppb)	0.6
C/N ratio	2.0	TN[%]	0.5
Na (ppb)	2.0	B (ppb)	0.5
Ni (ppb)	1.8	CEC [cmol/100g]	0.3
V (ppb)	1.7	Mg (ppb)	0.3
Mn (ppb)	1.7	Cd (ppb)	0.2
Olsen P[ppm]	1.6	K (ppb)	0.2
Ca (ppb)	1.6	As (ppb)	0.1
U (ppb)	1.5	Fe (ppb)	0.0
Tl (ppb)	1.4	Zn (ppb)	0.0
TC [%]	1.3	TSS [%]	0.0
In (ppb)	1.1		



(a) pH, R^2 = 3.7 %, S = 0.31



(b) TEB, R^2 = 5.0 %, S = 0.30



(c) Li, R^2 = 9.8 %, S = 0.30

Figure 4.22: Scatter plots of the three individual soil components that have the highest R^2 with F/G ratio, to demonstrate the low correlation.

taken from each region may improve the trend, but as the correlation is very weak, it can be concluded that the soil in which the mānuka trees grow has an insignificant effect on the F/G ratio found in the nectar.

As no strong linear correlations were observed between DHA/Tsugar, the F/G ratio and the soil components, combined with the lack of correlation in the scatter plots of the individual soil components with DHA/Tsugar and the F/G ratio, it was concluded that the soil has an insignificant effect on the nectar produced by the mānuka tree.

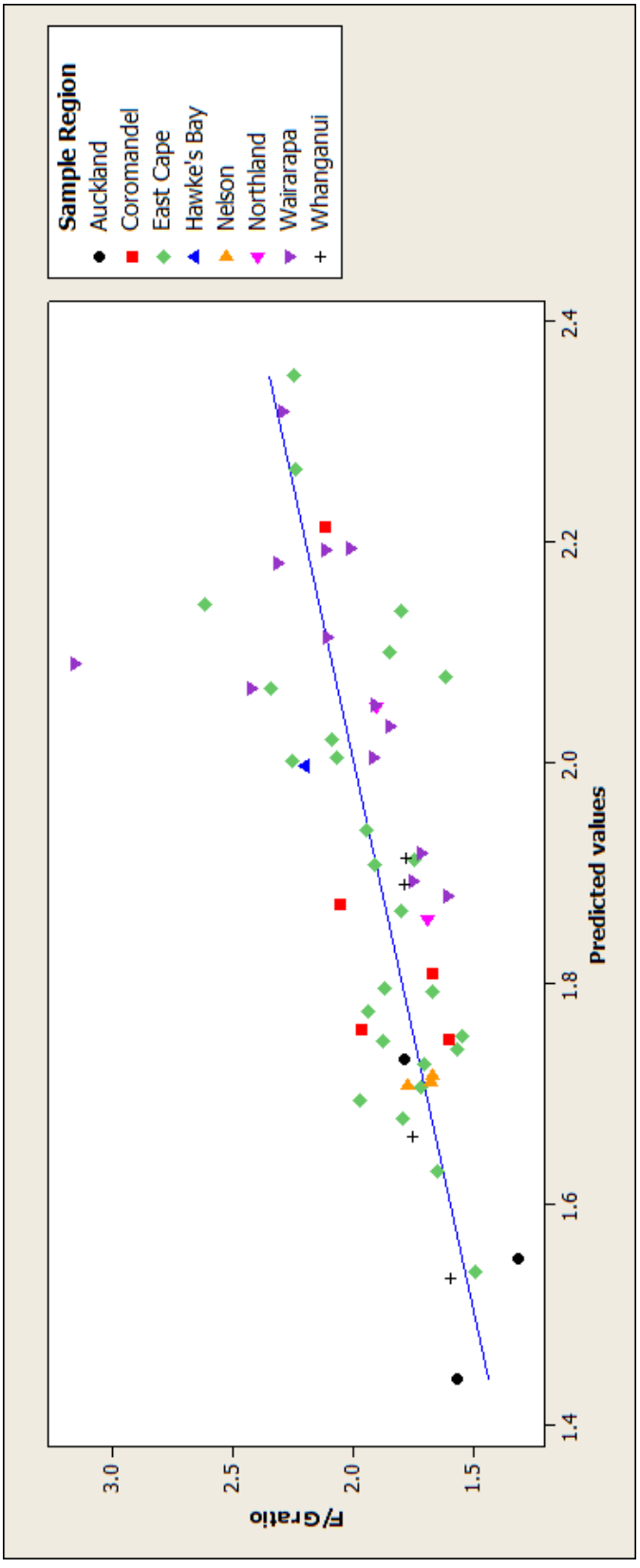


Figure 4.23: Scatter plot of the measured F/G ratio vs the values predicted by **Equation 4.6**, R^2(adj) = 37.3%, S = 0.24 using soil components across all regions.

4.10 Relationship between mānuka flower nectar and mānuka leaf oils

As part of the 2009 survey leaves were also collected from the mānuka trees sampled. The leaf oil was analysed by Janusch(2010).[8] Monoterpenes, esters, sesquiterpene and triketones were analysed.

It is possible that DHA may be formed in the leaves of the mānuka. If this is correct it may be possible to relate the different compounds in the leaves to DHA/Tsugar, thus allowing the DHA/Tsugar levels to be predicted when the tree is not in flower.

Because of regional differences, only the Coromandel, East Cape and Wairarapa samples were analysed in detail, as these have the most samples from one region for 2009. The % mass values were used for the oil compounds. These are based on the ratio of the oil compound in the total oil extract and therefore would not be affected by the amount of oil extracted.

4.10.1 DHA/Tsugar compared to leaf oil from all regions

To determine which oil compounds had the strongest correlation with the DHA/Tsugar over all the regions, an individual linear regression test was performed on the individual oil compounds to find those that had the greatest correlation with DHA/Tsugar (**Table 4.4**).

This allowed the top 20 oil compounds with a strong correlation to DHA/Tsugar to be selected from the initial 85 different oil compounds. A regression test using multiple variables was then carried out to determine what combination of variables gave the best correlation with DHA/Tsugar for all sampled regions.

Table 4.4: The 20 individual oil compounds that correlate the strongest with DHA/Tsugar from all regions. A complete table of all individual oil compounds can be found in **Appendix D**.

Oil Compound	R^2	Oil Compound	R^2
Cadina-3,5-diene	11.9	Benzyl-2-methyl butyrate	9.1
Benzyl isovalerate	8.2	Selinen-4α-ol	7.0
β-Selinene	6.3	α-Campholenal	6.2
R-Norinone	6.2	γ-Selinene	5.7
α-Selinene	5.0	α-Gurjunene	4.9
cis-Methyl cinnamate	4.9	Selina-3,7 (11)-diene	4.8
Isoamyl benzoate	4.8	α-Cubebene	3.8
Isoprenyl isovalerate	3.6	*trans*- Methyl cinnamate	3.5
Spathulenol	3.5	Grandiflorone	3.4
β-Pinene	3.2	Ledol	3.2

The multiple variable linear regression equation with the strongest correlation was **Equation 4.7** (R^2(adj) = 35.1%, S = 0.00064).

$$\begin{aligned} &0.00166 - 0.000072\,Grandiflorone + 0.000111\,Flavesone - 0.000380\,Cubenol \\ &+ 0.000194\,Selinen - 4\alpha - ol + 0.000089\,trans - Methyl\,cinnamate \\ &- 0.000352\,\alpha - Gurjunene - 0.000198\,Linalool \\ &-0.00405\,2 - Methyl\,butyl\,isovalerate - 0.00180\,Isoprenyl\,tiglate = DHA/Tsugar \end{aligned} \tag{4.7}$$

Figure 4.24 shows little correlation (R^2(adj) = 35.1%, S = 0.00064) between the values predicted by **Equation 4.7** and the measured values. The more variables used in a multiple variable linear regression equation, the greater the correlation that is expected to be seen. These results used a large number of variables with strong correlations, thus giving the best chance of correlation. A weak correlation (R^2 = 35.1 %(adj), S = 0.00064) was found,

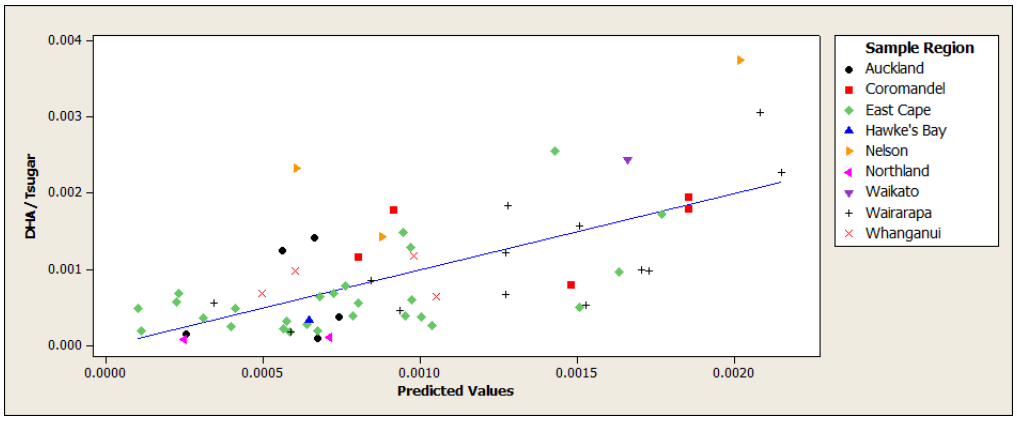


Figure 4.24: Scatter plot of the measured DHA/Tsugar vs the values predicted by **Equation 4.7**, R^2(adj) = 35.1%, S = 0.00064 for leaf oil from all regions.

therefore it can be concluded from these results that there is no one or set of oil compounds that correlates strongly with DHA/Tsugar, across all the regions sampled.

Previous studies have shown that the oil composition from the mānuka leaves does change around the country and that these different oil compositions have been divided up into oil chemotypes.[25] The studies found that mānuka exhibits different oil chemotypes in different regions and that these chemotypes are maintained when trees are transplanted into new regions.[5,25]

The Coromandel, East Cape and Wairarapa have been shown to possess different chemotypes,[25] so one might also expect that different combinations of components would correlate with DHA/Tsugar. As soil effects have been ruled out, a genetic or weather-based effect might be the cause of both the oil and DHA/Tsugar variation.

4.10.2 F/G ratio compared to leaf oil from all regions

Since correlation with DHA/Tsugar across all regions was poor, the F/G ratio was also tested for correlation, as this is another nectar component. To

determine which oil compounds had the greatest effect on the F/G ratio over all regions, an individual regression test was performed on the individual oil compounds to discover those that had the greatest correlation with the F/G ratio **Table 4.5**.

Table 4.5: The 20 individual oil compounds that correlate the strongest with F/G ratio from all regions. A complete table of all individual oil compounds can be found in **Appendix D**.

Oil Compound	R^2	Oil Compound	R^2
Aromadendrene	10.1	γ-Eudesmol	8.5
α-Eudesmol	7.3	β-Eudesmol	6.6
$C_{15}H_{24} - 1$	5.5	γ-Terpinene	3.5
Linalool	3.2	Cubenol	3.2
Humulene	3.0	*cis*-Methyl cinnamate	2.7
$C_{15}H_{24}$ (1486)	2.7	δ-Elemene	2.5
τ-Elemene	2.5	Germacrene D	2.0
β-Selinene	1.9	Elemol	1.8
Caryophyllene Oxide	1.7	*trans*-Citral	1.6
Geranyl acetate	1.4	Selina-3,7 (11)-diene	1.4

$$\begin{aligned} 1.88 + 0.0618\,Aromadendrene - 0.0201\,\alpha - Eudesmol \\ + 0.129\,C_{15}H_{24} - 1 + 0.295\,\gamma - Terpinene - 0.0580\,Linalool \\ - 0.113\,Cubenol - 0.639\,\delta - Elemene + 0.0731\,Elemol \\ = F/G\,ratio \end{aligned} \quad (4.8)$$

Like the DHA/Tsugar, the F/G ratio correlated across all regions poorly with the best multiple variable linear equation (**Equation 4.8**) gave R^2(adj) = 19.4%, S = 0.27. This shows that there is no one individual or set of oil

components that can explain the variation in nectar components across all the regions sampled.

4.10.3 DHA/Tsugar compared to leaf oil from the East Cape

To determine which oil compounds had the greatest effect on the DHA/Tsugar in the East Cape region, an individual regression test was performed on the individual oil compounds to find those that had the greatest correlation with DHA/Tsugar (**Table 4.6**).

Table 4.6: The 20 individual oil compounds that correlate the strongest with DHA/Tsugar from the East Cape region. A complete table of all individual oil compounds can be found in **Appendix D**.

Oil Compound	R^2	Oil Compound	R^2
Aromadendrene	25.2	*Allo*-Aromadendrene	13.4
β-Eudesmol	12.8	α-Eudesmol	12.5
Isoamyl isovalerate	12.2	γ-Eudesmol	11.8
Cadina-3,5-diene	11.0	2-Methyl butyl isovalerate	9.2
$C_{15}H_{24}$ (1517)	8.7	γ-Terpinene	8.0
p-Cymene	8.0	α-Ylangene	7.9
Humulene	6.9	Grandiflorone	6.7
Limonene	6.5	Eucalyptol	6.1
$C_{15}H_{24}$-3	5.7	δ-Elemene	5.5
Elemol	5.5	τ-Elemene	5.5

This allowed the top 20 oil compounds with a strong correlation to DHA/Tsugar to be selected from the initial 85 different oil compounds. A regression test using multiple variables was then carried out to determine which combination of variables gave the best correlation with DHA/Tsugar.

The oil compounds aromadendrene, limonene, α-gurjunene, caryophyllene, *allo*-aromadendrene, β-selinene and grandiflorone were found to have the greatest correlation with DHA/Tsugar, giving **Equation 4.9**.

$$\begin{aligned} 0.00263 + 0.000514\,Aromadendrene - 0.0147\,Limonene \\ - 0.00229\,\alpha - Gurjunene + 0.000326\,Caryophyllene \\ - 0.00127\,Allo - Aromadendrene - 0.000168\,\beta - Selinene \\ - 0.000034\,Grandiflorone = DHA/Tsugar \end{aligned} \quad (4.9)$$

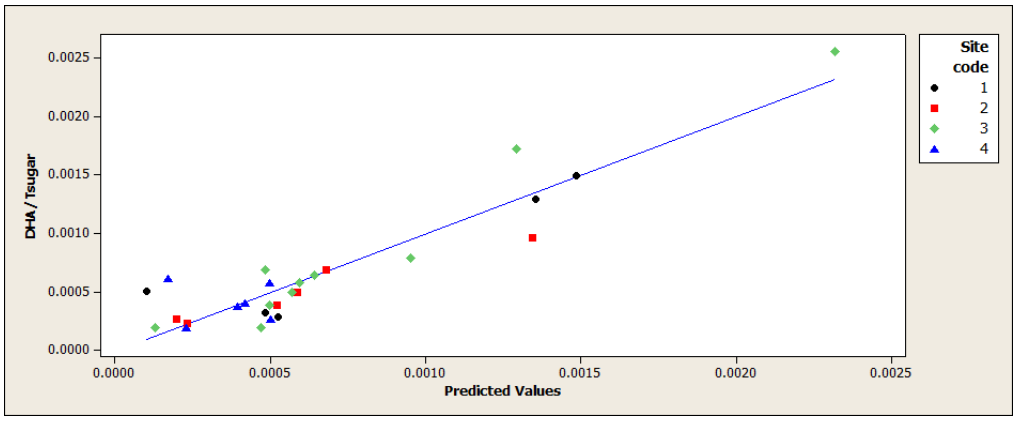


Figure 4.25: Scatter plot for the East Cape of the measured DHA/Tsugar vs the values predicted by **Equation 4.9**, R^2(adj) = 80.7%, S = 0.00024.

Figure 4.25 shows a moderate correlation (R^2(adj) = 80.7%, S = 0.00024) between the values predicted by **Equation 4.9** and the measured values.

4.10.4 F/G ratio compared to leaf oil from the East Cape

To determine which oil compounds had the greatest effect on the F/G ratio in the East Cape region, an individual linear regression test was performed on the individual oil compounds to find those that had the greatest correlation with F/G ratio (**Table 4.7**).

This allowed the top 19 oil compounds with a strong correlation to F/G ratio to be selected from the initial 85 different oil compounds. A regression test

Table 4.7: The 20 individual oil compounds that correlate the strongest with the F/G ratio from the East Cape region. A complete table of all individual oil compounds can be found in **Appendix D**.

Oil Compound	R^2	Oil Compound	R^2
γ-Terpinene	23.0	$C_{15}H_{24}-1$	22.7
p-Cymene	19.3	Isoprenyl isovalerate	15.4
α-Pinene	14.1	δ-Cadinene	12.0
Humulene	8.5	Limonene	8.2
Benzyl-2-methyl butyrate	8.2	γ-Muurolene	7.9
Prenyl isovalerate	7.9	β-Elemene	7.8
δ-Elemene	7.0	Elemol	7.0
τ-Elemene	7.0	α-Amorphene	7.0
Linalool	6.9	*trans*- Methyl cinnamate	6.6
Cubenol	6.5		

using multiple variables was then carried out to determine what combination of variables gave the best correlation with DHA/Tsugar.

The oil compounds γ-terpinene, $C_{15}H_{24}-1$, isoprenyl isovalerate, α-pinene, benzyl-2-methyl butyrate, β-elemene, germacrene D and benzyl isovalerate were found to have the greatest correlation with F/G ratio, giving **Equation 4.10**.

$$\begin{aligned} 1.89 - 3.02\,\gamma - Terpinene + 0.207\,C_{15}H_{24} - 1 + 0.824\,Isoprenyl\,isovalerate \\ - 0.374\,\alpha - Pinene + 1.62\,Benzyl - 2 - methylbutyrate - 0.282\,\beta - Elemene \\ + 0.420\,Germacrene\,D - 0.596\,Benzyl\,isovalerate \\ = F/G\,ratio \end{aligned} \quad (4.10)$$

Figure 4.26 shows a moderate correlation (R^2(adj) = 70.3%, S = 0.15) between the values predicted by **Equation 4.10** and the measured values.

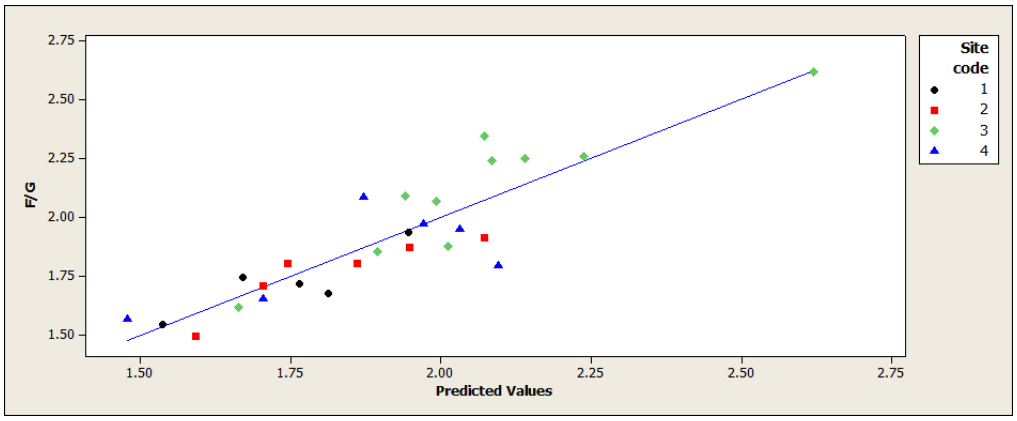


Figure 4.26: Scatter plot for the East Cape of the measured F/G ratio vs the values predicted by **Equation 4.10**, R^2(adj) = 70.3%, S = 0.15.

4.10.5 DHA/Tsugar compared to leaf oil from the Wairarapa

To determine which oil compounds had the greatest effect on the DHA/Tsugar in the Wairarapa region, an individual linear regression test was performed on the individual oil compounds to find those that had the greatest correlation with DHA/Tsugar (**Table 4.8**).

This allowed the top 20 oil compounds with a strong correlation to DHA/Tsugar to be selected from the initial 85 different oil compounds. A regression test using multiple variables was then carried out to determine what combination of variables gave the best correlation with DHA/Tsugar.

For the Wairarapa samples grandiflorone, cubenol, α-selinene, β-cubebene and *trans*-methyl cinnamate were found to have the best correlation with DHA/Tsugar, giving **Equation 4.11**.

$$\begin{aligned} 0.000385 + 0.000119\,Grandiflorone - 0.000918\,Cubenol - 0.000033\,\alpha - Selinene \\ + 0.000819\,\beta - Cubebene + 0.000285\,trans - Methyl\,cinnamate \\ = DHA/Tsugar \end{aligned} \quad (4.11)$$

Figure 4.27 shows a moderate correlation (R^2 = 88.9%(adj), S = 0.00027) between the values predicted by **Equation 4.11** and the measured values.

Table 4.8: The 20 individual oil compounds that correlate the strongest with DHA/Tsugar from the Wairarapa region. A complete table of all individual oil compounds can be found in **Appendix D**.

Oil Compound	R2	Oil Compound	R2
Cubenol	45.7	*trans*- Methyl cinnamate	32.9
β-Cubebene	28.4	β-Gurjunene	27.9
Calamenene	26.9	Grandiflorone	25.9
α-Gurjunene	24.5	*cis*-Methyl cinnamate	19.0
α-Muurolene	18.9	Ledol	16.9
Geranyl acetate	16.6	Isoamyl benzoate	16.2
α-Calacorene	15.5	Flavesone	15.5
Allo-Aromadendrene	13.7	Isoamyl acetate	13.0
Cadina-1,4-diene	13.0	Spathulenol	12.5
β-Elemene	11.3	Isoprenyl-2-methyl butyrate	11.2

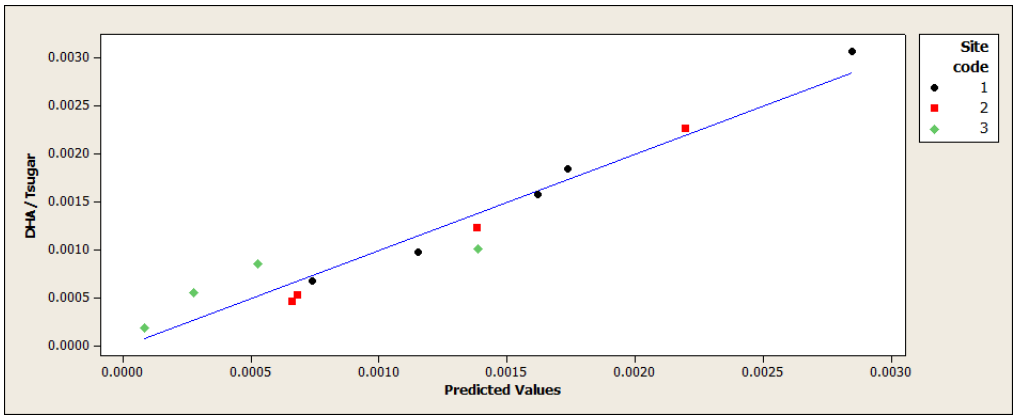


Figure 4.27: Scatter plot of the measured DHA/Tsugar 2009 Wairarapa samples vs the values predicted by **Equation 4.11**, R^2 = 88.9%(adj), S = 0.00027.

4.10.6 F/G ratio compared to leaf oil from the Wairarapa

To determine which oil compounds had the greatest effect on the F/G ratio in the Wairarapa region, an individual linear regression test was performed on the individual oil compounds to find those that had the greatest correlation with F/G ratio (**Table 4.9**).

Table 4.9: The 20 individual oil compounds that correlate the strongest with the F/G ratio from the Wairarapa region. A complete table of all individual oil compounds can be found in **Appendix D**.

Oil Compound	R2	Oil Compound	R2
α-Ylangene	29.9	Isoamyl isovalerate	15.9
2-Methyl butyl isovalerate	15.8	Selina-3,7 (11)-diene	13.1
$C_{15}H_{24}$-2	12.8	Isoprenyl isovalerate	11.7
$C_{15}H_{24}$ (1517)	9.3	α-Pinene	8.9
α-Cubebene	7.4	Ledol	7.1
δ-Cadinene	6.7	γ-Selinene	6.6
α-Bergamotene	6.2	Spathulenol	5.9
Linalool	5.6	γ-Cadinene	5.4
$C_{15}H_{24}$	5.0		

This allowed the top 20 oil compounds with a strong correlation to F/G ratio to be selected from the initial 85 different oil compounds. A regression test using multiple variables was then carried out to determine what combination of variables gave the best correlation with F/G ratio.

For the Wairarapa samples α-ylangene, isoamyl isovalerate, selina-3,7 (11)-diene, $C_{15}H_{24}$ (1517) and ledol were found to have the best correlation

with DHA/Tsugar, giving **Equation 4.12**.

$$0.132 + 0.767\,\alpha - Ylangene - 0.476\,Isoamylisovalerate$$
$$+2.56\,Selina{-}3,7(11){-}diene{+}0.782\,C_{15}H_{24}(1517){+}4.23\,Ledol = F/G\,ratio \tag{4.12}$$

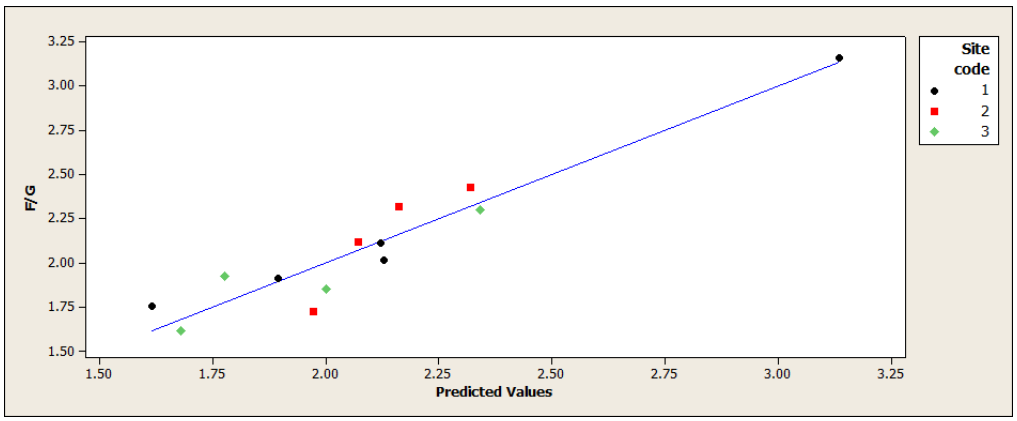


Figure 4.28: Scatter plot for the 2009 Wairarapa samples of the measured F/G ratio vs the values predicted by **Equation 4.11**, R^2 = 83.6%(adj), S = 0.16.

Figure 4.28 shows a moderate correlation (R^2 = 83.6%(adj), S = 0.16) between the values predicted by **Equation 4.12** and the measured values.

4.10.7 DHA/Tsugar compared to leaf oil from the Coromandel

To determine which oil compounds had the greatest effect on the DHA/Tsugar in the Coromandel region, an individual regression test was performed on the individual oil compounds to find, those that had the greatest correlation with DHA/Tsugar (**Table 4.10**).

Table 4.10 appears to demonstrate that the Coromandel samples' oil components, individually, have a stronger correlation than those from the East Cape and Wairarapa; though this stronger correlation, is due to the smaller number of samples taken from the Coromandel region.

Table 4.10: The 20 individual oil compounds that correlate the strongest with the DHA/Tsugar from the Coromandel region. A complete table of all individual oil compounds can be found in **Appendix D**.

Oil Compound	R^2	Oil Compound	R^2
β-Pinene	68.2	Cadina-3,5-diene	62.7
$C_{15}H_{24}$-2	61.2	Grandiflorone	61.0
α-Bergamotene	52.6	β-Cubebene	50.0
α-Selinene	47.6	β-Selinene	45.3
γ-Selinene	43.8	Benzyl-2-methyl butyrate	41.3
Eucalyptol	40.6	Selina-3,7 (11)-diene	40.1
Isoprenyl isovalerate	38.4	Isoprenyl-2-methyl butyrate	35.8
Limonene	27.9	Selinen-4α-ol	27.5
Flavesone	23.2	α-Cadinol	22.6
$C_{15}H_{24}$-3	21.4	Cadina-1,4-diene	20.9
$C_{15}H_{24}$ (1517)	6.3		

Figure 4.29 supports this conclusion, as the number of sample points do not represent a broad range of values. More samples are needed from the Coromandel to confirm any correlation observed. The Auckland, Northland and Whanganui samples will not be correlated to the oil compounds because the number of samples from these is also insufficient.

4.10.8 F/G ratio compared to leaf oil from the Coromandel

As the oil testing from the Coromandel showed, that there were insufficient samples for a correlation to be demonstrated sufficiently, The F/G ratio was not investigated for the Coromandel samples.

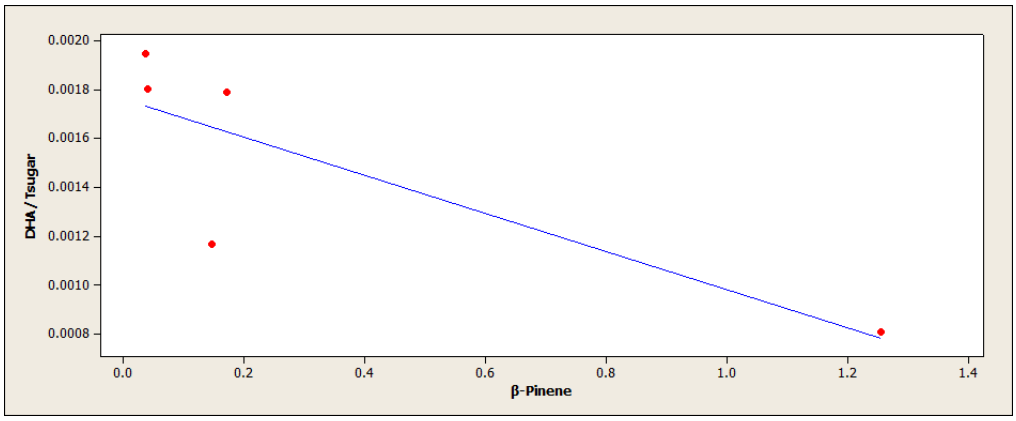


Figure 4.29: A plot of DHA/Tsugar vs β-pinene in the Coromandel, R^2 = 68.2%, S = 0.00032.

4.10.9 DHA/Tsugar, F/G ratio and oils summary

A moderate correlation was found for combination of selected leaf oil components and DHA/Tsugar for the East Cape region (R^2(adj) = 80.7%, S = 0.00024) and Wairarapa region (R^2(adj) = 88.9%, S = 0.00027) and a slightly worse correlation was found for the F/G ratio; East Cape (R^2(adj) = 70.3%, S = 0.15) and Wairarapa R^2(adj) = 83.6%, S = 0.16. There was insufficient Coromandel samples to conclude any correlation for DHA/Tsugar or F/G ratio. The East Cape and Wairarapa correlations were strong compared to the correlation across all regions for both DHA/Tsugar and F/G ratio. It may be possible to improve the correlation within a region by obtaining a better understanding of why DHA/Tsugar, F/G ratio and the leaf oils may vary. This understanding combined with a larger sample size from the region, may allow the R^2 value to be improved and thus the DHA/Tsugar and F/G ratio to be predicted from the leaves. Predicting the DHA/Tsugar from the leaf oils would be a more efficient method than from the flower, allowing trees to be sampled at any time of year. As multiple oil components had to be combined to give the regression equations, there is no single oil component that can be used to predict the DHA/Tsugar or F/G ratio expected from a mānuka tree using the leaves alone. The main oil type that correlated were the sesquiterpenes. This suggests that there could

possibly be a link between the metabolism responsible for formation of the sesquiterpenes and DHA/Tsugar. Different equations had to be used for each region reminiscent of the chemotypes reported by Douglas *et al.*(2005),[25] DHA/Tsugar and the F/G ratio were also found to vary between regions. The idea that DHA/Tsugar and F/G ratio is linked to a genetic difference between mānuka varieties, is thus supported. Nevertheless these results only show that there is a correlation between some of the leaf oil components and DHA/Tsugar and F/G ratio. As the oil correlates with both the DHA/Tsugar and F/G ratio, (two nectar components), there may be a link between the leaf oils and the nectar produced in the flowers. Although it is hypothesised that DHA may be produced in the leaves, this is not yet proven, nor is the direct relationship between DHA/Tsugar and the leaf oils clearly shown. Future investigations may seek to determine if DHA is found in the mānuka leaves, and if so, through what mechanism it relates to the leaf oils. Seasonal variations have been reported in *Leptospermum* varieties in Brazil.[89] If the leaf oil compositions are found to correlate with DHA/Tsugar, a further study would be to see if changes in leaf oil composition due to seasonal effects is also observed in the DHA/Tsugar. A genetics study could also discover if there are any genetic links between mānuka varieties and DHA/Tsugar, as well as between DHA/Tsugar and the leaf oils.

4.11 DHA/Tsugar and triketone oils

The triketone oils from mānuka on the East Cape have been found to have antibacterial properties. The ideal mānuka tree would produce both high levels of DHA/Tsugar and triketone enriched oil.

Using the 2009 oil and DHA/Tsugar values (**Figure 4.30**), demonstrates that VB193, VB195, L3962 and L3995 would be trees to consider breeding from as these exhibit both a high DHA/Tsugar as well as a large triketone oil content.

Such a tree could have its branches harvested every year for oil while keeping the tree small and bushy to produce a larger display of flowers. This would increase, the amount of nectar available to the bees.

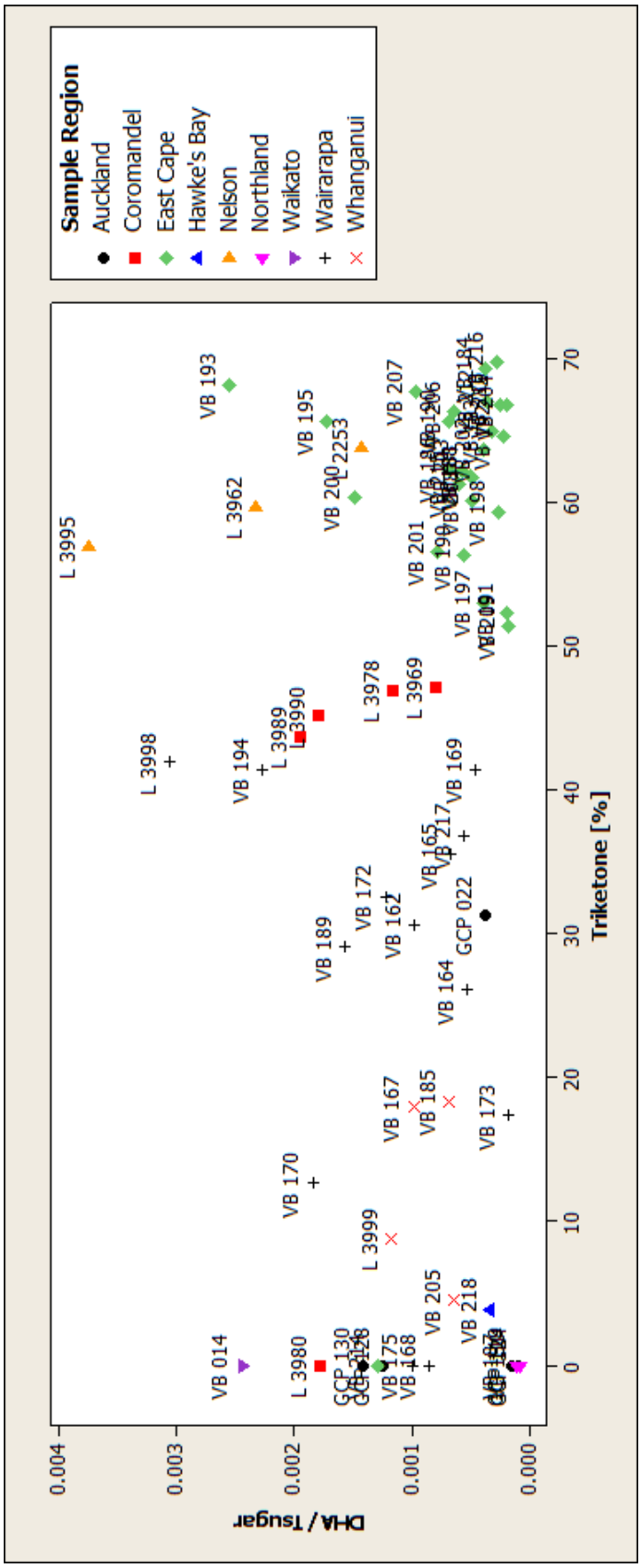


Figure 4.30: Scatter plot of the measured DHA/Tsugar vs Triketone(%).

Chapter 5

Conclusion and future work suggestions

5.1 Suggestions for future work

This section will bring together previous suggestions made throughout the thesis for possible future work.

5.1.1 Flowers and nectar

For the 20 flower method, any flaws in the flowers are averaged over all the flowers sampled. For the SFT this is not possible and any flaws may impact on the DHA/Tsugar measured from these flowers. Understanding how the DHA/Tsugar varies in an individual flower will aid in improve understanding when using the SFT and may help explain some of the variation in the 20 flower method.

The length of time a flower has been open may affect the DHA/Tsugar. A study that picked flowers that have been open for different time period would aid in this understanding. This could be further expanded by looking to see if DHA/Tsugar changes throughout the day.

Flowers were picked with no knowledge if they had been visited by pollinators or not. Pollinators have been shown to have an affect on nectar in other plants.[34–37] Future testing may wish to look at bagging flowers before they are picked to ensure pollinators are unable to visit the flower.

Mānuka flowers with a pink tinge to their white petals often exhibited a broad colour range of petals from white to full pink. The small study in this project did not find a strong correlation between colour and DHA/Tsugar. A similar study was carried out based on the centre of these flowers. This was undertaken as some flower centres were green while others were dark red. The cause of this different coloration was not investigated, but age or fertilisation might be responsible. A visual indication of high DHA/Tsugar through the colour of either the flowers centres or flower petals, would allow trees to be visually selected in the field for high or low DHA/Tsugar with a more detailed analysis carried out in the laboratory. Studies using a larger sample size than the one used in this project to confirm or deny a visual indicator of DHA/Tsugar could be carried out.

All samples used in this study were frozen before testing. It was assumed that there was not a difference between fresh and frozen samples in terms of DHA/Tsugar, although some physical differences were observed. A study testing this assumption would also aid in the DHA/Tsugar to NPA predictions as this would ensure that the prediction is based on fresh nectar from the mānuka flower.

It was shown that DHAP does not change into DHA during the analysis of the flowers. The methodology used did not favour the detection of DHAP; however, DHAP is linked to DHA in plants.[51–54,56] A project to develop a method to detect DHAP in mānuka flowers, would possibly confirm the presence or absence of DHAP in flowers and its relationship to DHA.

The research could also be expanded to the whole of the mānuka tree. A small survey of mānuka parts indicated that DHA may also be found in the

leaves. A larger sample size and modified method would be required to confirm this. The detection of DHA and possibly DHAP in different parts of the tree might allow the source of DHA to be found and therefore give a better understanding of why DHA is found in significant levels in mānuka. An advantage would be that DHA would also be able to be measured when the flowers are not present. High levels of DHA and MGO have been identified in unifloral honeys from six *Leptospermum* species,[90] a survey of other plants with a focus on the *Leptospermum* genera and the Myrtaceae family may aid in identification of a common factor that explains the presence of DHA in the nectar of mānuka.

5.1.2 Experimental

During analysis of the samples, possible improvements became apparent that could improve the methodology being used. To maintain consistency for this project, these were not incorporated into this work. These concepts will be listed now for possible inclusion into future methodologies.

The nectar was extracted from the flower by soaking the flowers (20) in water (4 mL). It was found during analysis that the size and shape of some flowers made this extraction difficult. 4 mL was originally chosen as this worked with the original test flowers, an increase in volume may make extraction of these flowers easier but this will need to be balanced by the increase in dilution of nectar components.

Ten replicates of 20 flowers each are used per tree in this study. A suggestion is to use five replicates of 40 flowers each, this could give a larger DHA signal in the chromatogram and a larger averaging effect that could reduce the % RSD per tree. It would also reduce the time required for an individual tree to be analysed.

A 20 min extraction time was used in this work. A longer extraction period was tested and this suggested that sugar was leaching from other parts of the plant. However, a larger amount of DHA was also extracted. Testing of DHA/Tsugar vs extraction time would allow the extraction time to be optimised to extract the maximum amount of DHA without risk of sugar leaching. This would have to be tested with different mānuka flower physiologies to ensure the extraction time is consistent across all types.

It is recommended to change the extraction to DCM (1 mL) from (2 mL) as the SFT demonstrated that DCM (1 mL) is sufficient for the testing. This should have the effect of increasing the LOD and LOQ of the method.

A brief look was taken in this work at the DHA that remained in the sample bag, after the flowers were removed. To gain a better understanding of the movement of nectar around the sample bag, further work should be carried out looking at both the DHA and Tsugar that remain in the bag and how this compares to the flowers in the bag.

A number of assumptions were made when converting DHA/Tsugar to NPA in honey. The largest one being the conversion of DHA to MGO. This is based on an initial amount of DHA added to clover honey and stored for 175 days, to allow the conversion of DHA to MGO before the final MGO levels are measured. A study of the kinetics of the conversion would improve the accuracy of the prediction.

5.1.3 Trees and regions

While undertaking the survey for this thesis, limitations were found in the collection process. These are discussed with suggested improvements for future projects.

The survey undertaken in this project, highlighted the information that future projects should ensure they collect and also ways to improve collection.

It was difficult to draw any complete conclusions due to the number of samples collected, as the variation between trees was much higher than expected. The major factors that were thought to have an effect on the measurement of DHA/Tsugar were age, micro-environments and genetics. Overall more samples need to be collected to provide a large enough pool of data to detect abnormal values, thus allowing conclusions to be drawn.

The effect of the tree age would be better understood by taking samples over more years as well as determining the age exactly of those trees sampled. This could be achieved by using an increment borer, but this may have the disadvantage of killing the tree as well. Therefore, a method that reduces the risk of killing the sampled trees needs to be sought.

The micro-environments in which the trees grow may have an effect on mānuka nectar as demonstrated in other plants.[32–35] To determine the effect of this, more trees need to be sampled that are closer together. This project sampled over a wider area though this gave an indication of site wide variation in DHA/Tsugar it did not grant understanding on why the variation was there.

There did not appear to be any correlation between DHA/Tsugar and soil components. The different regions did exhibit different oil compositions that correlated with DHA/Tsugar and F/G ratio. Using a larger survey focused on one region, would either; improve the correlation, confirming there is a link between leaf oils and DHA/Tsugar or reduce the correlation suggesting that the correlation is a statistically abnormality, and therefore there is no link. If the DHA/Tsugar does correlate with different oil compositions and therefore oil chemotypes, a possible genetic effect is suggested as oil chemotypes are linked to the tree genetics.[5] This is further seen by the different physical appearances observed in the mānuka trees in the regions surveyed. DNA testing is required to see if there is a genetic link to

DHA/Tsugar, if there is, this will aid in identifying the cause of the high DHA production and allow selective breeding to be carried out more easily.

A future study that may aid in answering these questions would involve a plantation of two mānuka cultivars at a number of test sites spread out in different environments and with the trees tested each year. This would allow the variation between trees to be better understood as the age of the trees would be known, the effect of micro-environments would be minimal allowing the environments at each site to be compared, and by using two cultivars the difference between cultivars and therefore genetics could also be compared.

5.2 Conclusion

The project has shown there is still a great deal not understood about the nectar and DHA production in mānuka trees and further work needs to be carried out to fully understand all the variables that contribute to the over-production of DHA.

This project has found that the DHA/Tsugar measured in the nectar of the mānuka flower does vary within and between the regions surveyed. Suggested causes of within region variation are the age of the tree, micro-environments and possibly genetics. Variation between regions is strongly suggested to be genetically linked. Only a weak correlation was found with the soil components measured by Kiefer(2010).[7] A weak correlation was found between DHA/Tsugar and the oil compounds measured by Janusch(2010)[8] when correlated across all the regions. However, a stronger correlation was found when each region was correlated individually, this suggests a link to the mānuka oil chemotypes. Using the results of these surveys mānuka trees have been identified for the purpose of breeding and on-going study.

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Appendix A

Raw data

All values in this section are recorded as peak area, unless otherwise stated.

- **Table A.1** Raw data on-column vs splitless.
- **Table A.2** Raw data LOD and LOQ.
- **Table A.3** Raw data comparing the initial method and the TMSI evaporation method.
- **Table A.4** Raw data for comparing different volumes of TMSI for silylation of DHA.
- **Table A.5** Raw data for addition of different volumes of TMSI to nectar sugar to determine if TMSI is in excess.
- **Table A.6** Raw data method comparison.
- **Table A.7** Raw data DHA extracted from empty sample bag.
- **Table A.8** Raw data for comparing the mānuka trees VB014 and VB190 using the soaking method 1 and the dipping method 2.
- **Table A.9**Raw data for effect of differing amounts of PFBHA upon derivatisation.

If the raw data is not presented here, check **Appendix E**.

Table A.1: Raw data for comparison of splitless and on-column injection

On-Column						Splitless					
Standard	HA	DHA	MGO	DHA/HA	MGO/HA	Standard	HA	DHA	MGO	DHA/HA	MGO/HA
1	24.4	91.4	11.2	3.75	0.46	1	20.8	103.5	41.85	4.98	2.01
2	35.5	44.5	21.5	1.25	0.61	2	22.5	109.1	46.78	4.85	2.08
3	23.8	79	6.85	3.32	0.29	3	23.9	121.5	53.35	5.08	2.23
4	32.4	92.2	21.2	2.85	0.65	4	22.3	106.8	45.66	4.79	2.05
5	25.4	101	22.3	3.98	0.88	5	23.4	118.6	55.55	5.07	2.37
Average	28.30	81.62	16.61	3.03	0.58	Average	22.58	111.90	48.64	4.95	2.15
Std Dev	5.30	22.18	7.10	1.08	0.22	Std Dev	1.19	7.77	5.67	0.13	0.15
% RSD	18.74	27.18	42.77	35.73	38.26	% RSD	5.27	6.94	11.65	2.64	7.04

Table A.2: Raw data LOD and LOQ

Replicates	HA Area	DHA Area	DHA/HA	Replicates	HA Area	MGO Area	MGO/HA
1	18.56	0.557	0.0300	1	20.7	0.349	0.0169
2	19.91	0.664	0.0334	2	20.99	0.327	0.0156
3	20.71	0.431	0.0208	3	21.15	0.454	0.0215
4	20.47	0.598	0.0292	4	22.14	0.527	0.0238
5	21.18	0.576	0.0272	5	20.72	0.505	0.0244
6	20.95	0.281	0.0134	6	23.44	0.538	0.0230
7	20.65	0.518	0.0251	7	21.16	0.617	0.0292
8	22.17	0.409	0.0184	8	24.61	0.655	0.0266
9	23.73	0.458	0.0193	9	23.46	0.578	0.0246
10	26.16	0.35	0.0134	10	22.56	0.658	0.0292
Average	21.4490	0.4842	0.0230	Average	22.0930	0.5208	0.0235
Std Dev	2.1369	0.1195	0.0070	Std Dev	1.3760	0.1162	0.0046
% RSD	9.96	24.69	30.39	% RSD	6.23	22.31	19.44
			DHA/HA Peak Ratio				MGO/HA Peak Ratio
LOD			0.0210				0.0137
LOQ			0.0700				0.0456
mg			Mass of DHA in 205 μL				Mass of MGO in 205 μL
LOD			6.16 ng				93.8 ng
LOQ			20.5 ng				313 ng

Table A.3: Raw data comparing the no-evaporation and the evaporation method for TMSI

no-evaporation method						evaporation method					
Sample	HA	DHA	MGO	DHA/HA	MGO/HA	Sample	HA	DHA	MGO	DHA/HA	MGO/HA
1	65.76	15.34	117.3	0.233	1.784	1	62.78	23.21	160.9	0.370	2.563
2	66.59	15.4	122.75	0.231	1.843	2	59.34	12.13	152.57	0.204	2.571
3	64.7	15.4	131.07	0.238	2.026	3	64.95	19.12	187.88	0.294	2.893
4	66.97	15.63	138.69	0.233	2.071	4	65.67	23.27	216.45	0.354	3.296
5	68.75	15.46	150.06	0.225	2.183	5	65.73	21.85	178.56	0.332	2.717
6	71.82	16.2	156.11	0.226	2.174	6	71.67	26.03	252.28	0.363	3.520
7	77.1	20.94	196.43	0.272	2.548	7	76.53	29.03	297.59	0.379	3.889
Average	68.81	16.33	144.63	0.237	2.090	Average	66.67	22.09	206.60	0.33	3.06
Std Dev	4.32	2.05	26.74	0.016	0.253	Std Dev	5.71	5.39	52.61	0.062	0.514
% RSD	6.29	12.55	18.49	6.75	12.11	% RSD	8.57	24.41	25.46	18.75	16.78

Table A.4: Raw data for comparing different volumes of TMSI for silylation of DHA

TMSI added	Replicate	HA	DHA	MGO	DHA/HA	MGO/HA
50	A	35.6	2.96	177.2	0.0831	4.97
	B	33.0	2.71	199.6	0.0822	6.05
	C	35.3	3.24	223.8	0.0920	6.35
	Average	34.6	2.97	200.2	0.0857	5.79
	Std Dev	1.4	0.27	23.3	0.0054	0.72
	% RSD	4.1	9.0	11.6	6.3	12.5
TMSI added	Replicate	HA	DHA	MGO	DHA/HA	MGO/HA
100	A	37.7	3.39	165.1	0.0899	4.38
	B	38.1	3.48	90.3	0.0914	2.37
	C	36.7	3.25	142.0	0.0887	3.87
	Average	37.5	3.38	132.5	0.0900	3.54
	Std Dev	0.7	0.12	38.3	0.0013	1.04
	% RSD	2.0	3.4	28.9	1.5	29.5
TMSI added	Replicate	HA	DHA	MGO	DHA/HA	MGO/HA
150	A	39.6	3.43	235.0	0.0866	5.93
	B	36.6	3.42	223.3	0.0934	6.10
	C	37.0	3.48	219.9	0.0941	5.94
	Average	37.7	3.44	226.0	0.0914	5.99
	Std Dev	1.6	0.03	7.9	0.0042	0.09
	% RSD	4.3	1.0	3.5	4.5	1.6
TMSI added	Replicate	HA	DHA	MGO	DHA/HA	MGO/HA
200	A	38.5	3.71	248.4	0.0963	6.45
	B	41.4	3.64	511.2	0.0881	12.36
	C	38.0	3.70	208.4	0.0974	5.48
	Average	39.3	3.69	322.7	0.0939	8.10
	Std Dev	1.8	0.04	164.5	0.0051	3.72
	% RSD	4.6	1.0	51.0	5.4	46.0

Summary					
TMSi Added	DHA/HA	STDev	STDev Error range		
50	0.0857	0.0054	0.0803	¡-¿	0.0911
100	0.0900	0.0013	0.0886	¡-¿	0.0913
150	0.0914	0.0042	0.0872	¡-¿	0.0955
200	0.0939	0.0051	0.0889	¡-¿	0.0990

Table A.5: Raw data for addition of different volumes of TMSI to nectar sugar to determine if TMSI is in excess

TMSI Added	Replicate	Fructose Peak Area	Glucose Peak Area	Mannitol Peak Area	Fructose/Mannitol Peak Area Ratio	Glucose/Mannitol Peak Area Ratio	Mass (mg) of Fructose in 20ul Fructose in 20ul	Mass (mg) Glucose in 20ul	Fructose /Glucose	Total Mass (mg) of F+G in 200uL
50	a	434.70	401.40	662.00	0.66	0.61	0.03144	0.01885	1.67	0.50
	b	448.90	395.10	655.90	0.68	0.60	0.03277	0.01873	1.75	0.51
100	a	440.30	391.80	633.10	0.70	0.62	0.03330	0.01924	1.73	0.53
	b	441.70	376.10	622.50	0.71	0.60	0.03398	0.01878	1.81	0.53
200	a	397.70	326.50	578.00	0.69	0.56	0.03295	0.01756	1.88	0.51
	b	406.20	335.10	559.10	0.73	0.60	0.03479	0.01863	1.87	0.53
	Average	428.25	371.00	618.43	0.69	0.60	0.03320	0.01863	1.78	0.52
	Std Dev	21.04	32.35	41.69	0.02	0.02	0.00114	0.00056	0.08	0.01
	% RSD	4.91	8.72	6.74	3.43	3.03	3.43	3.03	4.58	2.46

Table A.6: Raw data method comparison

Method	1			2			3		
Sample	HA	DHA	DHA/HA	HA	DHA	DHA/HA	HA	DHA	DHA/HA
1	65.4	4.7	0.071865	67.3	1.5	0.022288	68.3	1.2	0.01757
2	50.8928	6.2994	0.123778	32.7068	0	0	62.4985	4.69	0.075042
3	32.7179	3.34371	0.102198	36.3028	2.36808	0.065231	33.0324	0	0
4	29.8391	4.48682	0.150367	31.458	1.60787	0.051112	41.2	0	0
5	32.2576	4.25351	0.131861	29.8297	3.36279	0.112733	33.2169	2.93457	0.088346
6	30.1058	4.43645	0.147362	29.0197	1.94751	0.06711	28.7628	0	0
7	64.7628	11.3217	0.174818	30.6138	0	0	29.3631	0	0
Average	43.71086	5.548799	0.128893	36.74726	1.540893	0.045496	42.3391	1.260653	0.025851
Std Dev	16.30301	2.693064	0.033939	13.68184	1.219545	0.041003	16.35129	1.867399	0.038874
% RSD	37.29739	48.53418	26.33107	37.23227	79.14537	90.12329	38.61984	148.1295	150.3761

Table A.7: Raw data DHA extracted from empty sample bag

DHA Extracted from Bag			
Sample	HA	DHA	DHA/HA
1	29.898	1.086	0.036
2	28.485	1.254	0.044
3	28.615	2.353	0.082
Average	28.999	1.564	0.054
Std Dev	0.781	0.689	0.025
% RSD	2.69	44.01	45.39

Table A.8: Raw data for comparing the mānuka trees VB014 and VB190 using the soaking method 1 and the dipping method 2

Extraction method 1							
Samples	**HA**	**DHA**	**DHA/HA**	**Samples**	**HA**	**DHA**	**DHA/HA**
VB014-1	73.30	194.10	2.65	VB190-1	62.82	5.74	0.09
VB014-2	62.80	178.80	2.85	VB190-2	67.67	5.54	0.08
VB014-3	53.30	126.50	2.37	VB190-3	70.80	6.32	0.09
Average	63.13	166.47	2.61	**Average**	67.10	5.87	0.09
Std Dev	10.00	35.45	0.34	**Std Dev**	4.02	0.41	0.01
% RSD	15.85	21.29	12.83	**% RSD**	5.99	6.91	6.11

Extraction method 2							
Samples	**HA**	**DHA**	**DHA/HA**	**Samples**	**HA**	**DHA**	**DHA/HA**
VB014-1	62.20	15.40	0.25	VB190-1	72.52	3.53	0.05
VB014-2	61.20	18.30	0.30	VB190-2	77.69	3.44	0.04
VB014-3	68.70	30.20	0.44	VB190-3	67.70	3.50	0.05
Average	64.03	21.30	0.37	**Average**	72.64	3.49	0.05
Std Dev	4.07	7.84	0.10	**Std Dev**	5.00	0.05	0.01
% RSD	6.36	36.82	26.92	**% RSD**	6.88	1.31	10.93

Table A.9: Raw data for effect of differing amounts of PFBHA upon derivatisation.

PFBHA added (uL)	Replicate	HA	DHA	DHA/HA Ratio	Mass (mg) of DHA in 200uL
50	a	19.5	16.8	0.8615	0.0040
	b	22.1	18.3	0.8281	0.0039
100	a	19.3	18.5	0.9585	0.0045
	b	19.4	17.2	0.8866	0.0041
200	a	21.8	19.1	0.8761	0.0041
	b	20.5	17.2	0.8390	0.0039
	Average	20.4	17.9	0.8750	0.0041
	Std Dev	1.3	0.9	0.0465	0.0002
	% RSD	6.141	5.0947	5.31054	5.310541383

Appendix B

Collection notes

Sampling Procedure

Previously sampled Trees:

If the trees have been sampled previously

- 3 small bags of flowers
- DNA specimen
- Take photo
- Fill out specimen information form

New Trees:

Need to collect

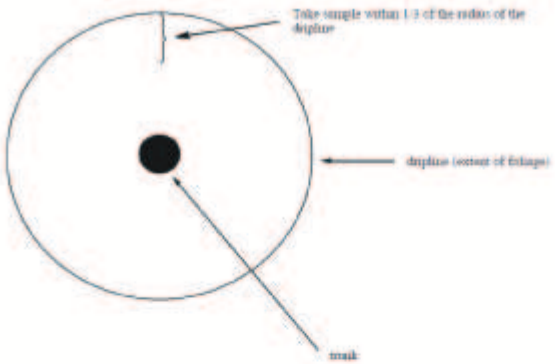
- 3 small bags of flowers
- DNA specimen
- Soil Sample
- Herbarium sample
- Take photo
- Fill out specimen information form

Samples

- Flower samples
  - Pick the flowers from the tree and place in bag
  - Avoid picking leaves and bugs
  - Mark the bag with the tree code

- DNA sample
  - Collect 2-3 branch tips (with lots of fresh growth) the size of your index finger
  - Place in coffee filter supplied and place this in the small Ziploc bag containing the blue granules of drying agent
  - **Press the bag flat to exclude air and seal immediately to keep the sample dry**
  - Mark the bag with the tree code

- Soil sample



Map to show location for soil sample

  - The soil sample should be collected in a large Ziploc bag and should approximately half fill the bag
  - Soil samples should be taken using a trowel or similar implement
  - Sample the top 10 cm (about 4 inches) of soil
  - It is OK to leave any vegetation that is tangled with the soil in the sample
  - Squeeze any air out of the bag before sealing
  - Label the bag with the tree code

- Herbarium sample
  - 3 branches of foliage about 20cm long
  - Foliage should include flowers or capsules
  - Mark the bag with the tree code

- Specimen information form
  - Last year's notes weren't best
  - Fill out form to best of ability
  - Not any additional significant information on form as well
- Photo
  - Try to include the whole tree in photo
  - Avoid people if possible to ensure most of the photo is used for the tree
  - Take photo before sampling so it gives an indication of how covered the tree is in flowers before sampling

Specimen Information (ver3)

Location Information

1. Tag number:_____
2. GPS coordinates: S_____

   E_____
3. Elevation:_____
4. Location:_____
5. Time collection begins: _____
6. Date:_____
7. Weather:

Pouring Down		Light Rain		50/50		Fine but overcast			Clear, Sunny
1	2	3	4	5	6	7	8	9	10

Pick a number

Tree Information

8. Tree Details

A	Single Mature Remnant Tree
B	Single Young Regenerating Tree
C	Mature Natural Stand
D	Young Regenerating Natural Sequence (e.g farm land going wild)
E	Mature Tree located within mixed Natural Bush Sequence (e.g forest)
F	Plantation Planting [Mature\Young\Mixed] (select one)
G	Garden planting [Mature\Young\Mixed] (select one)

9. Position in Stand

Inside Stand	Edge of Stand	Slightly Apart from Stand	Spread out Stand	Not in a Stand
1	2	3	4	5

10. Flower cycle:

Flower Buds only		50/50 Buds/Flowers		Fully in Flower			50/50 Flowers/Old Flower		Dead/Old Flowers
1	2	3	4	5	6	7	8	9	10

11. ~Height:_____
12. Flower colour

White	Strong Pink	Pale Pink	Mix Pink/White	Other(state)
A	B	C	D	E

13. Scent:

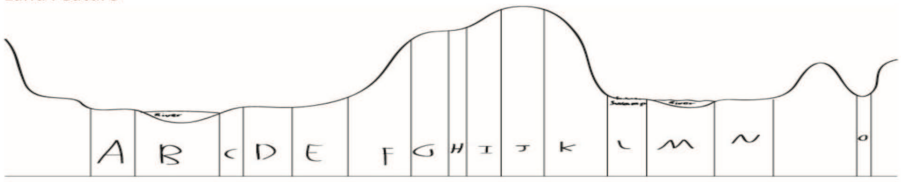
No Scent				Very Strong
1	2	3	4	5

14. Black Fungus covered

none	little			Thick layer of fungus
1	2	3	4	5

Natural Geophysical Features

15. Land Feature



16.

A	e.g. river terrace	I	e.g saddle between two tops
B	e.g. in river	J	e.g. hill top largest point in immediate area
C	e.g. on river bank	K	e.g. very steep slope
D	e.g. plain like flat land	L	e.g. swamp like land
E	e.g. transit from flat to hill country	M	
F	e.g. side of ridge, hill, valley	N	e.g. floodplain next to river
G	e.g. top of a ridge line going towards a hill top	O	e.g. bottom of valley/gully
H	e.g. flat area on top of a ridge		

17. Direction Slope facing

N	NE	E	SE	S	SW	W	NW

18. Fresh Water Source :

Pond	Swamp	Creek	Stream	River	None
A	B	C	D	E	F

19. Distance to Coast:_____

20. Exposed to Wind: y / n

Manmade Influences

21. Land treatment

A	Trees growing on rich fully managed farmland
B	Trees growing on semi managed farmland
C	Trees growing on impoverished unmanaged farmland
D	Trees growing on land left to grow wild
E	Trees growing on managed residential land
F	Trees growing on unmanaged residential land
G	Trees growing as garden cultivars
H	None of the above

22. Stock camping under Trees:

Cow	Sheep	Goat	All	None
A	B	C	D	E

23. Next to road(road defined as a public road): y / n

Appendix C

Enlarged graphs for results section

- **Figure C.1** Overview of DHA/Tsugar of trees sampled in 2009.
- **Figure C.2** Overview of DHA/Tsugar of trees sampled in 2010.
- **Figure C.3** Overview of F/G ratio of trees sampled in 2009.
- **Figure C.4** Overview of F/G ratio of trees sampled in 2010.
- **Figure C.5** DHA/Tsugar for trees sampled in both 2009 and 2010.
- **Figure C.6** F/G ratio for trees sampled in both 2009 and 2010.
- **Figure C.7** DHA/Tsugar for East Cape Trees.
- **Figure C.8** DHA/Tsugar for Wairarapa Trees.
- **Figure C.9** DHA/Tsugar for Waikato Trees.
- **Figure C.10** Individual value plot of DHA/Tsugars for trees, grouped according to soil order and sample region.

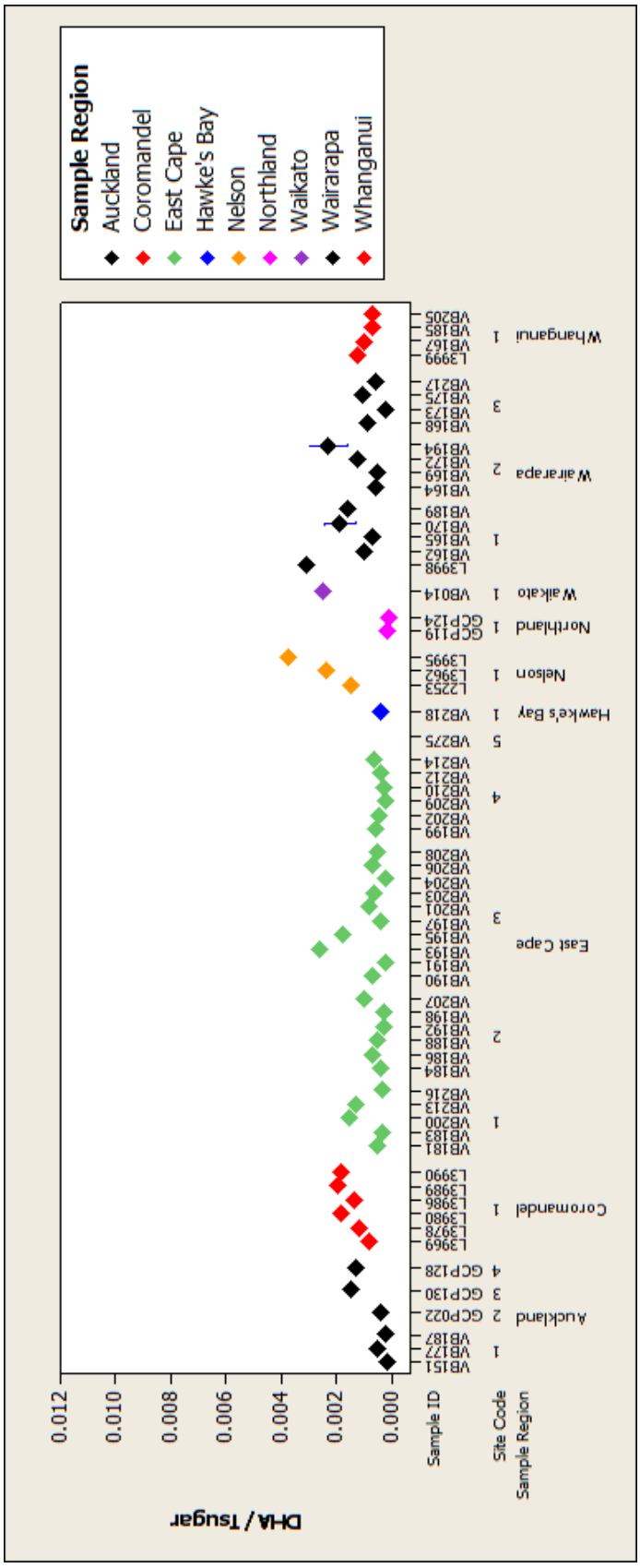


Figure C.1: Interval plot of DHA/Tsugar ratios for trees sampled in 2009, grouped by regions and with error bars defined by the 95% confidence interval.

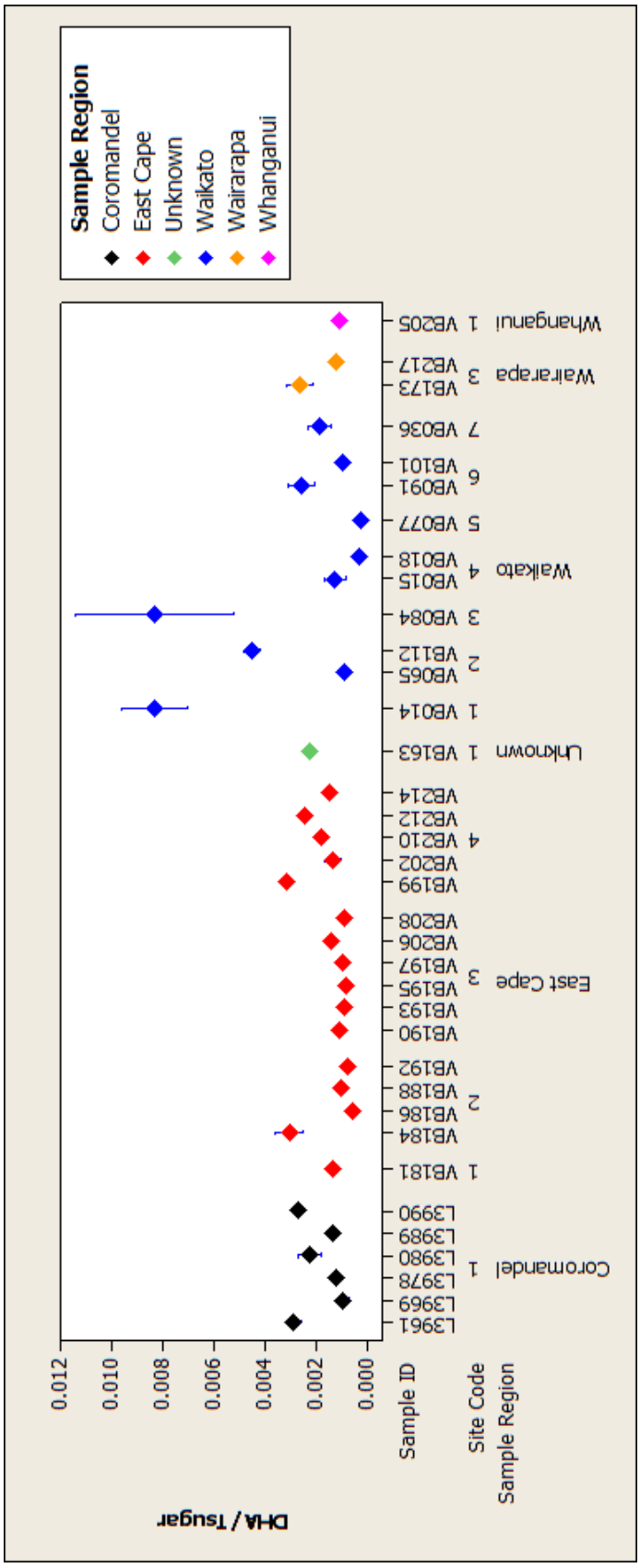


Figure C.2: Interval plot of DHA/Tsugar ratios for trees sampled in 2010, grouped by regions and with error bars defined by the 95% confidence interval.

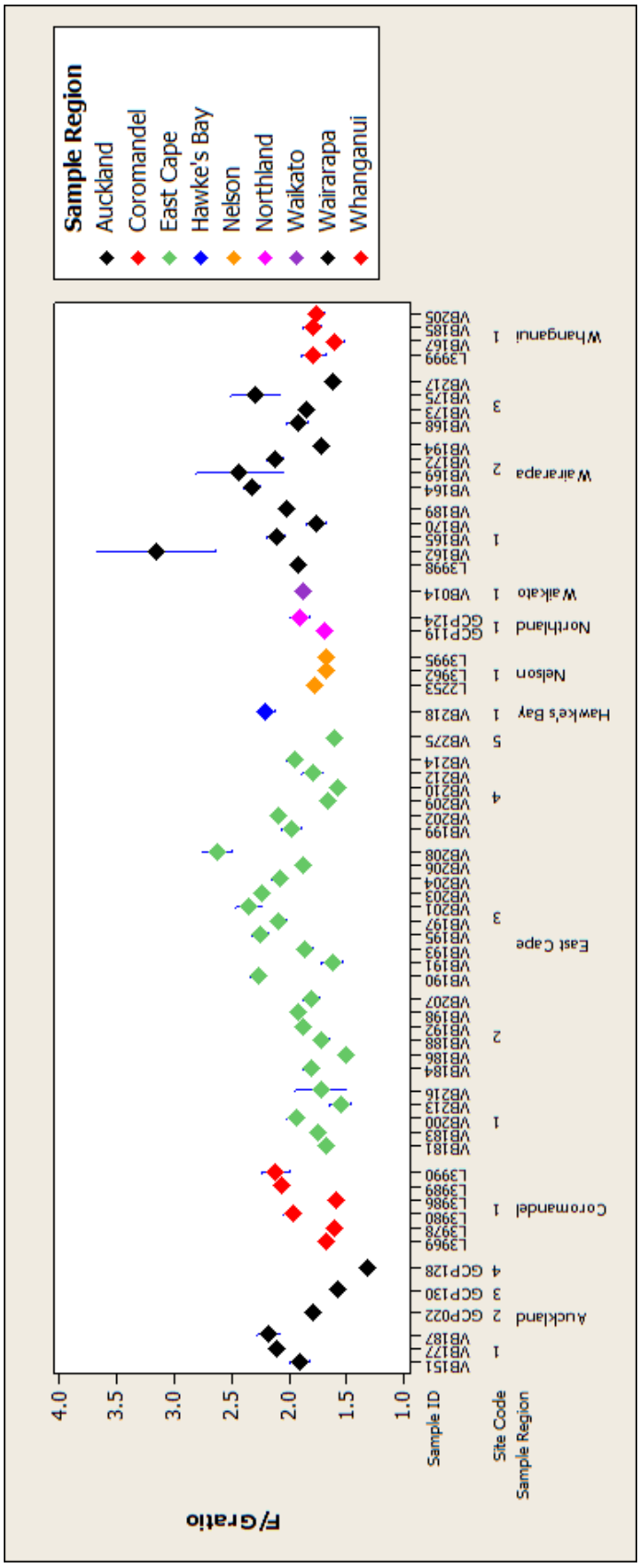


Figure C.3: Interval plot of F/G ratios for trees sampled in 2009, grouped by regions and with error bars defined by the 95% confidence interval.

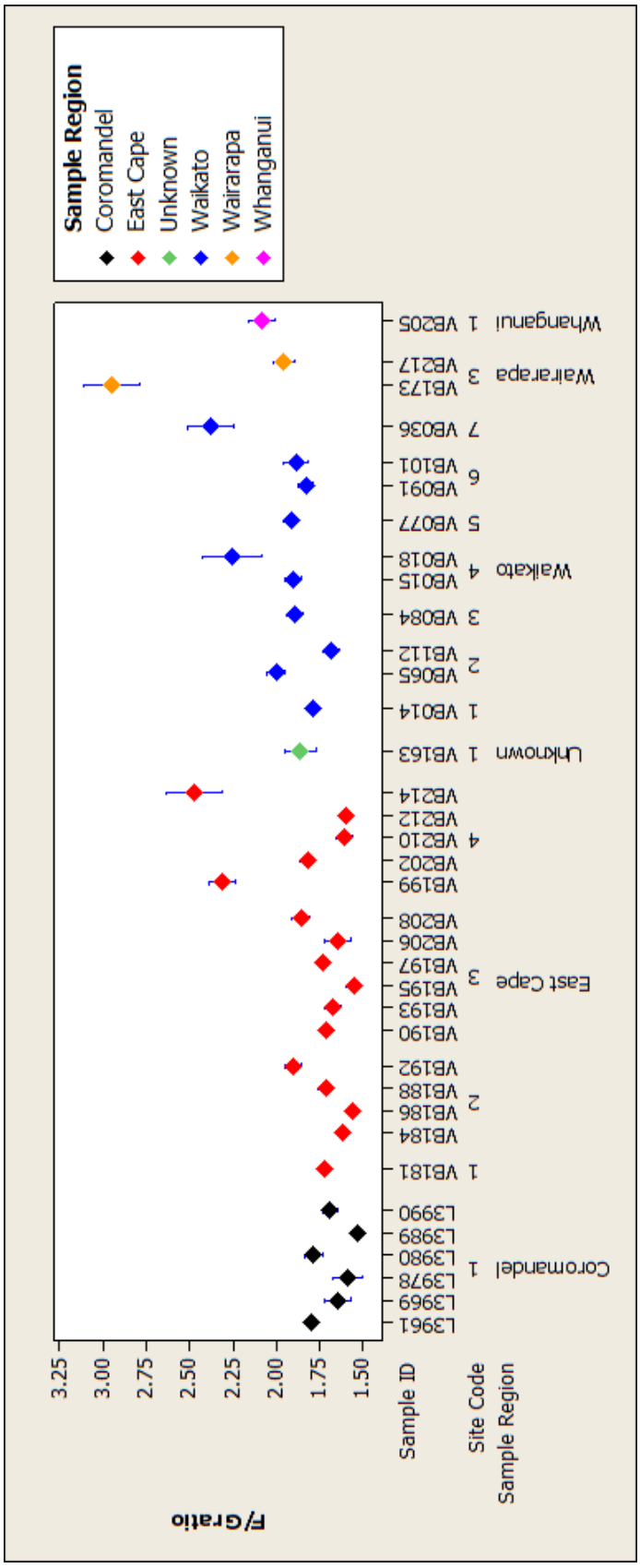


Figure C.4: Interval plot of F/G ratios for trees sampled in 2010, grouped by regions and with error bars defined by the 95% confidence interval.

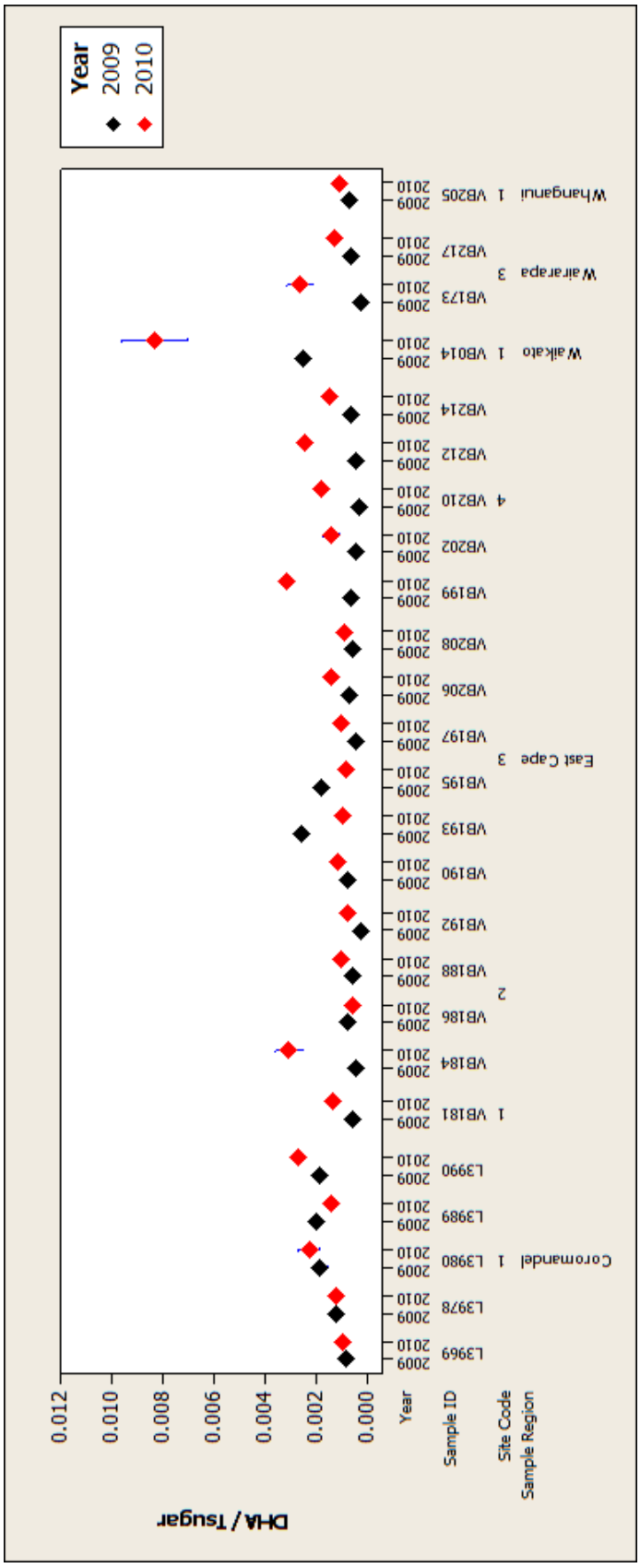


Figure C.5: Interval plot of the mean DHA/Tsugar values for trees sampled in 2009 and 2010 with error bars showing the 95% confidence interval.

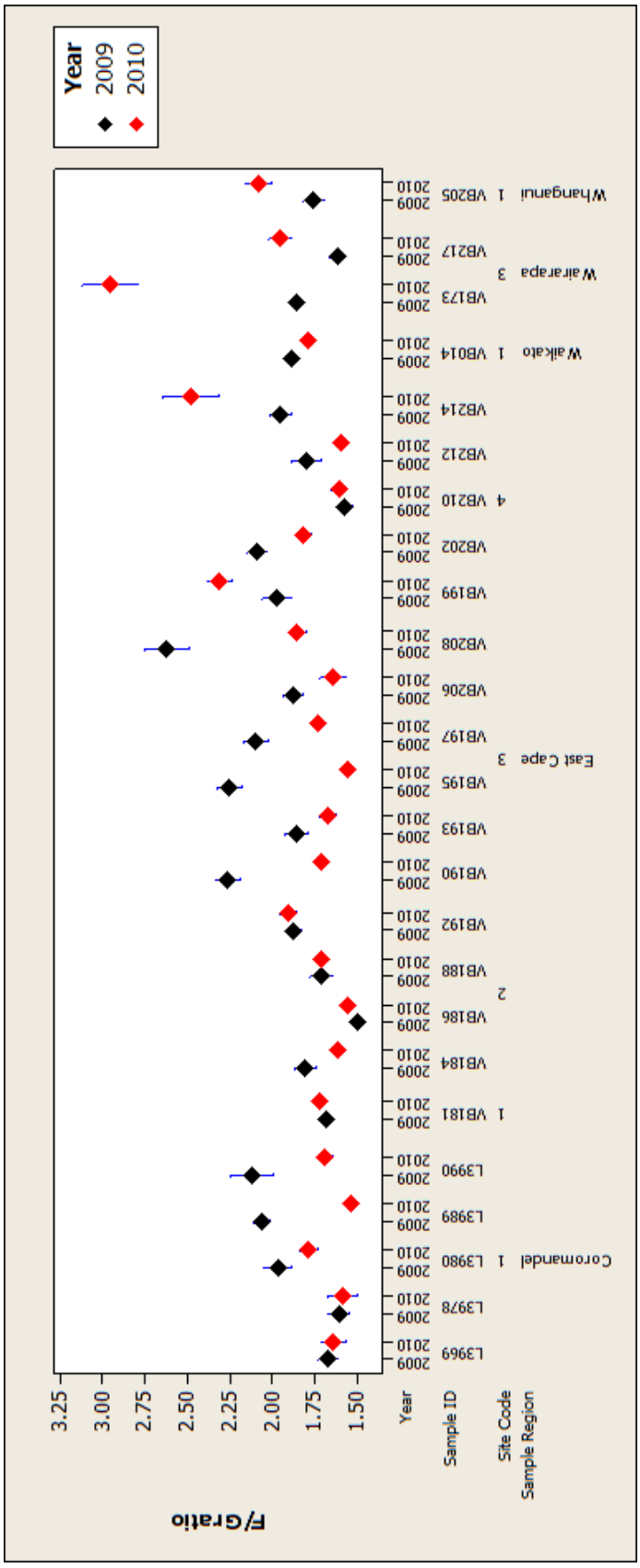


Figure C.6: Interval plot of the mean F/G ratos for trees sampled in 2009 and 2010 with error bars showing the 95% confidence interval.

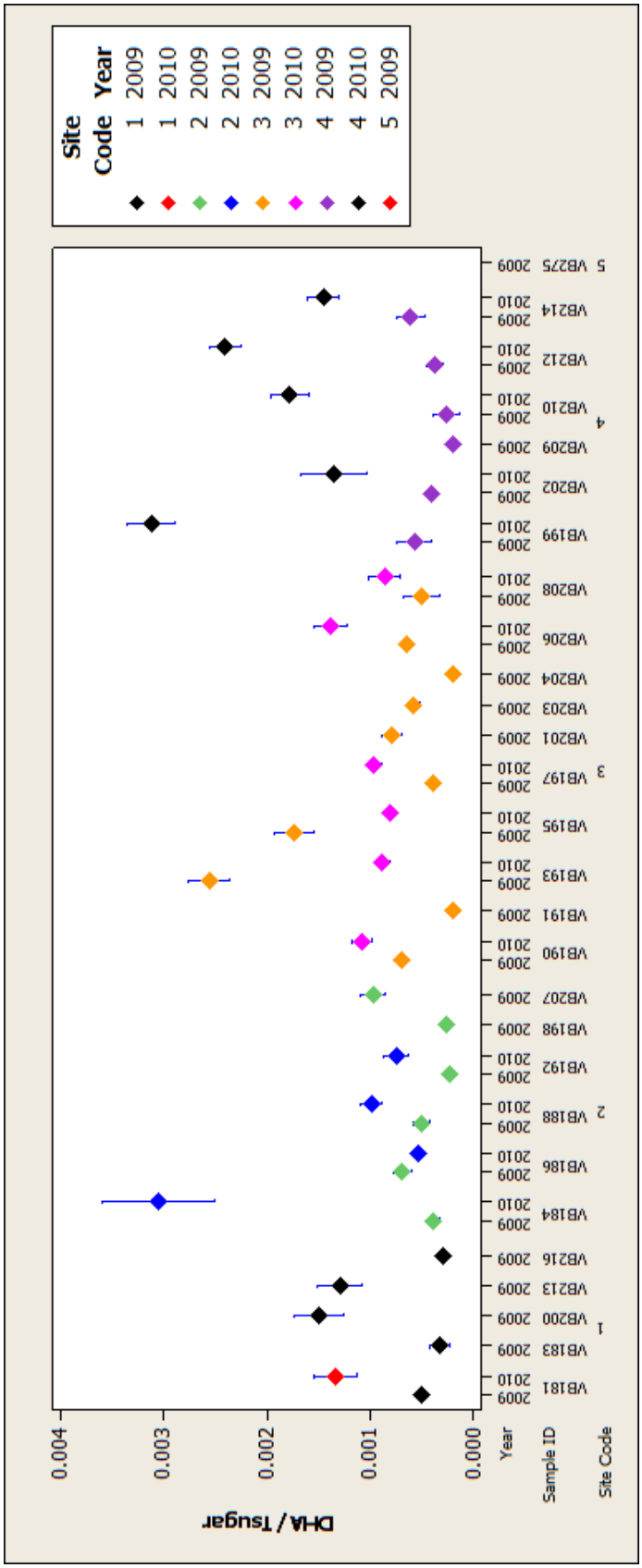


Figure C.7: Interval plot of the mean DHA/Tsugar values for the East Cape trees sampled in 2009 and 2010 with error bars showing the 95% confidence interval.

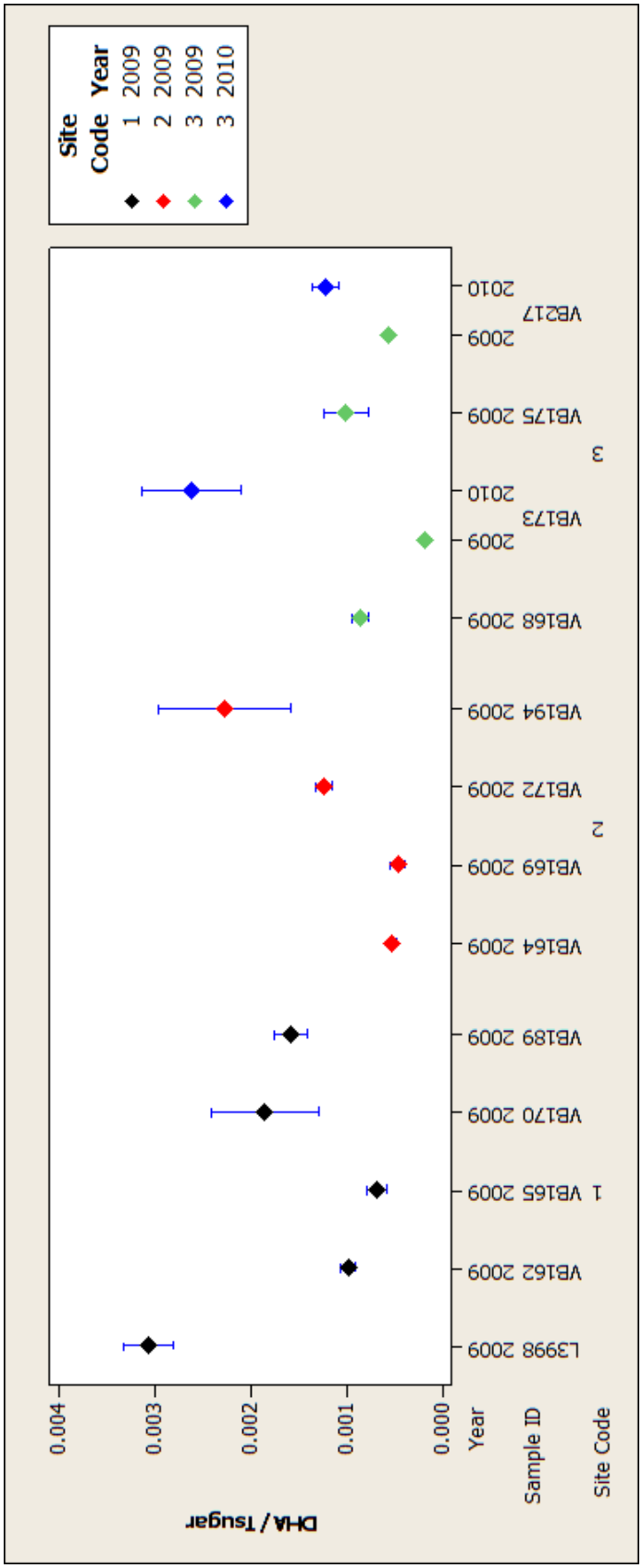


Figure C.8: Interval plot of the mean DHA/Tsugar values for the Wairarapa trees sampled in 2009 and 2010 with error bars showing the 95% confidence interval.

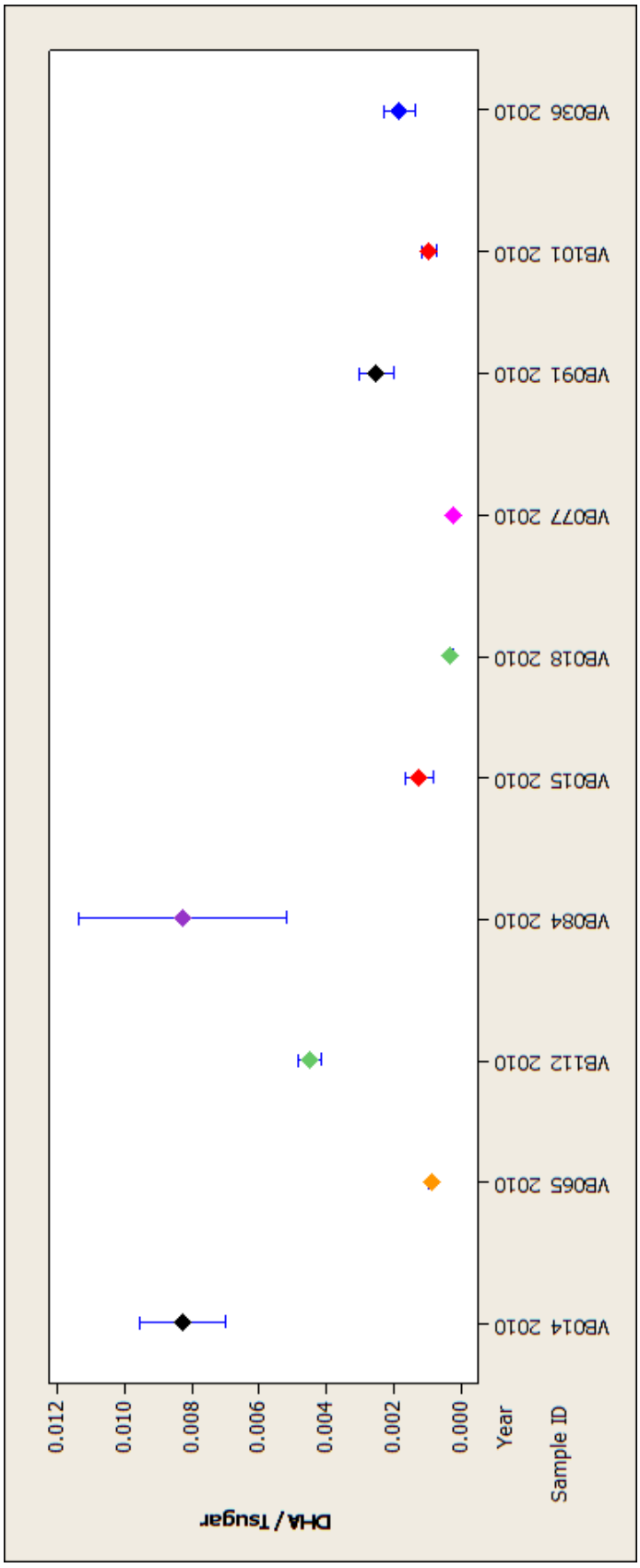


Figure C.9: Interval plot of the mean DHA/Tsugar values for the Waikato trees sampled in 2010 with error bars showing the 95% confidence interval.

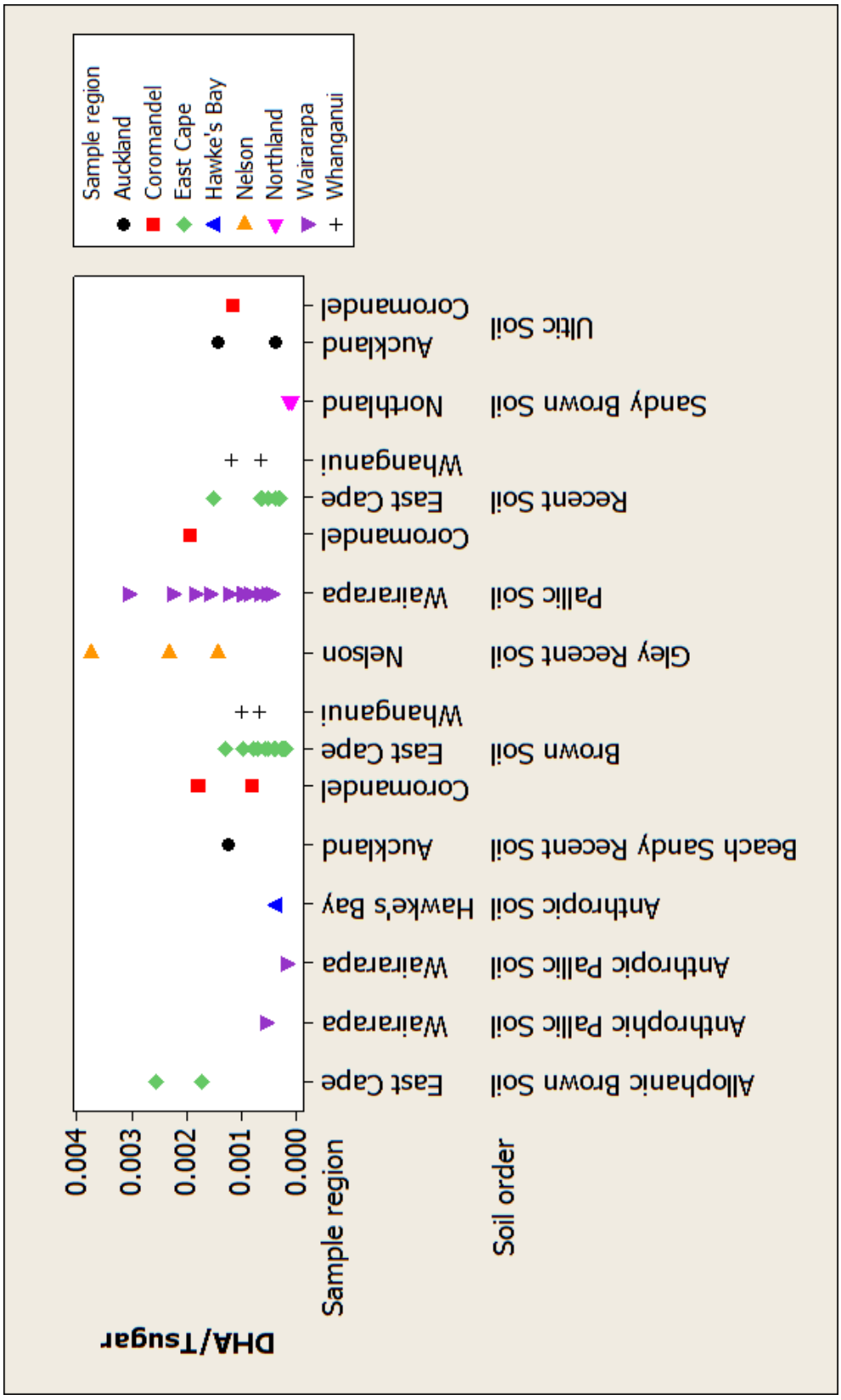


Figure C.10: Interval plot of DHA/Tsugars for trees, grouped according to soil order and sample region.

Appendix D

Full correlation tables relating DHA/Tsugar to individual oil compounds

- **Table D.1, D.2** Individual oil compounds that correlate with DHA/Tsugar from all regions.
- **Table D.3, D.4** Individual oil compounds to F/G ratio from all regions.
- **Table D.5, D.6** Individual the oil compounds to DHA/Tsugar for the East Cape trees.
- **Table D.7, D.8** Individual oil compounds to F/G ratio for the East Cape trees.
- **Table D.9, D.10** Individual the oil compounds to DHA/Tsugar for the Wairarapa trees.
- **Table D.11** Individual oil compounds to F/G ratio for the Wairarapa trees.
- **Table D.12, D.13** Individual the oil compounds to DHA/Tsugar for the Coromandel trees.

Table D.1: Individual oil compounds that correlate with DHA/Tsugar from all regions, **part 1**.

Oil Compound	R^2	Oil Compound	R^2
Cadina-3,5-diene	11.9	Isoprenyl-2-methyl butyrate	1.0
Benzyl-2-methyl butyrate	9.1	$C_{15}H_{24}$-1	0.9
Benzyl isovalerate	8.2	α-Farnesene	0.9
Selinen-4α-ol	7.0	α-Terpineol	0.8
β-Selinene	6.3	γ-Cadinene	0.8
α-Campholenal	6.2	Ocimene	0.6
R-Norinone	6.2	α-Copaene	0.6
γ-Selinene	5.7	α-Calacorene	0.6
α-Selinene	5.0	α-Amorphene	0.6
α-Gurjunene	4.9	Isoleptospermone	0.5
cis-Methyl cinnamate	4.9	Methyl geranate	0.5
Selina-3,7 (11)-diene	4.8	*Allo*-Aromadendrene	0.4
Isoamyl benzoate	4.8	MW 212	0.4
α-Cubebene	3.8	Isoamyl isovalerate	0.4
Isoprenyl isovalerate	3.6	δ-Cadinene	0.4
trans- Methyl cinnamate	3.5	α-Bergamotene	0.4
Spathulenol	3.5	α-Pinene	0.3
Grandiflorone	3.4	Prenyl tiglate	0.3
β-Pinene	3.2	δ-Elemene	0.3
Ledol	3.2	τ-Elemene	0.3
Cubenol	3.2	Humulene	0.3
$C_{15}H_{24}$ (1486)	3.1	$C_{15}H_{24}$	0.2

Table D.2: Individual oil compounds that correlate with DHA/Tsugar from all regions, **part 2**.

Oil Compound	R^2	Oil Compound	R^2
Terpinen-4-ol	3.1	Elemol	0.2
Calamenene	2.8	α-Muurolene	0.2
α-Cadinol	2.8	Aromadendrene	0.2
$C_{15}H_{24}$ (1517)	2.3	$C_{15}H_{24}$-2	0.1
β-Gurjunene	2.1	Isoprenyl benzoate	0.1
Myrtenal	2.0	2-Methyl butyl isovalerate	0.1
Flavesone	2.0	α-Ylangene	0.1
p-Cymene	1.9	$C_{15}H_{24}$-3	0.1
trans-Citral	1.9	Leptospermone	0.1
Citronellol	1.8	α-Eudesmol	0.1
cis-Citral	1.8	γ-Eudesmol	0.1
Prenyl isovalerate	1.6	γ-Terpinene	0.0
Isoamyl acetate	1.6	Caryophyllene	0.0
Isoprenyl tiglate	1.5	γ-Muurolene	0.0
Cadina-1,4-diene	1.4	Geraniol	0.0
Linalool	1.4	Caryophyllene Oxide	0.0
Eucalyptol	1.4	β-Eudesmol	0.0
Limonene	1.4	Germacrene D	0.0
Geranyl acetate	1.2	β-Elemene	0.0
β-Cubebene	1.1	Globulol	NC
δ-Selinene	1.0		

Table D.3: Correlation of the individual oil compounds to F/G ratio from all regions,**part 1**.

Oil Compound	R^2	Oil Compound	R^2
Aromadendrene	10.1	β-Pinene	0.4
γ-Eudesmol	8.5	α-Ylangene	0.4
α-Eudesmol	7.3	Methyl geranate	0.3
β-Eudesmol	6.6	$C_{15}H_{24}$-3	0.3
$C_{15}H_{24} - 1$	5.5	β-Gurjunene	0.3
γ-Terpinene	3.5	δ-Cadinene	0.3
Linalool	3.2	δ-Selinene	0.3
Cubenol	3.2	α-Calacorene	0.3
Humulene	3.0	α-Pinene	0.3
cis-Methyl cinnamate	2.7	Cadina-1,4-diene	0.3
$C_{15}H_{24}$ (1486)	2.7	Leptospermone	0.3
δ-Elemene	2.5	Spathulenol	0.2
τ-Elemene	2.5	Benzyl isovalerate	0.2
Germacrene D	2.0	MW 212	0.2
β-Selinene	1.9	Isoprenyl-2-methyl butyrate	0.2
Elemol	1.8	Geraniol	0.2
Caryophyllene Oxide	1.7	$C_{15}H_{24}$	0.1
trans-Citral	1.6	Limonene	0.1
Geranyl acetate	1.4	Isoprenyl tiglate	0.1
Selina-3,7 (11)-diene	1.4	*trans*-Methyl cinnamate	0.1
α-Selinene	1.3	Isoamyl isovalerate	0.1
γ-Selinene	1.1	Calamenene	0.1

Table D.4: Correlation of the individual oil compounds to F/G ratio from all regions, **part 2**.

Oil Compound	R^2	Oil Compound	R^2
Isoleptospermone	1.1	α-Bergamotene	0.1
Selinen-4α-ol	1.0	Eucalyptol	0.1
Caryophyllene	1.0	Isoamyl acetate	0.1
Citronellol	0.9	Terpinen-4-ol	0.1
cis-Citral	0.9	α-Amorphene	NC
Isoprenyl benzoate	0.8	α-Muurolene	NC
2-Methyl butyl isovalerate	0.8	γ-Cadinene	NC
$C_{15}H_{24} - 2$	0.8	Ocimene	NC
Cadina-3,5-diene	0.8	α-Campholenal	NC
Allo-Aromadendrene	0.7	R-Norinone	NC
α-Gurjunene	0.7	α-Farnesene	NC
Isoamyl benzoate	0.7	Flavesone	NC
Ledol	0.6	Benzyl-2-methyl butyrate	NC
Isoprenyl isovalerate	0.6	Myrtenal	NC
γ-Muurolene	0.6	β-Cubebene	NC
α-Cubebene	0.6	β-Elemene	NC
α-Cadinol	0.5	p-Cymene	NC
$C_{15}H_{24}$ (1517)	0.5	α-Terpineol	NC
Prenyl isovalerate	0.4	Grandiflorone	NC
Prenyl tiglate	0.4	Globulol	NC
α-Copaene	0.4		

Table D.5: Correlation of individual the oil compounds to DHA/Tsugar for the East Cape trees, **part 1**.

Oil Compound	R^2	Oil Compound	R^2
Aromadendrene	25.2	Cubenol	1.6
Allo-Aromadendrene	13.4	β-Gurjunene	1.4
β-Eudesmol	12.8	γ-Cadinene	1.3
α-Eudesmol	12.5	Isoprenyl-2-methyl butyrate	1.3
Isoamyl isovalerate	12.2	Calamenene	1.1
γ-Eudesmol	11.8	Prenyl tiglate	1.1
Cadina-3,5-diene	11.0	Ledol	1.0
2-Methyl butyl isovalerate	9.2	Caryophyllene	1.0
$C_{15}H_{24}$ (1517)	8.7	α-Selinene	0.9
γ-Terpinene	8.0	$C_{15}H_{24}$ (1486)	0.9
p-Cymene	8.0	Isoleptospermone	0.9
α-Ylangene	7.9	MW 212	0.7
Humulene	6.9	$C_{15}H_{24}$-2	0.4
Grandiflorone	6.7	α-Pinene	0.4
Limonene	6.5	α-Amorphene	0.4
Eucalyptol	6.1	α-Cadinol	0.3
$C_{15}H_{24}$-3	5.7	*cis*-Methyl cinnamate	0.2
δ-Elemene	5.5	$C_{15}H_{24}$-1	0.2
Elemol	5.5	Prenyl isovalerate	0.2
τ-Elemene	5.5	α-Farnesene	0.1
Selinen-4α-ol	5.4	Caryophyllene Oxide	0.1

Table D.6: Correlation of individual the oil compounds to DHA/Tsugar for the East Cape trees, **part 2**.

Oil Compound	R^2	Oil Compound	R^2
γ-Selinene	5.4	Benzyl-2-methyl butyrate	0.1
$C_{15}H_{24}$	5.3	Cadina-1,4-diene	0.1
β-Selinene	5.3	δ-Cadinene	0.1
α-Bergamotene	5.3	α-Muurolene	0.1
Flavesone	5.2	Selina-3,7 (11)-diene	0.0
Linalool	5.2	Spathulenol	0.0
β-Elemene	5.2	Isoamyl acetate	NC
trans-Methyl cinnamate	4.8	α-Campholenal	NC
Methylgeranate	4.5	R-Norinone	NC
Ocimene	4.1	Terpinen-4-ol	NC
β-Cubebene	4.0	α-Terpineol	NC
Isoprenyl tiglate	3.9	Myrtenal	NC
β-Pinene	3.5	Citronellol	NC
Isoprenyl benzoate	3.4	*cis*-Citral	NC
Germacrene D	3.4	Globulol	NC
α-Cubebene	3.3	α-Calacorene	NC
α-Copaene	3.0	Geraniol	NC
α-Gurjunene	2.4	*trans*-Citral	NC
Benzyl isovalerate	2.4	δ-Selinene	NC
Leptospermone	2.3	Isoamyl benzoate	NC
γ-Muurolene	2.2	Geranyl acetate	NC
Isoprenyl isovalerate	1.8		

Table D.7: Correlation of the individual oil compounds to F/G ratio for the East Cape trees, **part 1**.

Oil Compound	R^2	Oil Compound	R^2
γ-Terpinene	23.0	Prenyl tiglate	2.8
$C_{15}H_{24} - 1$	22.7	Flavesone	2.4
p-Cymene	19.3	Grandiflorone	2.0
Isoprenyl isovalerate	15.4	γ-Cadinene	2.0
α-Pinene	14.1	Isoprenyl-2-methyl butyrate	1.8
δ-Cadinene	12.0	*cis*-Methyl cinnamate	1.6
Humulene	8.5	Cadina-1,4-diene	1.2
Limonene	8.2	Caryophyllene Oxide	1.0
Benzyl-2-methyl butyrate	8.2	Caryophyllene	0.7
γ-Muurolene	7.9	α-Copaene	0.3
Prenyl isovalerate	7.9	Cadina-3,5-diene	0.3
β-Elemene	7.8	Selina-3,7 (11)-diene	0.3
δ-Elemene	7.0	β-Cubebene	0.2
Elemol	7.0	β-Pinene	0.2
τ-Elemene	7.0	Isoleptospermone	0.2
α-Amorphene	7.0	$C_{15}H_{24} - 2$	0.2
Linalool	6.9	α-Cubebene	0.1
trans-Methyl cinnamate	6.6	α-Farnesene	0.1
Cubenol	6.5	Ocimene	0.1
Aromadendrene	6.5	α-Ylangene	0.1
α-Gurjunene	6.4	β-Gurjunene	0.1
Germacrene D	6.3	Spathulenol	0.1

Table D.8: Correlation of the individual oil compounds to F/G ratio for the East Cape trees, **part 2**.

Oil Compound	R^2	Oil Compound	R^2
Benzyl isovalerate	6.0	Calamenene	0.0
γ-Eudesmol	5.6	Isoamyl isovalerate	0.0
Methyl geranate	5.6	Selinen-α-ol	0.0
γ-Selinene	5.6	2-Methyl butyl isovalerate	0.0
α-Eudesmol	5.3	Isoprenyl tiglate	0.0
$C_{15}H_{24}$ (1486)	5.2	Isoamyl acetate	NC
β-Eudesmol	5.1	α-Campholenal	NC
MW 212	4.6	R-Norinone	NC
Isoprenyl benzoate	4.2	Terpinen-4-ol	NC
α-Selinene	4.1	α-Terpineol	NC
$C_{15}H_{24} - 3$	4.1	Myrtenal	NC
Allo-Aromadendrene	4.0	Citronellol	NC
α-Bergamotene	3.9	*cis*-Citral	NC
Eucalyptol	3.9	Globulol	NC
$C_{15}H_{24}$	3.7	α-Calacorene	NC
$C_{15}H_{24}$ (1517)	3.2	Geraniol	NC
α-Muurolene	3.2	*trans*-Citral	NC
Leptospermone	3.2	δ-Selinene	NC
β-Selinene	3.0	Isoamyl benzoate	NC
Ledol	3.0	Geranyl acetate	NC
α-Cadinol	2.9		

Table D.9: Correlation of the individual oil compounds to DHA/Tsugar for the Wairarapa trees, **part 1**.

Oil Compound	R2	Oil Compound	R2
Cubenol	45.7	α-Copaene	3.8
trans-Methyl cinnamate	32.9	α-Bergamotene	3.7
β-Cubebene	28.4	Germacrene D	3.7
β-Gurjunene	27.9	Isoprenyl isovalerate	3.4
Calamenene	26.9	Isoprenyl tiglat	3.2
Grandiflorone	25.9	Leptospermone	3.0
α-Gurjunene	24.5	Methylgeranate	2.7
cis-Methyl cinnamate	19.0	γ-Muurolene	2.6
α-Muurolene	18.9	γ-Cadinene	2.4
Ledol	16.9	Prenyl tiglate	2.4
Geranyl acetate	16.6	$C_{15}H_{24}$-1	2.4
Isoamyl benzoate	16.2	Cadina-3,5-diene	1.6
α-Calacorene	15.5	Humulene	1.4
Flavesone	15.5	MW 212	1.3
Allo-Aromadendrene	13.7	Selinen-4α-ol	1.3
Isoamyl acetate	13.0	α-Ylangene	1.2
Cadina-1,4-diene	13.0	Benzyl isovalera	0.7
Spathulenol	12.5	Aromadendrene	0.7
β-Elemene	11.3	γ-Selinene	0.5
Isoprenyl-2-methyl butyrate	11.2	β-Selinene	0.5
α-Pinene	9.8	Isoprenyl benzoate	0.2
β-Pinene	8.9	$C_{15}H_{24}$-2	0.1
Linalool	8.5	Isoleptospermone	0.1

Table D.10: Correlation of the individual oil compounds to DHA/Tsugar for the Wairarapa trees, **part 2**.

Oil Compound	R2	Oil Compound	R2
α-Cubebene	7.7	$C_{15}H_{24}$ (1517)	0.1
2-Methyl butyl isovalerate	7.4	Eucalyptol	0.1
p-Cymene	7.2	Terpinen-4-ol	0.0
Caryophyllene Oxide	6.8	α-Terpineol	0.0
$C_{15}H_{24}$-3	6.8	α-Farnesene	0.0
α-Amorphene	6.6	Geraniol	0.0
α-Cadinol	6.5	α-Selinene	0.0
Elemol	6.1	α-Campholenal	NC
γ-Eudesmol	6.1	R-Norinone	NC
β-Eudesmol	6.1	Prenyl isovalerate	NC
α-Eudesmol	6.1	Myrtenal	NC
trans-Citral	5.7	Citronellol	NC
Ocimene	5.5	*cis*-Citral	NC
Limonene	5.3	δ-Elemene	NC
$C_{15}H_{24}$	5.2	Benzyl-2-methyl butyrate	NC
Isoamyl isovalerate	5.2	$C_{15}H_{24}$ (1486)	NC
Caryophyllene	4.9	δ-Selinene	NC
Selina-3,7 (11)-diene	4.9	τ-Elemene	NC
δ-Cadinene	4.8	Globulol	NC
γ-Terpinene	4.2		

Table D.11: Correlation of oil compounds to F/G ratio for the Wairarapa trees.

Oil Compound	R2	Oil Compound	R2
α-Ylangene	29.9	*Allo*-Aromadendrene	2.4
Isoamyl isovalerate	15.9	*cis*-Methyl cinnamate	1.9
2-Methyl butyl isovalerate	15.8	α-Gurjunene	1.8
Selina-3,7 (11)-diene	13.1	β-Gurjunene	1.7
$C_{15}H_{24}$-2	12.8	Selinen-4α-ol	1.6
Isoprenyl isovalerate	11.7	β-Cubebene	1.5
$C_{15}H_{24}$ (1517)	9.3	Cubenol	1.4
α-Pinene	8.9	Caryophyllene	1.3
α-Cubebene	7.4	Aromadendrene	1.0
Ledol	7.1	β-Pinene	0.9
δ-Cadinene	6.7	Limonene	0.9
γ-Selinene	6.6	*trans*-Methyl cinnamate	0.9
α-Bergamotene	6.2	β-Elemene	0.8
Spathulenol	5.9	Calamenene	0.8
Linalool	5.6	α-Copaene	0.6
γ-Cadinene	5.4	Benzyl isovalerate	0.4
$C_{15}H_{24}$	5.0	γ-Terpinene	0.3
α-Selinene	4.8	$C_{15}H_{24}$-3	0.2
γ-Muurolene	4.8	Humulene	0.2
β-Selinene	4.5	α-Cadinol	0.2
$C_{15}H_{24}$-1	3.9	Leptospermone	0.1
MW 212	3.6	α-Muurolene	0.1
Isoleptospermone	3.4	Cadina-1,4-diene	0.1
α-Farnesene	3.2	Grandiflorone	0.1
Caryophyllene Oxide	3.0	Eucalyptol	NC
α-Amorphene	2.8	Flavesone	NC
p-Cymene	2.6		

Table D.12: Correlation of the individual oil compounds to DHA/Tsugar for the Coromandel trees, **part 1**.

Oil Compound	R^2	Oil Compound	R^2
β-Pinene	68.2	Isoamyl isovalerate	5.6
Cadina-3,5-diene	62.7	γ-Muurolene	5.3
$C_{15}H_{24}$-2	61.2	β-Elemene	4.5
Grandiflorone	61.0	Spathulenol	3.8
α-Bergamotene	52.6	$C_{15}H_{24}$-1	2.4
β-Cubebene	50.0	Benzyl isovalerate	2.3
α-Selinene	47.6	2-Methylbutyl isovalerate	1.7
β-Selinene	45.3	*Allo*-Aromadendrene	1.3
γ-Selinene	43.8	MW 212	1.3
Benzyl-2-methyl butyrate	41.3	Isoprenyl tiglate	1.2
Eucalyptol	40.6	α-Ylangene	1.0
Selina-3,7 (11)-diene	40.1	Isoleptospermone	0.4
Isoprenyl isovalerate	38.4	Humulene	0.4
Isoprenyl-2-methyl butyrate	35.8	α-Cubebene	0.2
Limonene	27.9	α-Amorphene	0.2
Selinen-4α-ol	27.5	$C_{15}H_{24}$	0.0
Flavesone	23.2	Isoamyl acetate	NC
α-Cadinol	22.6	Ocimene	NC
$C_{15}H_{24}$-3	21.4	γ-Terpinene	NC
Cadina-1,4-diene	20.9	α-Campholenal	NC

Table D.13: Correlation of the individual oil compounds to DHA/Tsugar for the Coromandel trees, **part 2**.

Oil Compound	R^2	Oil Compound	R^2
Ledol	20.1	R-Norinone	NC
Caryophyllene	19.9	Prenyl isovalerate	NC
γ-Cadinene	19.4	Terpinen-4-ol	NC
α-Muurolene	19.0	α-Terpineol	NC
Linalool	15.5	Myrtenal	NC
Prenyl tiglate	14.4	Citronellol	NC
Methyl geranate	14.4	*cis*-Citral	NC
Isoprenyl benzoate	14.4	Geraniol	NC
α-Gurjunene	12.0	*trans*-Citral	NC
δ-Cadinene	11.7	*cis*-Methyl cinnamate	NC
Calamenene	11.2	δ-Elemene	NC
trans-Methyl cinnamate	10.6	Geranyl acetate	NC
α-Calacorene	10.6	Isoamyl benzoate	NC
β-Gurjunene	10.0	Germacrene D	NC
α-Farnesene	9.6	$C_{15}H_{24}$ (1486)	NC
Cubenol	9.2	δ-Selinene	NC
Leptospermone	8.7	Elemol	NC
p-Cymene	8.5	τ-Elemene	NC
α-Pinene	7.7	Globulol	NC
Aromadendrene	7.5	γ-Eudesmol	NC
Caryophyllene Oxide	6.4	β-Eudesmol	NC
$C_{15}H_{24}$ (1517)	6.3	α-Eudesmol	NC
α-Copaene	5.7		

Appendix E

Data CD

- **Appendix E.1** Tree sample data.
- **Appendix E.2** Nectar and leaf oil.
- **Appendix E.3** Nectar and soil samples.
- **Appendix E.4** Predicted NPA vs actual NPA.
- **Appendix E.5** Raw sugar response factor data.
- **Appendix E.6** Raw data for the number of flowers required for extraction.
- **Appendix E.7** Raw data for the SFT.
- **Appendix E.8** Raw data for the SFT, petal colour.
- **Appendix E.9** Raw data for the SFT, flower centre colour.
- **Appendix E.10** Raw data for the SFT, relating SFT to standard method.
- **Appendix E.11** Raw data for period of extraction.
- **Appendix E.12** Raw data for using a second extraction.
- **Appendix E.13** Raw data for testing of sequence stability.

- **Appendix E.14** Raw data for single nectar extract repeated 5 times compared to the variation within the standard method of 10 nectar extracts each repeated once.

- **Appendix E.14** Raw data for comparing tree samples that have been repeated on different days.