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Determination of a Chemical and Biological Profile of Honey from the Chatham Islands (Rēkohu/Wharekauri)

A thesis

submitted in partial fulfilment

of the requirements for the degree

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Simon Winship



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Abstract

Honey is predominantly sugar and water. Enzymes, minerals and bioactive compounds such as phenolics are present in small quantities. These components give each variety a unique composition, often representative of its floral origin and geographic location. Chatham Island (Rēkohu/Wharekauri) and neighbouring Pitt Island (Rangiauria/Rangiaotea), collectively known as the Chatham Islands (Rēkohu), are home to native plants which are not found anywhere else in the world. This provides an untapped resource for unique honey types, which may have important chemical and bioactive properties yet to be discovered. This investigation determined the various properties of honey produced from Rēkohu, including sugars, phenolic compounds, bioactivity, residues detection (tutin and glyphosate), elemental composition, quality parameters, pollen, and physicochemical properties such as colour, moisture, pH and conductivity. This research was intended to profile Chatham Islands honey to support the growing apiculture industry on the islands. Rēkohu honey was found to be similar to some mainland New Zealand honey varieties in its physicochemical parameters and carbohydrate composition. The pollen analysis and elemental profiling showed unique aspects of Rēkohu honey, useful for its floral and geographic characterisation. C4 adulteration, HMF, glyphosate and tutin were below the maximum limits required for export, while diastase activity was above the minimum limit. It was demonstrated that Rēkohu honeys have only low or zero levels of MGO and DHA since bees do not have access to mānuka. Total phenolics, antioxidant capacity and total antibacterial activity were higher compared to mainland clover honey but lower than mānuka honey.

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List of Abbreviations

Abs	Absorbance
ACN	Acetonitrile
AHS	Artificial honey solution
AMPA	Aminomethylphosphonic acid
Asp	Aspartic Acid
Codex	Codex Alimentarius
CuPRAC	Cupric reducing antioxidant capacity
DHA	Dihydroxyacetone
DN	Diastase Number
DOC	Department of Conservation
DPPH	2,2-diphenyl-1-picrylhydrazyl
E. coli	*Escherichia coli*.
f	Furanose
F/G ratio	Fructose/Glucose ratio
FC	Folin-Ciocalteu
FRAP	Ferric reducing antioxidant power
Fru	Fructose
GA	Gallic Acid
GAE	Gallic Acid Equivalent
Gal	Galactose
GC-MS	Gas Chromatography Mass Spectrometry
Glc	Glucose
Glu	Glutamic Acid
HA	Hydroxyacetone
HMF	5-Hydroxymethylfurfural
HPLC	High Performance Liquid Chromatography
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
IHC	International Honey Commission
IR-MS	Isotope Ratio Mass Spectrometry
LC/MS	Liquid Chromatography Mass Spectrometry

LoQ	Limit of Quantification
LoD	Limit of Detection
MGO	Methylglyoxal
MIC	Minimum Inhibitory Concentration
MPI	Ministry for Primary Industries
MS	Mixed Standard
n.d.	Not detected
OPD	*o*-phenylenediamine
p	Pyranose
PFBHA	*O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine
QC	Quality Control
RLP	Recognised Laboratory Practice
S. aureus	*Staphylococcus aureus*
SPE	Solid-phase extraction
TEAC	Trolox equivalent antioxidant capacity
UMF	Unique Manuka Factor®
UV-Vis	Ultraviolet-visible light
ZOI	Zone of Inhibition Radius

Chapter 1- Literature Review

1.1 Introduction

New Zealand (Aotearoa) is home to a wide variety of native plants from which the introduced honeybee (*Apis mellifera*) harvests nectar and produces honey.[1] Many of these plants are unique to New Zealand, making these honey types exclusive and desirable in a global market for potential novel organoleptic and bioactive properties. In a market dominated by mānuka honey, other native New Zealand honey varieties are under-researched, and their beneficial properties are unknown.[2] This is especially so for the Chatham Islands (Rēkohu/Wharekauri)[a], with an emerging apiculture industry and a collection of indigenous plants, found only on the islands.[3] The composition of Rēkohu honeys is previously unstudied. With the information reported in this thesis, a unique profile can be created for Rēkohu honeys, distinguishing them from the honey of mainland New Zealand. The quality and bioactivity of Rēkohu honeys is also explored.

1.2 Honey

1.2.1 Honey Production

Honey contains a wide variety of compounds that contribute to its taste, aroma, and texture. These primarily result from floral nectar or honeydew, used by bees to make honey. Depending on the origin of the starting material, honey is classified as floral or honeydew. Each plant has a unique nectar composition that contributes many substances to the honey blend. Floral nectar is a mixture of sucrose, fructose, and glucose. The concentrations of these can vary, with sucrose being dominant in some cases or fructose and glucose in other cases. Chalcoff et.al. (2006)[4] analysed nectar from 26 species of plant in South America. *Berberis darwinii* nectar was comprised of 91.5% sucrose, 1.2% fructose, 3.3% glucose and 4.7% unknown sugars, whereas *Rhodophiala mendocina* nectar contained 1.4% sucrose, 38.7% fructose and 59.9% glucose. Floral honeys are derived from flowering plants and nectar collection is often limited by seasonal and environmental changes. Honeydew is a concentrated sugary liquid excreted from small insects such as aphids, which live upon the

[a] From this point, the Chatham Islands will be referred to as Rēkohu

plant. Bees will collect honeydew and use it to make honey. As such, honeydew honeys have slightly different compositions compared to floral honeys.[5]

When collecting nectar, the forager bees store it in a special storage organ known as a honey stomach, which can hold up to one third of the bee's weight in nectar.[6] Upon returning to the hive, the forager bee regurgitates the nectar mixture into the mouth of another worker bee. They continue to circulate it between each other using the body temperature of their mouths to reduce the moisture content and saturate the solution. It is then placed in a cell, and the bees further evaporate the solution, by fanning their wings. Once it has reached the correct moisture content, the bees cap the cell with beeswax and store it for later consumption by the colony. Collection of this stored nectar by apiarists yields honey. During the mouth-to-mouth transfer, peptides and enzymes from the salivary glands are incorporated; these result in many chemical reactions within the honey whilst it is maturing in the comb and after harvest.[7]

1.2.2 Physical Characteristics

Honey consists principally of sugar and water, with minor amounts of minerals, enzymes, organic acids and other trace compounds.[8] This aqueous carbohydrate solution is super-saturated and can crystallize under the correct conditions. A typical honey composition consists of 80-85% carbohydrates, 15-17% water, 0.3% protein and 0.2% minerals and trace elements. The remaining ~4.5% is comprised of polyphenols, vitamins and organic acids.[9] Due to the presence of organic acids, honey has an acidic pH of 3-4. While the physical properties of honey can vary, the Codex Alimentarius International Food Standard (Codex)[10] sets limitations upon which many countries' import requirements are based. The Codex states that moisture content must not be greater than 20%, except for heather honey which can be up to 23%. The fructose and glucose content must collectively be greater than 60%, with the exception of honeydew honey, in which it can be as low as 45%. Sucrose content in must not be greater than 5%, however, several specific floral varieties can have up to 15%.

Several physical characteristics for a number of floral and honeydew varieties from across the world are shown in **Table 1.1** and **Table 1.2**, respectively.

Table 1.1. Physical properties of floral honey from various geographical origins

	New Zealand[11-14]	United States of America[15, 16]	Montenegro[17, 18]	Brazil[19, 20]	Iran[21-25]	China[26-28]
Glucose (%)	28.9-34.0	22.03-40.75	26.41-36.08	28.60-36.08	21.11-33.87	27.66-32.10
Fructose (%)	34.7-38.9	27.25-44.26	31.45-41.27	33.42-42.66	26.65-41.93	42.00-48.26
Sucrose (%)	n.d.[b]	0.25-7.57	0.51-3.35	0.75-3.32	0.13-5.53	0.82-3.82
Maltose (%)	n.d.[b]	2.74-15.98	0.30-2.57	0.96-4.67	1.6-3.8	0.80-1.93
Moisture (%)	16.5-17.4	13.40-22.90	14.85-17.15	16.00-19.98	14.4-16.33	17.76-19.66
Elements (%)	0.01-0.19	0.02-1.03	0.09-0.28	0.01-0.71	0.17-0.8	0.028-0.032
pH	3.57-4.78	3.42-6.10	3.87-4.49	3.80-5.29	3.60-6.98	3.86-4.06
Diastase (DN)	4.4-18.8	2.1-61.2	19.77-46.11	3.53-27.44	7.60-46.43	49.34-64.94
Total Phenolics (mgGAE/kg)	1867-2373	584.5-815.1	391.6–1106.5	131.2-1085.0	711.6-2062.2	242.9-297.2

[b] No data available

Table 1.2. Physical properties of honeydew honey from various geographical origins

	New Zealand[29, 30]	United States of America[15]	Spain[31, 32]	Brazil[19, 33]	India[34, 35]	Turkey[36-38]
Glucose (%)	19.42-20.29	19.23-31.86	26.6-34.3	20.84-26.38	31.63-34.05	16.29-41.52
Fructose (%)	35.82-36.47	2.91-38.12	36.0-42.9	33.43-41.07	30.69-33.23	19.54-44.72
Sucrose (%)	0.40-0.77	0.44-1.14	0.05-1.87	3.56-6.40	1.08-1.12	n.d.-7.87
Maltose (%)	1.9-6.19	5.11-12.48	3.85-6.74	0-3.97	0.96-1.08	0.19-0.54
Moisture (%)	15.70-16.10	12.20-18.20	15.70-18.60	15.20-18.40	18.08-18.32	13.00-21.90
Elements (%)	0.26-0.28	0.21-1.19	0.38-1.13	0.01-0.02	0.33-0.37	0.21-1.85
pH	n.d.[c]	3.90-4.88	3.87-6.91	4.0-4.9	3.67-3.89	3.33-5.54
Diastase (DN)	n.d.[c]	6.7-48.4	4.70-25.80	4.6-22.8	23.2-28.78	4.10-32.36
Total Phenolics (mgGAE/kg)	560.4-2109.0	n.d.[c]	500.4-2438.6	767.6-1307.7	595.1-601.7	558.3-670.1

[c] No data available

1.3 Rēkohu and Apiculture

1.3.1 Geography

The Chatham Islands (Moriori: Rēkohu; Māori: Wharekauri) are an archipelago located 800 kilometres offshore from the east coast of the South Island of New Zealand. They comprise a collection of seven islands and three island clusters, shown in **Figure 1.1**. Only the largest two islands, Chatham Island and Pitt Island, are inhabited and collectively have a population of approximately 700 permanent residents. South-East Island, Māngare Island, Little Māngare Island, Star Keys, The Sisters, The Forty-Fours, The Castle and The Pyramid are monitored as nature reserves by the New Zealand Department of Conservation (DOC). Seven percent of the total area of Rēkohu is DOC land, consisting of historic reserves, scenic reserves and conservation areas.[39]

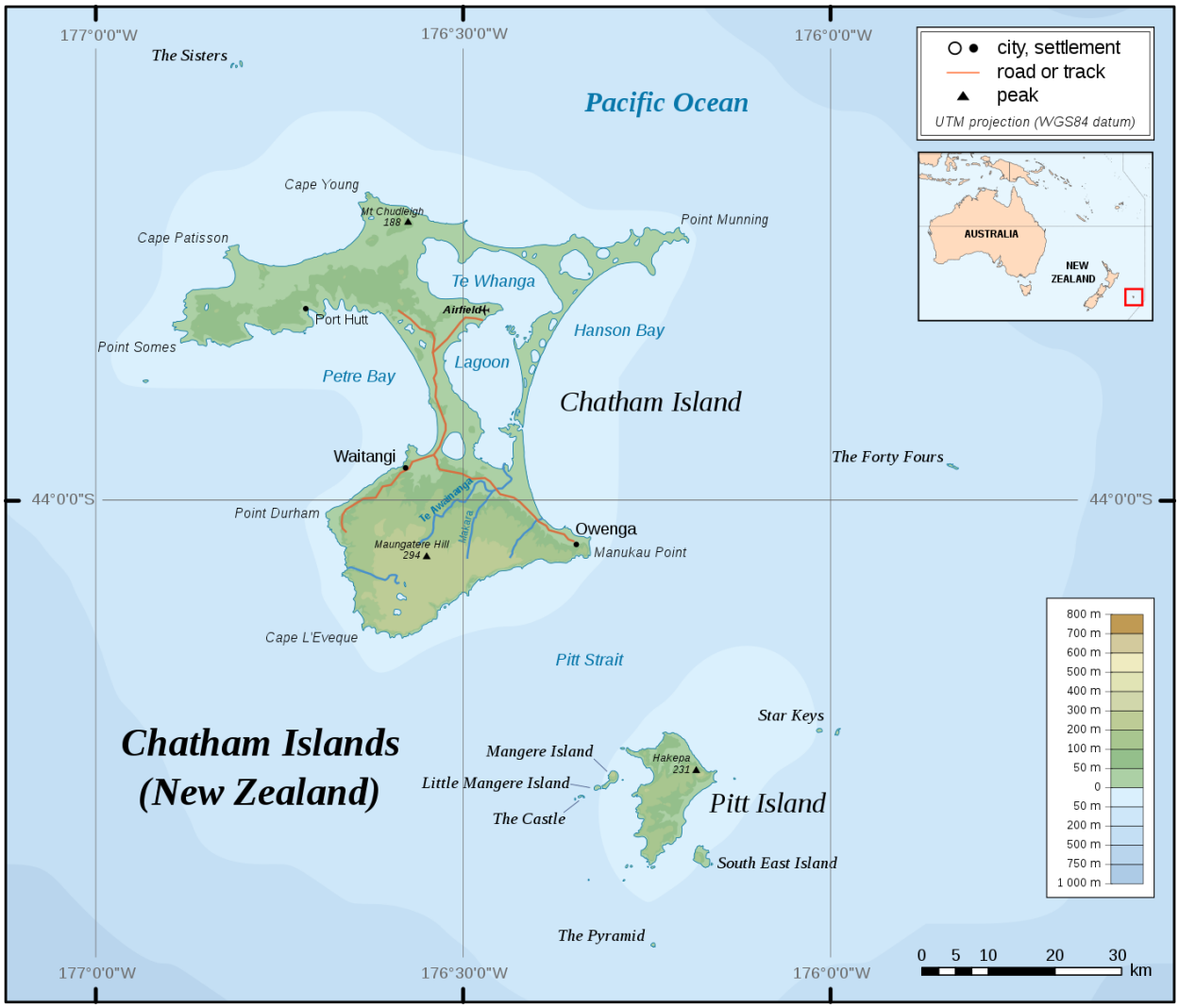


Figure 1.1. Topographical map of Rēkohu[40]

The largest island, Chatham Island is divided into six main regions, shown in **Figure 1.2**.[41]

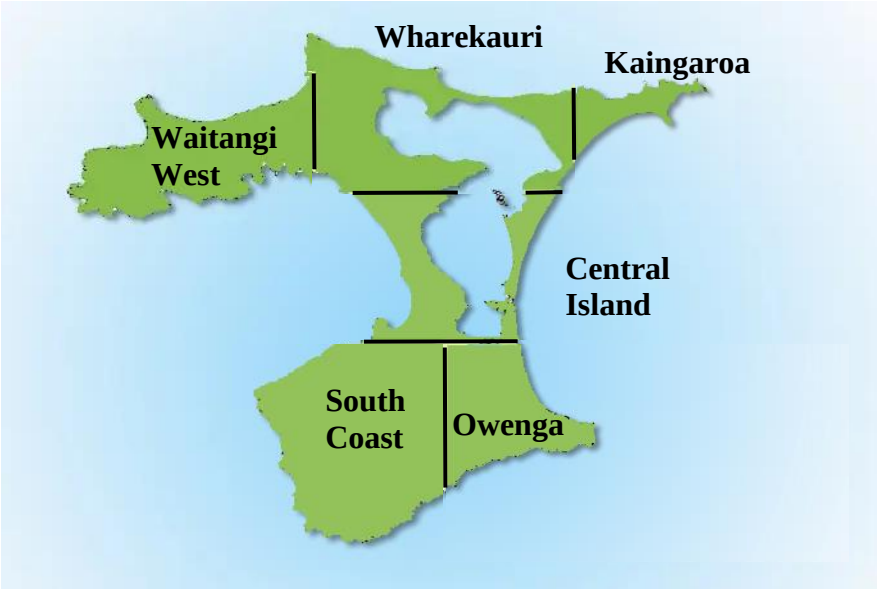


Figure 1.2. Regions of Chatham Island[41]

The main settlement on Chatham Island, Waitangi, is located on the eastern coast of the central island. There are also small settlements in Kaingaroa and Owenga. Apart from DOC land, the rest of Rēkohu's land mass is private land, used primarily for farming sheep and cattle. For Pitt Island, with 40 permanent residents, the majority of the land is DOC administered land with the main settlement being located at the northern end of the island.[42]

1.3.2 Flora

Since the formation of the islands as a result of tectonic activity, Rēkohu has been surrounded by the open ocean and isolated from the rest of New Zealand. However, this has not prevented the growth of plants on the islands. Wind and migratory seabirds, such as the albatross, carried seeds from the mainland, and over time, it flourished, with most of the landscape covered in flora. After the arrival of humans, initially Moriori, then Māori, then Europeans, exotic plants were imported for human usage.

The flora present on Rēkohu can be classified as either native, where it is naturally present on both mainland New Zealand and Rēkohu; endemic, where it is exclusively found on Rēkohu; indigenous naturalised, where it is native to mainland New Zealand

but was introduced to Rēkohu; and exotic naturalised, where it is not native to New Zealand and was introduced to Rēkohu.[3] Some examples of common plant species on Rēkohu are given in **Table 1.3**.

In 1864, a floral classification of plants from Rēkohu was published by botanist, Ferdinand von Mueller, without ever setting foot on the islands. He documented 87 species across 67 genera.[43] While many of these have been reclassified, Mueller's work provided a foundation for botanical documentation on Rēkohu. A report resulting from Leonard Cockayne's visit to the islands was published in 1928 and built upon Mueller's flora, establishing that 257 species of vascular plant were present.[44] De Lange *et al.* (2011) published a checklist of vascular plants on Rēkohu, which catalogued 902 species present on Rēkohu, with 465 listed as indigenous to New Zealand and 437 listed as naturalised to New Zealand. Within those that are native to New Zealand, 40 are known to be endemic specifically to Rēkohu, and as many as 13 other species may be categorised as endemic with further investigation.[3]

Table 1.3. Examples of plant species found on Rēkohu and their origin

Photograph of plant[45-55]	Species	Common Name	Origin
	*Corynocarpus laevigatus*	Kopi	Endemic to mainland NZ and naturalised on Rēkohu
	*Dracophyllum arboreum*	Tarahina	Endemic to Rēkohu

Photograph of plant[45-55]	Species	Common Name	Origin
	*Leptecophylla robusta*	Pouteretere	Endemic to Rēkohu
	*Piper excelsum excelsum*	Kawakawa	Endemic to NZ and Rēkohu
	*Muehlenbeckia australis*	Pouhuehue	Endemic to NZ and Rēkohu
	*Pseudopanax chathamicus*	Hoho	Endemic to Rēkohu

Photograph of plant[45-55]	Species	Common Name	Origin
	*Astelia chathamica*	Moriori Flax	Endemic to Rēkohu
	*Leucopogon parviflorus*	Mingimingi	Endemic to NZ and Rēkohu
	*Lotus corniculatus*	Trefoil	Naturalised to NZ and Rēkohu
	*Ulex europaeus*	Gorse	Naturalised to NZ and Rēkohu
	*Trifolium repens*	Clover	Naturalised to NZ and Rēkohu

Clover, Trefoil and Gorse are examples of species introduced by European settlers[3]. These have come to dominate the floral diversity of Rēkohu due to their association with agriculture and rapid spread. Kopi is an example of a species that was native to mainland New Zealand and was supposedly introduced to Rēkohu by early Polynesian settlers.[42] Kawakawa is an example of species found on Rēkohu that is identical to its counterpart found on mainland New Zealand. This indicates that these species made their way to Rēkohu via natural means such as sea and wind dispersal[3].

1.3.3 Apiculture on Rēkohu

Rēkohu's economy relies heavily on primary industries such as farming and fishing. Early European settlers brought honeybees to the islands and apiculture on Rēkohu has shown significant growth in recent years. Of the 28 native bees found in New Zealand, only one species is present on Rēkohu - *Hylaeus relegatus*.[56, 57] However, the introduction of western honeybees has significantly increased pollination across the islands, allowing non-native floral species to thrive. It is unknown how many native bees still inhabit the islands and how honeybees have impacted their survival.

With the arrival of European settlers, the British black honeybee was introduced to Rēkohu in 1890. Unfortunately, attempts to establish colonies on the islands were unsuccessful, most likely due to high wind speeds; lack of food, with many native species providing little nectar and pollen; and inexperienced beekeepers.[58] In 1957, five hives were flown to Chatham Island to help pollinate pasture land and this began frequent introductions of hives across Chatham Island.[59-62]. Macfarlane[57] reported the unsuccessful introduction of bumblebees to Chatham Island to boost pollination and support the existing honeybee hives. Beekeepers on the island became interested in the potential of apiculture on the islands, likening the quality of Chatham Island clover honey to that of clover honey produced in Canterbury[63]. In 1983, 130 hives were managed by 38 beekeepers, producing 150 kg of honey. Some of the white clover honey produced was considered some of New Zealand's purest.[64] In the years following, hive numbers dwindled, however apiculture began to grow once again thanks to Michele Anderson and Mana Cracknell. The pair coordinated beekeeping on the islands and raised the number of hives from 2 to 70, reported in 2010. It was also reported that the honeybees on Rēkohu, at this time, were thought to be a hybridisation of the British

black bee, Buckfast honeybees, Italian honeybees and Carniolan honey bees, from Europe. [65]

In 2019, Kaai Silbery and Francesca Bonaventre established Go Wild Apiary and began producing honey thought to be derived from the endemic tree, tarahina (*Dracophyllum arboretum*). However, no analysis has been conducted prior to this study to confirm this. A freeze-dried honey product was also developed and has become the main product for the company. The work of Go Wild Apiary continues to grow apiculture on Rēkohu, with hopes of marketing unique varieties of honey produced by the islands.[66]

The importance of establishing bees on the island not only stemmed from their agricultural benefit to the islands, but also because these bees are free of the diseases and pests that affect bees in almost all other parts of the world. This includes American foulbrood, caused by the bacterium, *Paenibacillus larvae*; and *Varroa destructor* mites, which feed off the larvae of bees and carry diseases such as deformed wing virus. It should be noted that while Rēkohu honeybees are free of these introduced threats, they are still affected by common diseases found in all honeybees. It has been found by the New Zealand Ministry for Primary Industries (MPI)[67] that Rēkohu bees were exposed to the fungus, *Nosema apis*; the gut parasite, *Lotmaria passim*, and Black Queen Cell Virus. These diseases are not linked to the significant colony losses seen with other pathogens. Although Rēkohu bees are reputed to be disease-free, this is not entirely true.[68]

1.4 Honey Analysis

1.4.1 Pollen

As part of the honeybee's foraging, pollen is collected as an additional food source, rich in protein. It is mixed with nectar or honey to form bee bread, also known as ambrosia, which is used to feed workers and larvae.[69] Simultaneously, bees aid in the pollination of plants, by transferring pollen from one plant to another as they search for nectar. Honeybees have specialised cavities on their legs called corbicula or honey baskets.[70] As the bee flies, its fuzzy body becomes positively charged through static electricity in the air. When bees rub over the stamen of the flower, the negatively-charged pollen becomes stuck all over their body.[71] Any residual pollen on the bee's body pollinates the next flower that they visit.

Bees do not deliberately add pollen to honey. Pollen in honey originates from pollen falling into the nectar of a flower and remains in the honey as nectar is collected. Additionally, pollen, on bees returning to the hive, inadvertently spreads from bee to bee around the hive, ending up in the honey product.[72] This unintentional cross contamination has become a useful tool for identifying the nectar origin of honey. Identifying the pollen grains in honey can indicate which plants bees are visiting. Bees typically will only visit one species per foraging trip, which is an evolutionary benefit for successful pollination.[73] However, the pollen that returns to the hive from all foragers is varied and as a result, honey will contain pollen from a number of species.

Melissopalynology is the science of identifying floral origin of honeys by means of pollen identification. Pollen can be identified because of each species' unique morphology. Pollen shape is described from both equatorial (E) and polar (P) view, in varying degrees of elongation about the central point, from oblate, which is horizontally elongated, to prolate, which is vertically elongated.[74] Additionally, pollen grains may contain apertures in their surface. The number and shape of these can be used to distinguish pollen species.[75]

The pollen shape classes are shown in **Figure 1.3**.

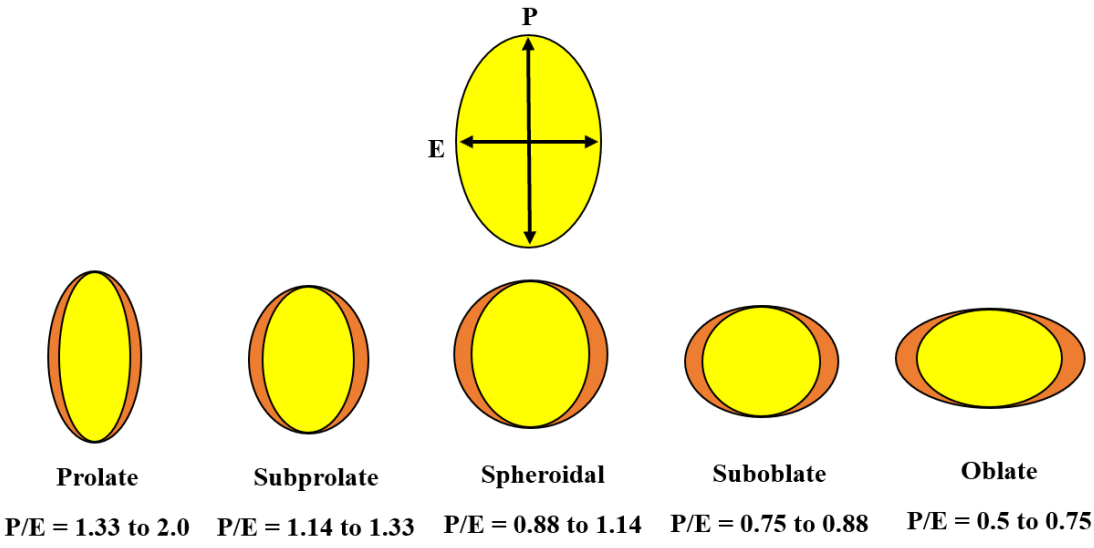
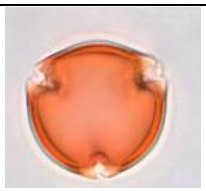
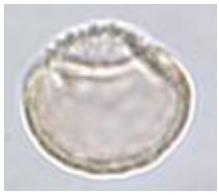


Figure 1.3. Five classes of pollen shapes. Adapted from Roubik *et al.*[74]

Some examples of pollen from common plant species on Rēkohu are given in **Table 1.4**.

Table 1.4. Pollen characteristics of common plant species found on Rēkohu Retrieved from the NZ Bee Pollen Catalogue[76]

Equatorial View	Polar View	Species	Characteristics
		White clover *(Trifolium repens)*	Elliptical (equatorial), round (polar), three apertures
		Gorse *(Ulex europaeus)*	Round (equatorial), round (polar), three apertures
		Kawakawa (*Piper excelsum excelsum*)	Round (equatorial), round (polar), no apertures

Traditionally, pollen determination is performed by palynologists who are trained to distinguish pollen types by eye, under a microscope. Studies such as Allen *et al.* (2006)[77] and Holt *et al.* (2011)[78] have proposed the use of machine learning to identify pollen. The Classifynder system[79] used at Massey University in New Zealand is one example of computational pollen detection. By providing the algorithm with an extensive database of 3380 identified pollen grains from 15 species, Lagerstrom *et al.* (2015)[80] demonstrated the system's ability to correctly match pollen 75% of the time. This has since been improved to 98.85% accuracy for 46 pollen types by Sevillano *et al.* (2020)[81].

1.4.2 3 in 1 Assay for Methylglyoxal, Dihydroxyacetone and Hydroxymethylfurfal

1.4.2.1 Methylglyoxal and Dihydroxyacetone

Mānuka (*Leptospermum scoparium*) is well known for the potent antibacterial properties of its products. The plant, itself, has been widely used in traditional Māori medicine and many studies have shown the benefits of mānuka products such as essential oils[82, 83] and honey[84, 85]. All honey has an antibacterial effect due to the production of hydrogen peroxide by the enzyme, glucose oxidase. This enzyme is

incorporated by the bee into the honey during production to prevent bacterial growth over the storage period.[86] However, it was observed in a 1983 study by Russell[87] that mānuka honey has a much more potent bactericidal effect against *Staphylococcus aureus* (*S. aureus*), likely attributed to one or more compounds found naturally in the honey. Molan *et al.* (1988)[88] used catalase to destroy hydrogen peroxide and investigated the non-peroxide antibacterial activity of mānuka honey. Adams *et al.* (2008)[89] isolated the bioactive fraction of mānuka honey and characterised it as methylglyoxal (MGO), a reactive ketoaldehyde, shown in **Figure 1.4**. MGO was previously shown to be present in honey by Weigel *et al.* (2004)[90], in the range of 0.4 to 5.4 mg/kg, attributing its formation to sugar degradation via Maillard reactions. Since its identification as a key antibacterial ingredient, the quantity of MGO has become one of the standards for the quality of mānuka honey.

In a study by Adams *et al.* (2009)[91], dihydroxyacetone (DHA), shown in **Figure 1.4**, was identified as a precursor to MGO and found in high quantities in mānuka nectar. It is also found in large amounts in freshly harvested honey. However, when stored at 37 ^{o}C, the concentration of DHA decreased while MGO increased. The concentration of DHA in honey can be an indication of the potential to produce MGO, under the right conditions. The conversion of DHA to MGO was modelled by Grainger in 2014[92].

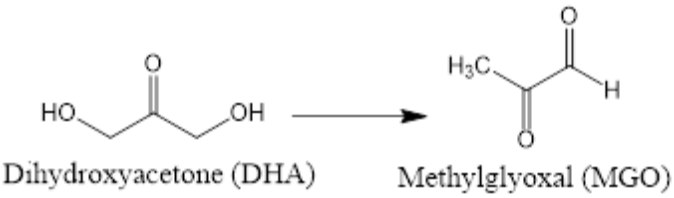


Figure 1.4. Structures of MGO and its precursor, DHA

1.4.2.2 5-Hydroxymethylfurfural

Another important carbonyl found in honey is 5-hydroxymethylfurfural (HMF), shown in **Figure 1.5**. It is produced through the chemical decomposition of sugars via the Maillard reaction. These reactions are accelerated at high temperatures. HMF, which has been labelled as a potential carcinogen, is used as a quality indicator for time and temperature of storage. This has led to the implementation of limits of 40 mg/kg for the export of honey from New Zealand.[93] Fresh honey usually contains little to no HMF but over time, levels increase. Any honey with HMF concentrations above 40 mg/kg is deemed "Baker's honey"[94] and cannot be imported by many countries under the traditional definition of honey.[95]

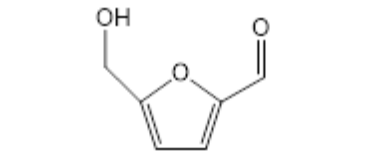


Figure 1.5. Structure of HMF

1.4.2.3 Analysis of MGO, DHA and HMF

Due to their importance in commercial food testing, two 3-in-1 methods have been devised for the quantification of MGO, DHA and HMF using a derivatising agent. The first is *o*-phenylenediamine (OPD), and the derivatisation scheme is shown in **Figure 1.6.**

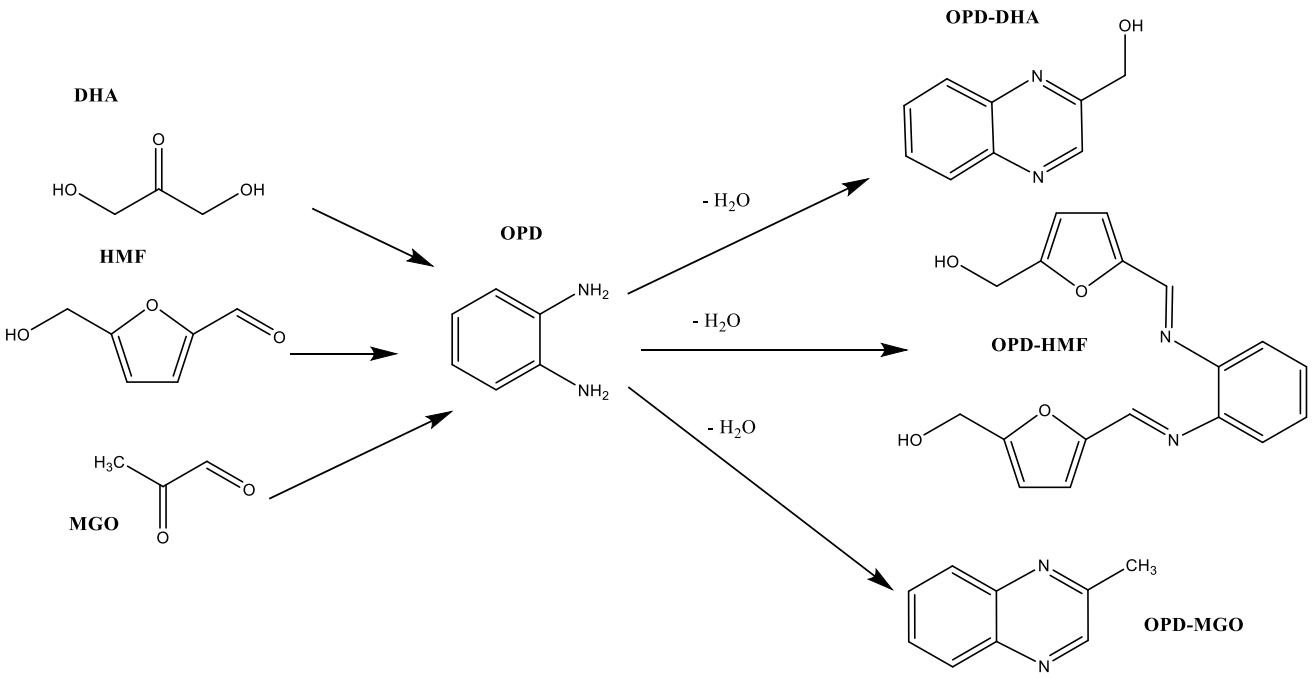


Figure 1.6. Derivatisation of DHA, HMF and MGO with OPD

For the case of MGO, each carbonyl reacts with one of the amines in OPD, forming the di-imine derivative, 2-methylquinoxaline. DHA, which is a monocarbonyl, first undergoes a single reaction with OPD, forming an imine which then undergoes rearrangement of the imine double bond, one of the two hydroxyls is converted to a carbonyl, which reacts with the other amine of the OPD, forming hydroxymethylquinoxaline. Quinoxaline derivatives are UV-Vis active so can easily be detected using High Performance Liquid Chromatography (HPLC) with ultraviolet-

visible light (UV-Vis) detection. HMF, when derivatised with OPD, does not form a quinoxaline derivative. Because rearrangement of the double bond would break the aromaticity of the furan ring, HMF reacts with each amine in OPD, forming a pair of di-imine isomers. While this is still UV active, the formation of isomers limits their quantification. Therefore, only DHA and MGO can be reliably analysed with OPD derivatisation[96]. OPD was first used for carbonyl detection in honey by Weigel *et al.* (2004)[90]. HMF is recommended to be quantified without derivatisation with OPD by HPLC-UV at 285 nm or a spectrophotometer at 284 nm. Alternatively, a reaction with barbituric acid and *p*-toluidine allows for spectrophotometric detection at 550 nm.[97] For many years, these methods were standard for food analysis laboratories.

An alternative derivatisation method, utilising the compound, *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBHA), was first used by Windsor *et al.* (2012)[98]. The derivatisation scheme is shown in **Figure 1.7**. The hydroxylamine in PFHBA reacts with the carbonyls in MGO, DHA and HMF to form an oxime derivative. Due to the aromaticity of the benzene ring, PFBHA derivatives absorb in the UV-Vis range.

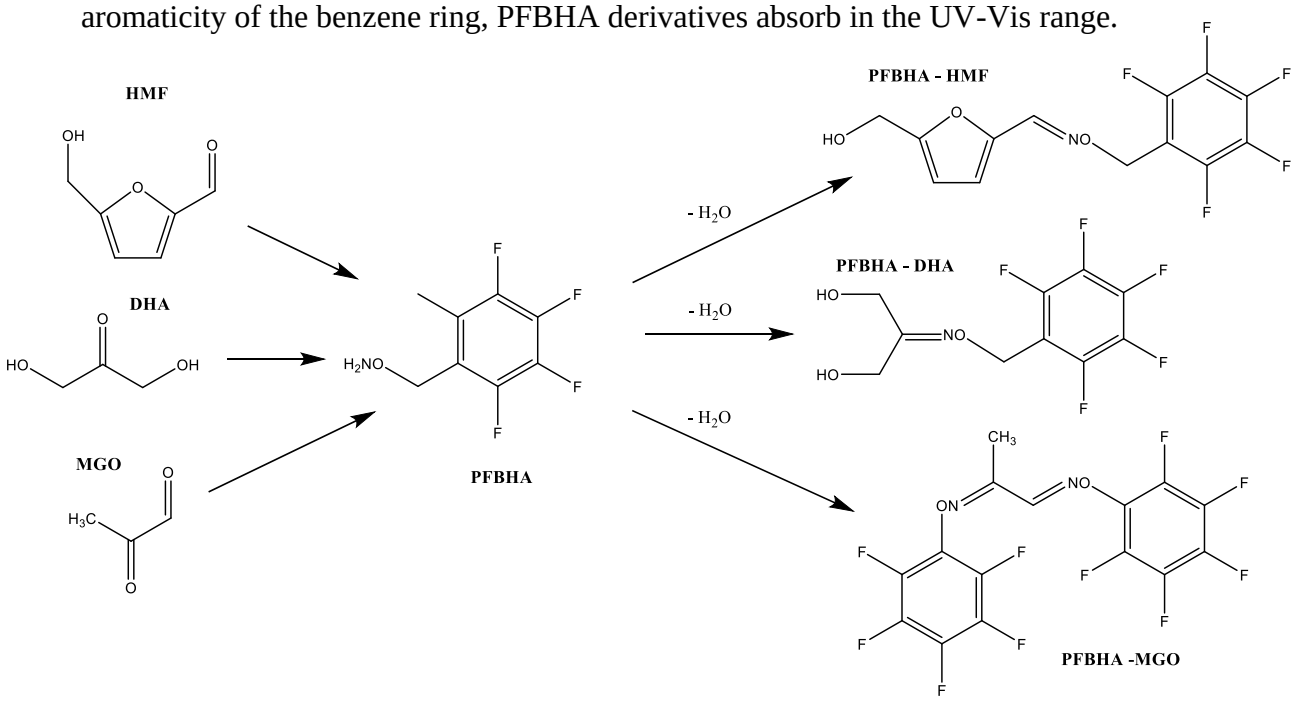


Figure 1.7. Derivatisation of DHA, HMF and MGO with PFBHA

The derivatisation step is much quicker for PFBHA, taking 1 hr according to Windsor *et al.* (2012)[98], compared to 6 hr for OPD, according to Weigel *et al.* (2004)[90]. Using OPD forms a UV active quinoxaline, however unreacted OPD is not detected by UV-Vis spectroscopy. PFBHA is UV-active both before and after derivatisation, allowing the detection of excess PFBHA to indicate successful derivatisation. The oxime derivatives

that PFBHA produces are a set of isomers with two peaks for each of MGO, DHA and HMF, whereas the quinoxaline derivatives only result in one peak per compound. The drawback to use of PFBHA is its cost.

1.4.3 Physicochemistry

1.4.3.1 Colour

The colour of honey is commonly assumed to be yellow or gold but, it can vary significantly from almost clear white to dark brown. It is classified using the Jack's scale, shown in **Figure 1.8**, using the measurement of refractive index diversion from a given standard.



Figure 1.8. Jack's scale for colour classification in honey

A Pfund colour grader uses a graduated amber colour glass wedge as a reference point. The distance required to match the colour along the wedge in mm is a mmPfund measurement. This is then converted to the Jack's scale for colour classification. The Pfund method is standard for colour in honey, but other methods exist to differentiate colour. The easiest method, used by beekeepers and honey tasters, is colour classification by eye, using a pre-established colour graded Jack's scale. While not scientifically rigorous, it provides an initial indication to inform further observations. In analytical food testing laboratories, two reproducible measurements are used. One is optical density, introduced by Townsend[99]. This method used a Coleman colorimeter and a series of optical densities were devised in relation to the Pfund scale. The second, and more common, technique for colour determination is using absorbance. White[100] demonstrated the use of spectrophotometry to determine honey colour. The conversion from absorbance at 635 nm to the Pfund scale is shown in **Equation 1.1**.

$$mm\ Pfund = -38.70 + 371.39 \times Abs_{635nm} \qquad \textbf{Equation 1.1}\ ^{100}$$

A study by Misto *et al.* (2019)[101] demonstrated that colour in honey can be analysed using the absorbance at 560 nm. The corresponding Coleman optical density from Townsend[99], absorbance at 560 nm from Misto *et al.* (2019)[101] and 635 nm from White[100] and Pfund measurement for each classification on the Jack's scale are given in **Table 1.5**.

Table 1.5. Relationship between optical density, absorbance at 560 and 635 nm, Pfund measurement and corresponding Jack's scale classification for honey[99-101]

Optical Density	Abs 560 nm	Abs 635 nm	Pfund (mm)	Jack's Scale Classification
< 0.08	< 0.030	< 0.126	< 8	Water White
0.12	0.060	0.150	9 - 17	Extra White
0.24	0.120	0.196	18 – 34	White
0.33	0.189	0.239	35 – 50	Extra Light Amber
0.55	0.441	0.333	51 – 85	Light Amber
0.73	0.955	0.411	86 – 114	Amber
> 0.73	> 0.955	> 0.411	> 114	Dark Amber

Colour can be linked to other properties in honey such as HMF content, total phenolic content, disaccharide content, pH and conductivity. Darker honeys often visually indicate high values of HMF and phenolics, while exhibiting a less acidic pH and a higher conductivity.[102]

1.4.3.2 pH

Honey exhibits a pH of 3-4, due to the presence of organic acids such as gluconic, citric, acetic and formic acids. The organic acid content of honey averages about 0.6% by weight. The major organic acid is gluconic acid, produced by the oxidation of glucose in honey. This reaction occurs because of the enzyme, glucose oxidase, which is introduced by the bee. It has also been found that amino acids are present in trace amounts in honey. Proline is the most prevalent amino acid in honey and is derived from the bee's haemolymph, an insect's equivalent of blood.[103] The acidity of the honey creates an environment in which many bacteria and fungi cannot survive, contributing to honey's antibacterial properties and long shelf-life. Honeydew honey has been shown to have a higher pH compared to blossom honey.[5] pH can be measured directly by a pH meter in dilute honey solution or alternatively by using titration.[97]

1.4.3.3 Moisture

Aside from carbohydrates, the second biggest component in honey is water, contributing to around 15-17% of the total mass.[104] This originates from nectar, which is primarily aqueous. In the process of producing honey, bees will fan honey to reduce the moisture content. Bees will cap the honey cell when the moisture content reaches a suitable level of dryness, however it is unknown how they know when this point is achieved. Following harvest, honey can be dried further to discourage bacterial and fungal growth during storage. When the honey cell is capped with wax, it is important that it is airtight as honey is hygroscopic, absorbing moisture from the atmosphere.[105] If honey moisture exceeds 20%, there is a possibility that it will ferment. Honey above this limit is considered of lesser quality and will not be sold in many countries. The exception to this is heather honey, which has a higher natural moisture content.[10] This variety is thixotropic meaning it becomes less viscous when shaken or stirred. This property makes it difficult for bees to reduce the moisture level. It is though that the thixotropic nature of heather honey reduces the likelihood of fermentation[106], and so for heather honey, a limit of 23% is implemented.[10] Moisture can be determined by measuring the refractive index of the honey. Using a refractometer, light is shone through the sample and a refractive index is calculated. The presence of moisture increases the refractive index.[10]

1.4.3.4 Conductivity

Conductivity is the measurement of how well a material conducts electricity. This is used as an indication of the botanical origin and quality of honey. It is derived from minerals, acids and other electrically charged compounds naturally present in the honey. Honeydew honeys have a higher percentage of minerals resulting in a higher conductivity compared to floral honey.[5] The Codex Alimentarius[10] outlines that honeydew honey conductivity must be greater than 800 µS/cm. When a honey is heated or aged, some of these degradation products are charged, increasing the conductivity. As a result, the Codex Alimentarius[10] states the conductivity of honey must not exceed 800 µS/cm for floral honey excluding honey derived from honey and chestnut, which must have a conductivity greater than 800 µS/cm. The International Honey Commission recommends that conductivity is measured using a conductivity meter.[97]

1.4.4 Phenolics

Phenolics are phenol-containing compounds that are important biomolecules. They are found in the nectar of plants and react with each other and enzymes in the bee, to form the more complex phenolics found in honey. Phenolics can be categorized into many different categories, including, but not limited to, phenolic acids, flavonoids, and polyphenols. Phenolic acids are the most common phenolic compounds found in honey and are derived from benzoic acid or cinnamic acid. Flavonoids consist of three phenolic rings and are the most varied class of phenolics. Polyphenols, such as such as tannins and lignans, are usually found in tree bark and wood and are formed by the reaction of smaller phenols with one another.[107, 108]

Phenolics are polar so are readily water soluble, and are antioxidants, actively scavenging free radicals. As a result of this bioactivity, substances high in phenolics have high nutritional value and are beneficial for health. Phenolic compounds are responsible for much of the colour and flavour profiles of honey and are often botanical markers for the floral origins of the honey. The structures of some phenolic compounds found in honey are given in **Table 1.6 - Table 1.11.**

Table 1.6. Structures of flavones found in honey

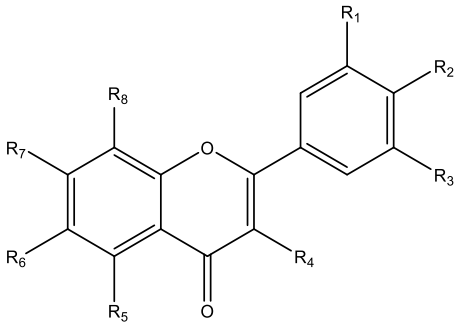
General Structure	Name	Side Chains (except H)	References
	Chrysin	R_5, R_7 = OH	109-112
	Apigenin	R_2, R_5, R_7 = OH	38, 109, 111-113
	Catechin	R_2, R_3, R_4, R_5, R_7 = OH	38, 113
	Galangin	R_4, R_5, R_7 = OH	93, 110-112
	Kaempferol	R_2, R_4, R_5, R_7 = OH	38, 109-113
	Luteolin	R_1, R_2, R_5, R_7 = OH	109, 111, 112
	Isorhamnetin	R_2, R_4, R_5, R_7 = OH R_1 = OCH_3	38, 111, 112
	Myricetin	R_1, R_2, R_3, R_4, R_5, R_7 = OH	109, 113
	Quercetin	R_1, R_2, R_4, R_5, R_7 = OH	38, 109, 111-113

Table 1.7. Structures of flavanones found in honey

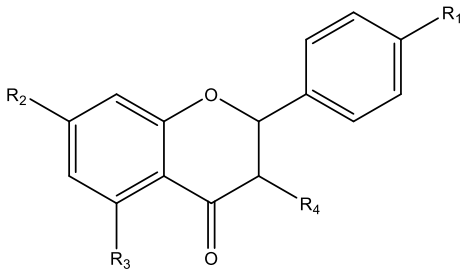
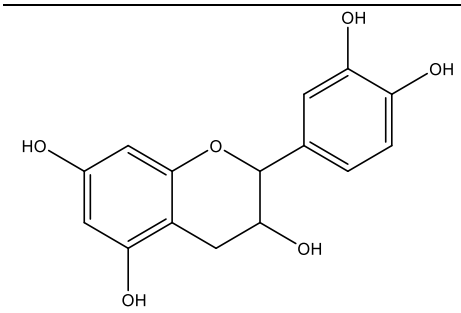
General Structure	Name	Side Chains (except H)	References
	Pinocembrin	R_2, R_3 = OH	109-112
	Naringenin	R_1, R_2, R_3 = OH	109, 112
	Pinobanksin	R_2, R_3, R_4 = OH	109-112
	Epicatechin	-	38, 114

Table 1.8. Structures of flavone glycosides found in honey

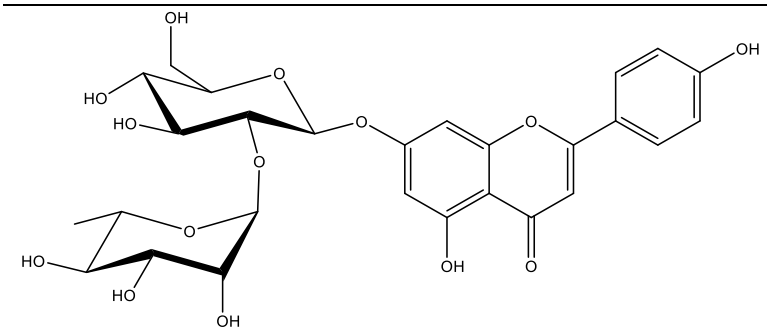
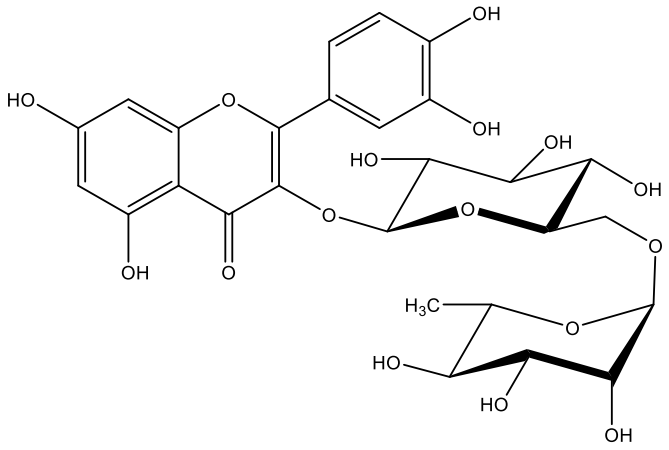
Structure	Name	References
	Naringin	109
	Rutin	38

Table 1.9. Structures of cinnamic acid and derivatives found in honey

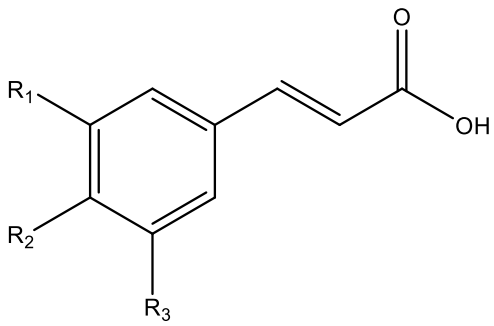
General Structure	Name	Side Chains (except H)	References
	Cinnamic Acid	-	110, 113
	p-Coumaric Acid	R_2 = OH	38, 109, 110, 113, 114
	Caffeic Acid	R_1, R_2 = OH	38, 109, 110, 113, 114
	Ferulic Acid	R_1 = OCH_3 R_2 = OH	38, 109, 110, 113, 114

Table 1.10. Structures of phenolic acids found in honey

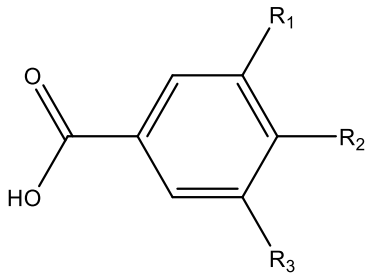
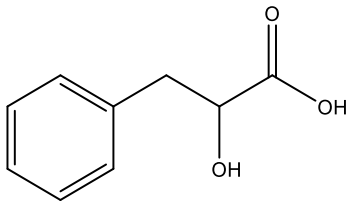
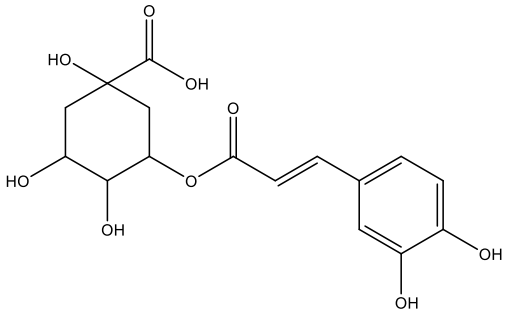
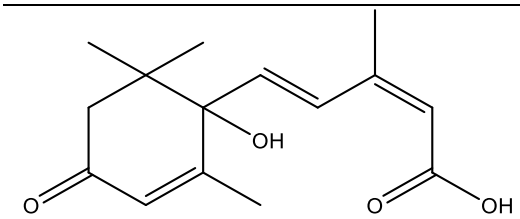
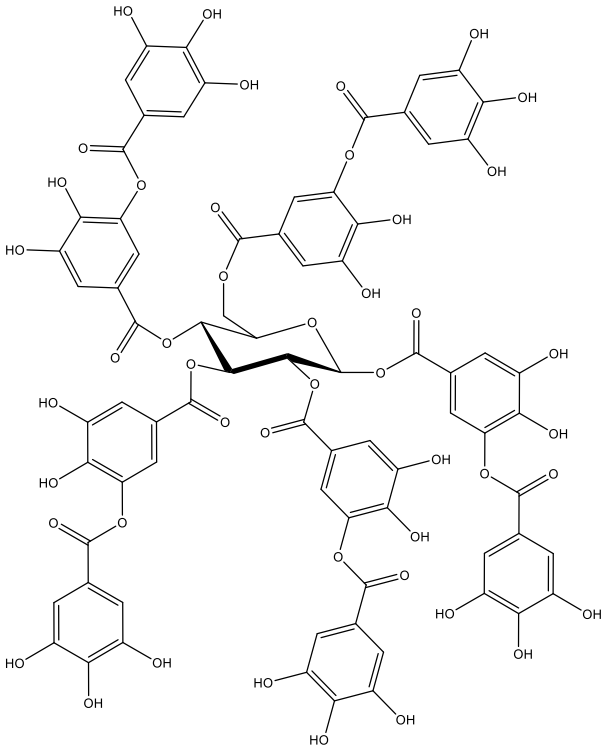
General Structure	Name	Side Chains (except H)	References
	Gallic Acid	R_1, R_2, R_3 = OH	38, 109, 113-115
	Syringic Acid	R_1, R_3 = OCH_3 R_2 = OH	38, 113-115

Table 1.11. Structures of miscellaneous phenolics found in honey

Structure	Name	References
	Phenyllactic Acid	115
	Chlorogenic Acid	109, 113

Structure	Name	References
	Abscisic Acid	110, 115
	Tannic Acid	114

To measure the total phenolic content of honey, a colorimetric method, known as the Folin-Ciocalteu method, is used. It involves Folin-Ciocalteu-Denis reagent, which was first used to determine tyrosine content in proteins[116], and later expanded to include tryptophan[117]. Folin *et al.* (1912)[116] developed a phenol reagent consisting of phosphotungstic acid ($H_3[PW_{12}O_{40}]$) and phosphomolybdic acid ($H_3[PMo_{12}O_{40}]$), which are reduced by antioxidants such as phenolic compounds. The reduced solution appears deep blue and the intensity of this colour is used to determine phenolic content in a sample. Folin *et al.* (1915)[118] first tested this method on phenols in urine, and it is has since been expanded for a variety of matrices including wine[119] and honey[38, 110, 113, 115, 120, 121]. The phenolic content is measured against known concentrations of a representative phenolic compound. For the Folin-Ciocalteu assay, gallic acid is used and total phenolic content is given in gallic acid equivalents (GAE). In some cases, when analysing flavonoids specifically, catechin is used and results are given in terms of catechin equivalents.

The detection of specific phenolics by HPLC or Liquid Chromatography with Mass Spectrometry (LC/MS) can be used to distinguish honey derived from different plants and determine if the honey has been adulterated or promoted as the incorrect variety. While a manufacturer may claim their product is derived from a particular plant, it might not contain the phenolic compounds that would be expected in honey of that type.[122, 123] For example, four compounds are used by MPI to determine if a sample is mānuka honey. If honey contains 3-phenyllactic acid, 2'-methoxyacetophenone, 2-methoxybenzoic acid and 4-hydroxyphenyllactic acid above specified minimum concentrations, it is considered monofloral mānuka honey. If they are present but in lower concentrations than those required for monofloral classification, it can be categorised as multifloral mānuka honey.[124] Alongside other methods, the analysis of phenolics in honey is useful in quality assurance for commercial honey production, especially for monofloral products, which are considered to be purer and of better quality than multifloral honey, since they contain higher amounts of the beneficial compounds uniquely derived from a single plant.[123]

1.4.5 Minerals and Trace Elements

Honey contains a wide variety of minerals and trace elements, totalling approximately 1% of its mass. These have been shown to be good indicators of geographic origin, with Grainger *et al.* (2023)[125] showing that New Zealand honey was distinct in its elemental profile compared to other international honeys. Solayman *et al.* (2016)[8] showed that the elemental content can also indicate botanical origin, with honeydew honeys having higher mineral contents. Environmental pollution can also be identified through elemental analysis of honey[126]. The most common elements found in honey are potassium (K), sodium (Na), calcium (Ca), magnesium (Mg) and phosphorus (P), comprising of more than 90% of the element composition. Other essential micronutrients in honey include cobalt (Co), nickel (Ni), copper (Cu), chromium (Cr), zinc (Zn), sulphur (S), and manganese (Mn). Non-essential elements such as aluminium (Al), strontium (Sr), vanadium (V) and barium (Ba) are naturally occurring in very low concentrations. Toxic elements including arsenic (As), cadmium (Cd), lead (Pb) and thallium (Tl), are also present in low amounts but are usually linked with contamination from industry and agriculture.[127] Concentrations of the elements found in honeys of various origins are shown in **Table 1.12**.

Table 1.12. Elemental concentrations reported in honey from Australia, USA and New Zealand

Element	Concentration (mg/kg)			
	Australia[128]	USA[126]	North Island, NZ[129]	South Island, NZ[11]
Na	3.7 - 382.7	14.3 - 15.8	9.9 - 488.7	1.1 - 110.0
Mg	6.0 – 190.0	n.d.	10.2 - 77.3	7.52 - 86.30
Al	0.05 - 14.00	n.d.	0.32 - 19.32	0.21 - 21.30
K	202 - 4600	360 - 450	230 - 2030	34.8 - 3640.0
Ca	21 - 270	n.d.	23.9 - 173.1	7.21 - 94.30
P	12 - 920	n.d.	n.d.	29.5 – 255.0
S	n.d.	n.d.	n.d.	13.4 - 93.9
V	0.005 - 0.020	n.d.	n.d.	n.d.
Cr	0.005 - 0.060	0.058 - 0.071	0.009 - 0.127	0.12 - 0.55
Mn	0.38 - 38.00	n.d.	0.31 - 18.90	0.18 - 4.75
Co	0.005 - 0.480	0.067 - 0.210	0.0060 - 0.0228	n.d.
Ni	0.005 - 1.400	n.d.	0.00015 - 0.79300	0.02 - 0.65
Cu	0.05 - 4.80	n.d.	0.130 - 1.760	0.09 - 0.70
Zn	0.16 – 120.00	1.9 - 3.2	0.35 - 5.97	0.20 - 2.46
Sr	0.08 - 2.30	n.d.	0.04 - 0.61	n.d.
As	0.0025 - 0.0090	n.d.	0.04 - 0.17	n.d.
Cd	0.0025 - 0.0530	n.d.	0.006 - 0.052	0.01 - 0.45
Ba	0.04 - 1.70	n.d.	0.05 - 1.10	n.d.
Tl	n.d.	n.d.	0.006 - 0.153	n.d.
Pb	0.003 - 0.690	n.d.	0.0095 - 0.0765	0.01 - 0.04

Several spectroscopic methods are utilised for the elemental analysis of honey. A study by Moniruzzaman *et al.* (2014)[113] reported three methods used for the analysis of different elements in Malaysian honeys, including flame atomic absorption spectroscopy (FAAS) for four elements; flame atomic emission spectroscopy (FAES) for two elements; and graphite furnace atomic absorption spectroscopy (GFAAS) for five elements. Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used by Hungerford *et al.* (2020)[128] for detecting five elements in Australian honey, whereas Vanhanen *et al.* (2011)[130] reported the use of ICP-OES for 18 elements in New Zealand honey. Hungerford *et al.* (2020)[128] also reported using inductively coupled plasma mass spectrometry (ICP-MS) to detect 21 elements in Australian honey.

Grainger *et al.* (2020)[131] developed a method for the analysis of 39 elements in New Zealand honey using ICP-MS. Elements in honey have also been detected through nuclear methods; Iskander *et al.* (1996)[126] reported 23 elements in US honey analysed by instrumental neutron activation analysis (INAA).

1.4.6 Diastase

Alpha-amylase (diastase) is an enzyme responsible for breaking down starch into individual maltose units. In humans, diastase is well-studied. However, its equivalent in *Apis mellifera* (Western Honeybee) is largely unstudied, despite its importance in honey production.[132] When nectar is collected from a flower, it is mixed with the bee's saliva, which contains many different enzymes such as diastase and glucose oxidase. This process is repeated as bees pass the honey to one another, each adding more and more enzymes. Bees cannot absorb long-chain starches or sucrose from the digestive tract so use diastase and other biomolecules to break them down into the readily absorbed monosaccharide sugars, fructose and glucose.

Honeybee diastase does not have an experimentally determined structure. It has a computationally predicted Alphafold structure[133], shown in **Figure 1.9**. It is 493 amino acids long[4] and consists of three domains[133-135], A, B and C. The B domain contains a beta sheet barrel which is the enzyme's active site. Near to this active site are binding sites for chlorine and calcium. The binding of chlorine allows calcium to bind which stabilises the active site in an open conformation.[135, 136] It is also thought that the C domain, which contains a second beta sheet barrel, acts as a second binding site for the starch substrate.[137] The predicted structure also contains a signal peptide (magenta), which consists of a number of hydrophobic residues, indicating that, upon translation, it is destined for transport across the cellular membrane. This makes sense as, in honeybees, diastase is secreted from salivary cells. The active site, shown in **Figure 1.10**, contains acidic residues, aspartic acid 206, aspartic acid 308, and glutamic acid 250 to help facilitate the hydrolysis of starch.[138]

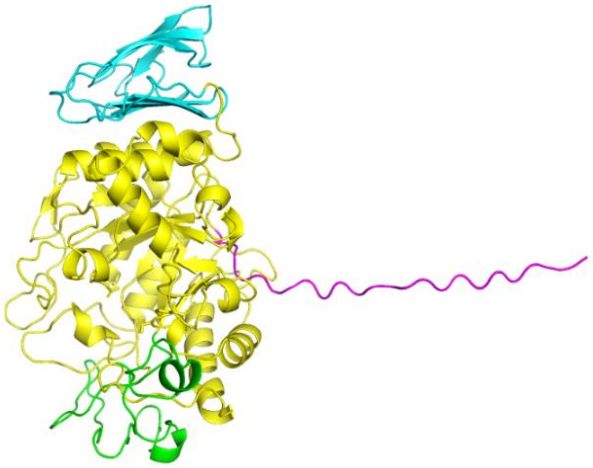


Figure 1.9. Alphafold predicted structure of diastase (yellow represents Domain A, green represents Domain B, cyan represents Domain C, magenta represents signal peptide). Adapted from UniProt structure[133]

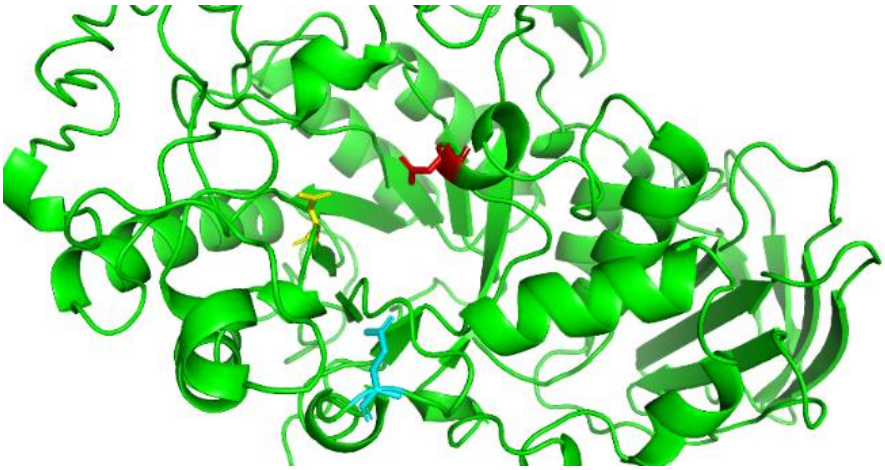


Figure 1.10. Active site of diastase (yellow represents Asp206, red represents Asp308, cyan represents Glu250). Adapted from UniProt structure[133]

In food testing, diastase activity is used as a quality indicator. Sometimes, honey is heated to facilitate processing or, in the case of mānuka honey, to accelerate the production of MGO. This comes with the caveat of the formation of HMF, when honey is heated or stored for a long time. Heating can denature enzymes, including diastase and, honey with low diastase activity is considered lesser quality. So much so, that in many parts of the world, honey with a diastase number (DN) of less than 8, cannot be imported under the traditional definition of honey. Low diastase honey can only be imported as baker's honey.[138] To measure diastase activity in honey, two main methods are employed and recommended by the International Honey Commission (IHC)[97]. The first is the Schade method.[139] It involves measuring the amount of time for a honey to

decompose a starch solution, using the reduction of iodine to iodide. Iodine reacts with starch to form a deep blue coloured product. As starch is broken down by diastase in honey, the iodine is reduced, and the solution becomes clear. The absorbance of the starch iodine solution is measured over specific intervals and the results are plotted against time. The time taken to reach the endpoint is inversely proportional to the diastase activity. The unit for diastase activity is determined to be the amount of enzyme required to convert 0.01 g of starch to an absorbance of 0.235 in 1 hr at 40 ^{o}C, referred to as the diastase number (DN).

In 1970, an alternative method was developed by Pharmacia Diagnostics called the Phadebas Amylase Test. The name Phadebas is an abbreviation of Pharmacia Diagnostics Biologically Active Substances, and the product is now marketed by Phadebas AB as Phadebas®, using a manufactured tablet containing starch cross-linked to a blue dye. As the tablet is broken down by diastase in the honey, the dye is released into solution. The solution is filtered to remove any dye still crosslinked and the absorbance at 620 nm is measured.[97, 140] This is converted to DN using **Equation 1.2**. If the first equation results in a diastase number lower than 8, **Equation 1.3** is used instead.

$$DN = 28.2 \times Abs_{620nm} + 2.64 \qquad \textbf{Equation 1.2}^{97, 140}$$

$$DN = 35.2 \times Abs_{620nm} - 0.46 \qquad \textbf{Equation 1.3}^{97, 140}$$

1.4.7 Sugars

Carbohydrates in honey are categorised as monosaccharides and oligosaccharides of increasing length; occasionally polysaccharides may be found. The most commonly occurring monosaccharides are D-glucose and D-fructose, shown in **Figure 1.11**. It is known that each of these sugars exists in a constant five-way equilibrium involving the two chair forms of the alpha and beta pyranose rings, the alpha and beta furanose rings and the acyclic form. As free molecules, fructose and glucose most commonly occur in the beta pyranose form. In larger molecules such as sucrose and inulin, fructose is found in its furanose form.[141]

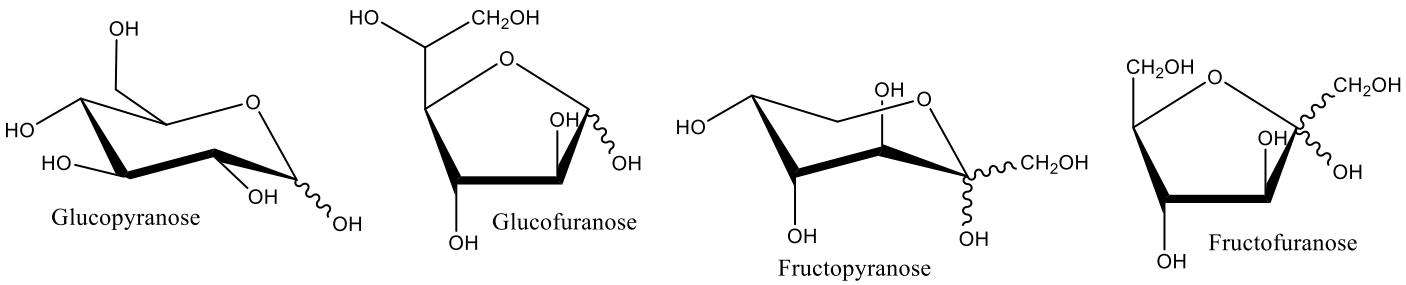


Figure 1.11. Pyranose and furanose structures of glucose and fructose

Oligosaccharides are comprised of combinations of the α and β pyranose configurations of glucose and fructose; and furanose conformations of fructose with a variety of glycosidic linkages between them. The most common oligosaccharides are sucrose, comprised of α-D-glucopyranose and β–D-fructofuranose, and maltose, comprised of α–D-glucopyranose and a second D-glucopyranose that can be α or β. These are shown in **Figure 1.12**.

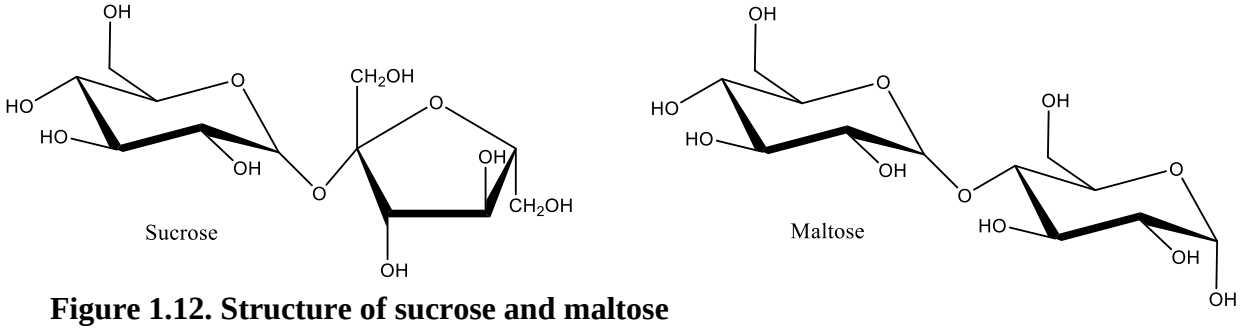


Figure 1.12. Structure of sucrose and maltose

Carbohydrates are transported around most plants as sucrose. Enzymes in the bee or insect will break down this sucrose into fructose and glucose, hence a high proportion of monosaccharides are present in honeys. When high concentrations of monosaccharides are present, as a result of concentration of the nectar, reversion can occur in glycoside hydrolases causing the formation of di-, tri- and higher oligosaccharides comprising glucose and fructose moieties variously linked, thus producing the wide variety of oligosaccharides found in honeys. As the nectar used for honeydew honey is processed by hydrolases from other insects besides bees, the more diverse range of enzymes yields a more varied carbohydrate profile characterised by oligosaccharides.

Oligo- and monosaccharides can be analysed by HPLC using a refractive index detector[142] or GC-MS following derivatization[143]. The sugars found in floral honey are given in **Table 1.13** and those found in honeydew honey are given in **Table 1.14.**

Table 1.13. Sugars found in floral honeys with nomenclature.[144-146]

Length of sugar	Name	Nomenclature
Monosaccharide	Glucose	Glc
	Fructose	Fru
Disaccharide	Sucrose	α–D-Glc*p*-(1↔2)-β-D-Fru*f*
	Maltose	α-D-Glc*p*-(1→4)-D-Glc*p*
	Maltulose	α-D-Glc*p*-(1→6)-D-Glc*p*
	Turanose	α-D-Glc*p*-(1→3)-D-Fru*p*
	α,β-Trehalose	α-D-Glc*p*-(1↔1)-β-D-Glc*p*
	Palatinose	α-D-Glc*p*-(1→3)-D-Fru*p*
	Laminaribiose	α-D-Glc*p*-(1→2)-D-Glc*p*
	Melibiose	α-D-Gal*p*-(1→6)-D-Glc*p*
	Isomaltose	α-D-Glc*p*-(1→6)-D-Glc*p*
	Gentiobiose	β-D-Glc*p*-(1→6)-D-Glc*p*
Trisaccharide	Raffinose	α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*
	Neo-kestose	β-D- Fru*f*-(2→6)-α-D-Glc*p*-α-D-Fru*f*
	1-Kestose	β-D-Fru*f*-(2↔1)-β-D- Fru*f*-α-D-Glc*p*
	Erlose	α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*
	Melezitose	α-D-Glc*p*-(1→3)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*
	Maltotriose	α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*
	Panose	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p*

Table 1.14. Sugars found in honeydew honeys with nomenclature.[29, 146]

Length of sugar	Name	Nomenclature
Monosaccharide	Glucose	Glc
	Fructose	Fru
Disaccharide	Sucrose	α-D-Glc*p*-(1↔2)-β-D-Fru*f*
	α,β-Trehalose	α-D-Glc*p*-(1↔1)-β-D-Glc*p*
	Cellobiose	β-D-Glc*p*-(1→4)-D-Glc*p*
	Turanose	α-D-Glc*p*-(1→3)-D-Fru*p*
	Nigerose	α-D-Glc*p*-(1↔1)-α-D-Glc*p*
	Maltose	α-D-Glc*p*-(1→4)-D-Glc*p*
	Gentiobiose	β-D-Glc*p*-(1→6)-D-Glc*p*
	Palatinose	α-D-Glc*p*-(1→3)-D-Fru*p*
	Isomaltose	α-D-Glc*p*-(1→6)-D-Glc*p*
Trisaccharide	Erlose	α-D-Glc*p*-(1→4)-α-D-Glc*p*- (1↔2)-β-D-Fru*f*
	Melezitose	α-D-Glc*p*-(1→3)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*
	Maltotriose	α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*
	Panose	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p*
Tetrasaccharide	Maltotetraose	α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*
Pentasaccharides		α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*
		α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*
		α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*
		α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*
Hexasaccharides		α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*

While there is significant variation in honeys depending on the origin of the honey, the basic carbohydrate composition is noticeably different in floral honeys compared to honeydew honeys. For example, the fructose, glucose and sucrose levels in a selection

of Spanish[145] and French floral honeys[144] averaged around 40%, 30% and 0.1% compared to around 30%, 23% and 0.67% in several New Zealand honeydew honeys[29].

The F/G ratio of honey typically ranges from 0.4 to 1.6.[147] While floral honeys are around 1:1, honeydew honeys can be up to 2:1[148] and are shown to have significantly higher levels of oligosaccharides. Of particular interest are tri and tetrasaccharides present in honeydew honeys but not in floral honeys.[29]

1.4.8 C4 Detection

To produce cheaper honey, there have been many instances of adulteration of honey with artificial syrups such as high fructose corn syrup and cane sugar to increase a batch size. Additionally, in larger scale apiary operations, during harsher winter months, bees may be fed with artificial feed comprised of 1:1 sucrose:water. Sometimes, this can be up to 2:1. The honey produced from bees fed with syrups is considered adulterated with a different composition to naturally produced honey, generally elevated sucrose. If adulteration is detected in honey, it is not considered fit for sale. Sugar additives can be detected using various analytical methods. Sugars derived from the Gramineae plant family including sugar cane and corn (maize) are formed via the C4 photosynthetic pathway, whereas sugars in flowering plant families are formed via the C3 pathway. The C4 and C3 pathways are photosynthetic routes where CO_2 is converted to a storable carbohydrate via four carbon intermediates and three carbon intermediates, respectively. There is a noticeable difference between the amount of ^{13}C isotope incorporated in each pathway, and this is measured to detect if a pure honey sample has been adulterated with C4 sugars.[22]

C4 sugars are analysed using Official Method 998.12[149] developed by AOAC International[d]. This method involves the extraction of protein from honey, from which the carbon isotope ratio between ^{12}C and ^{13}C is measured using Isotope Ratio Mass Spectrometry (IRMS). This is compared against the isotopic ratio of the honey itself, also measured by IRMS. If the honey has been tainted with C4 sugars, the two ratios will be different. The level of adulteration can be calculated using **Equation 1.4** and **Equation 1.5**.

[d]Previously known as the Association of Analytical Chemists (AOAC)

$$\delta^{13}C = \frac{{}^{13}C/_{12}C\ (test)}{{}^{13}C/_{12}C\ (standard)} \times 1000$$ **Equation 1.4** [149]

$$C4\ sugars\ \% = \frac{\delta^{13}C_{Protein} - \delta^{13}C_{Honey}}{\delta^{13}C_{Protein} - (-9.7)} \times 100$$ **Equation 1.5** [149]

It has been observed that mānuka honey from New Zealand can produce false positives when analysing C4 content. Grainger[150] proposed that this is caused by the presence of MGO, which binds to protein and affects the proportion of ^{12}C to ^{13}C in the protein. Additionally, protein degrades over time and as mānuka honey is usually stored to increase the levels of MGO, the C4 analysis proves ineffective at determining if a honey is indeed adulterated.

1.4.9 Residues

1.4.9.1 Tutin

Tutin is a neurotoxic sesquiterpenoid that is produced in *Coriaria spp*, which is native to New Zealand. Locally, this species is known as tutu, and all parts of the plant are poisonous to some extent. The biosynthesis of tutin has been tracked using tritium and carbon-14 labelled mevalonic acids[151, 152]. Additional work[153] highlighted the conversion of coperborneol to tutin, an important step in its formation. Tutin was first isolated in 1900 by Easterfield *et al.* (1901)[154] and its structure is shown in **Figure 1.13**. Hyenanchin is a less toxic derivative of tutin and its structure is also shown in **Figure 1.13**.

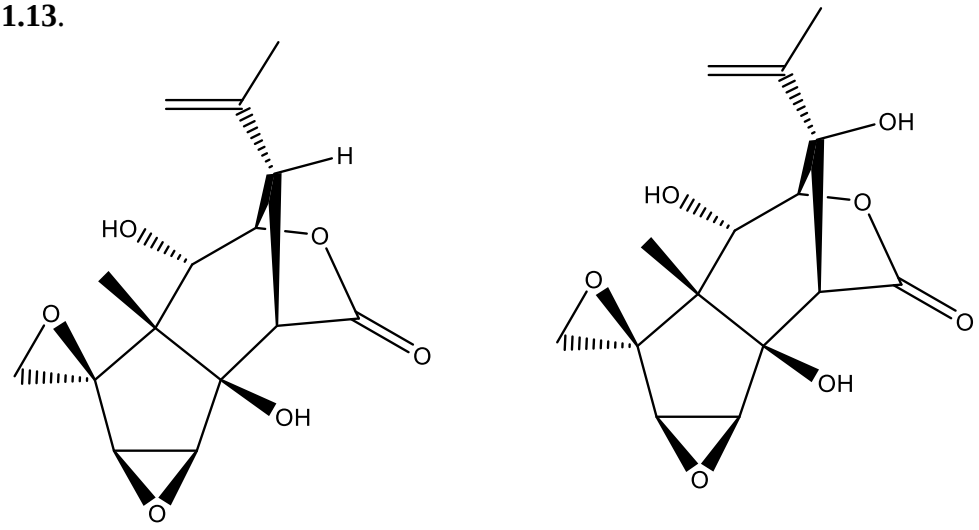


Figure 1.13. Structures of tutin and hyenanchin

The passion vine hopper (*Scolypopa australis*) feeds on the tutu plant and produces a honeydew that contains tutin, as well as hyenanchin, formed by the insect's metabolism. As native bees in New Zealand do not produce honey, this process only became an issue when European settlers brought honeybees to New Zealand. Honeybees will collect honeydew as an alternative source of sugar. The bees go on to create honey containing tutin, which can be lethal in high quantities.[155] Reports of toxic honey in New Zealand attributed to tutin presented symptoms such as headaches and vomiting.[156]

Several methods have been used to detect tutin in honey. Clinch *et al.* (1969)[157] and Palmer-Jones[156] incorporated animal models. Swallow *et al.* (1980)[158] suggested an alternative gas chromatography method which gave higher recovery for tutin spiked samples compared to the mouse bioassay suggested by Clinch *et al.* (1969)[157]. However, accuracy for hyenanchin spiked samples was much lower in gas chromatography, as hyenanchin becomes unstable under the conditions of gas chromatography.

Love *et al.* (1986)[159] introduced the use of HPLC with UV detection for analysing tutin and hyenanchin in honey. This method demonstrated agreement with the previous GC method for tutin but was significantly more accurate for hyenanchin. One of the issues with UV detection for tutin and hyenanchin is interference. As these compounds lack strong chromophores in the UV-Vis absorbance range, they are difficult to detect as other trace compounds are detected in the same absorbance region.

To overcome the issues of HPLC, tutin is usually analysed by LC-MS. Watkins *et al.* (2018)[160] found that honey from Te Puke, New Zealand contained 7.51 mg/kg of tutin and 7.94 mg/kg of hyenanchin. Larsen *et al.* (2015)[*161*] detected 50.79 mg/kg of tutin and 385.59 mg/kg of hyenanchin in honeycomb honey from Coromandel, New Zealand. This honey was linked to an outbreak in 2008 which initially saw three people from one family hospitalised as well as the beekeeper, who provided the honey, reporting gastrointestinal illness five weeks earlier. An investigation of the outbreak by Beasley *et al.* (2018)[162] identified eleven confirmed cases, nine probable cases and two possible cases as a result of the tutin contaminated honeycomb.

As a result of the 2008 cases, the Ministry for Primary Industries implemented a maximum residue limit of 0.7 mg/kg for tutin in honey. This means that any honey that is exported from New Zealand must comply with this limit, under the Food Standards

Australia New Zealand Act 1991.[163] Excluding the 2008 outbreak, the last reported outbreak occurred in 1974 and beekeepers in areas of known tutu growth are much more vigilant about testing for tutin in their honey.[162]

1.4.9.2 Glyphosate

N-(phosphonomethyl)glycine, more commonly known as, glyphosate is a primary component of many herbicides. It is an organophosphate, that acts by inhibiting the enzyme, 5-enolpyruvylshikimate-3-phosphate synthase, which is essential for the shikimate pathway and the production of aromatic amino acids in plants. This pathway is found in some bacteria, but not in animals. As a result of this, glyphosate is considered one of the safest pesticides used in agriculture, but there has been growing concern that bee health is inadvertently affected by glyphosate spraying on crops. Motta *et al.* (2018)[164] investigated the effect of glyphosate on the gut microbiome of honeybees. It was found that glyphosate is toxic to the eight primary species of bacteria found in the honeybee's gut. This results in reduced growth during development and susceptibility to pathogens. Similar observations were seen for another herbicide called glufosinate.

In addition to impacts on honeybee health, glyphosate and glufosinate have been found as contaminants in honey. Like honeybees, the ingestion of these herbicidal compounds by humans can lead to the disruption of gut bacteria and have significant health impacts. Glyphosate metabolises to aminomethylphosphonic acid (AMPA) and this is an indication of glyphosate contamination. Glyphosate and, less commonly, glufosinate and AMPA, shown in **Figure 1.14**, have been identified in honey in multiple studies using a variety of methods. Thompson *et al.* (2019)[165] derivatised honey with 9-fluorenylmethoxycarbonyl chloride, before solid-phase extraction (SPE) and analysis by LC/MS/MS. It was reported that 63% of honey samples in Canada contained glufosinate with concentrations up to 0.05 mg/kg. In the same study, it was reported that 99% of samples contained AMPA with concentrations up to 0.05 mg/kg. Chiesa *et al.* (2019)[166] utilised extraction in acidified water followed by ion-chromatography-high resolution mass spectrometry. Panseri *et al.* (2020)[167] used liquid extraction before analysis was carried out by tandem gas-chromatography. Neither study found traces of glyphosate, glufosinate or AMPA in Italian honey across 10 and 98 samples, respectively.[166, 167] Studies showing the detection of glyphosate in honey are summarised in **Table 1.15.**

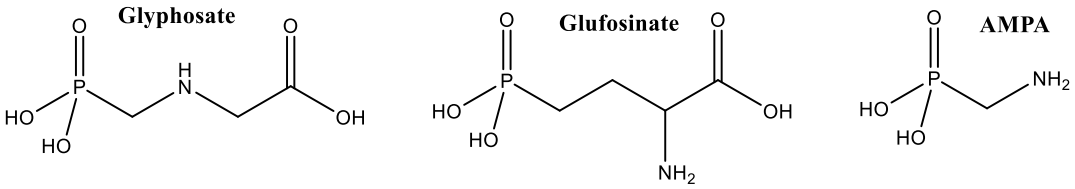


Figure 1.14. Structure of glyphosate, glufosinate and AMPA

Table 1.15. Summary of methods for the detection of glyphosate in honey

Sample Origin	Total samples	Positive detection	LOQ (mg/kg)	Concentration (mg/kg)	Sample Preparation	Analysis Method	Reference
New Zealand	233	50	0.01	0.011 – 1.100	- [a]	LC/MS/MS	[168]
USA (Philadelphia)	69	41	0.015	0.017 – 0.163	Acid digestion	ELISA	[169]
USA (Hawaii)	59	16	0.015	0.015 – 0.342	Acid digestion	ELISA	[170]
Switzerland	16	15	0.001	0.003 – 0.016	SPE	LC/MS/MS	[171]
Argentina	30	15	0.001	0.002 – 0.028	Aqueous extraction, FMOC derivatization	LC/MS/MS	[172]

[a] sample preparation was not disclosed in report.

1.4.10 Bioactivity

1.4.10.1 Antibacterial Activity

Honey has been used as a medical therapeutic for thousands of years. An Egyptian text, dating back to between 2600 and 2200 BCE, recorded a topical wound dressing containing honey. Similar records also indicated the antibacterial properties of honey in its use for embalming the dead.[173]. Hippocrates, an ancient Greek physician, prescribed honey to treat symptoms of bacterial infection such as sepsis and sore throat, [173] and Avicenna, a physician of ancient Iran, recommended treating tuberculosis, a bacterial infection of the lungs, with honey[174]. In modern hospitals and medical facilities, mānuka honey has been approved as a medicinal grade honey. It is used as a natural wound and burn treatment to prevent infection and improve wound healing.

The antibacterial activity of honey stems from several factors. The low moisture content of honey creates adverse osmotic pressure effects on bacteria.[175] Hydrogen peroxide is produced by the enzyme, glucose oxidase, and oxidizes the cell wall, destroying the structural integrity of the cell. When water is converted to hydrogen peroxide, glucose is converted to gluconic acid, adding to the acidic environment of honey, which is a deterrent to most bacterial growth.[176] Hydrogen peroxide is decomposed by catalase, an enzyme derived from pollen, however, the latter is not present in high enough quantities to significantly reduce the effects of glucose oxidase.[177]

A common method for testing the antibacterial properties of honey is a well-diffusion assay, and comparison with a set of phenol standards. Wells are created in a plate of agar previously inoculated with bacteria and honey is placed in the well. The plate is incubated, and zones of inhibition form based on the strength of the honey's antimicrobial activity. Alternatively, instead of a well, a disc soaked in the antibacterial substance may be placed on the agar to observe inhibition. Diffusion assays face the difficulty of having to rely on the diffusion of the substance through the agar matrix.[178] Broth dilution methods are also implemented to overcome this and use a bacterial broth added to the substance. The growth of the bacteria clouds the mixture and the turbidity of the sample indicates the antibacterial strength.[179]

Allen *et al.* (1991)[180] reported testing 345 samples of New Zealand honeys against the bacterium, *S. aureus*, with kānuka, mānuka, ling heather and kāmahi demonstrating the highest bactericidal effect. The effect of honey against *S. aureus* is of particular interest as it is estimated that 30% of the human population is colonised with *S. aureus* and it is one of the main causes of bacteraemia and infective endocarditis. Infection by *S. aureus* can range from mild skin infections to life threatening illnesses so treatments for this bacterium is very important. When catalase was added prior to the assay, all of the honeys except those derived from mānuka and viper's bugloss, demonstrated no antibacterial activity. Subsequent testing found that MGO was the main antimicrobial component in mānuka honey[89] and 1,4-dihydroxybenzene, in viper's bugloss honey[181]. A further survey of 179 non-mānuka honeys from New Zealand showed their effect on *S. aureus* and *Escherichia coli* (*E. coli*). While none exhibited non-peroxide activity, 50% of samples inhibited *S. aureus* by peroxide acitvity and 30% inhibited *E. coli*.[182]

1.4.10.2 Antioxidant Activity

Oxidants such as oxygen, OH^- and superoxide, induce oxidative stress on cells, causing cancer and ageing. To counteract this, cells utilise biomolecules such as phenolics and ascorbic acid. Foods such as honey contain natural antioxidants and have been shown to exhibit anti-inflammatory and anti-microbial properties because of this antioxidant capacity. The total phenolic content can be linked to the antioxidant capacity of honey [183]. There are several methods to measure antioxidant capacity, each utilising a slightly different reagent capable of indicating the presence of radical species and reducing compounds. These are outlined in **Table 1.16**.

Table 1.16. Various methods for the determination of antioxidant capacity in honey.

Reagent	Assay Name	Absorbance	Reaction Diagram	Reference
2,2-diphenyl-1-picrylhydrazyl (DPPH)	DPPH	519 nm	**Figure 1.15**	[184]
copper (II)-neocuproine (2,9-dimethyl- 1,10-phenanthroline)	Cupric reducing antioxidant capacity (CuPRAC)	450 nm	**Figure 1.16**	[185]
ferric-tripyridyltriazine	Ferric reducing antioxidant power (FRAP)	595 nm	**Figure 1.17**	[186]

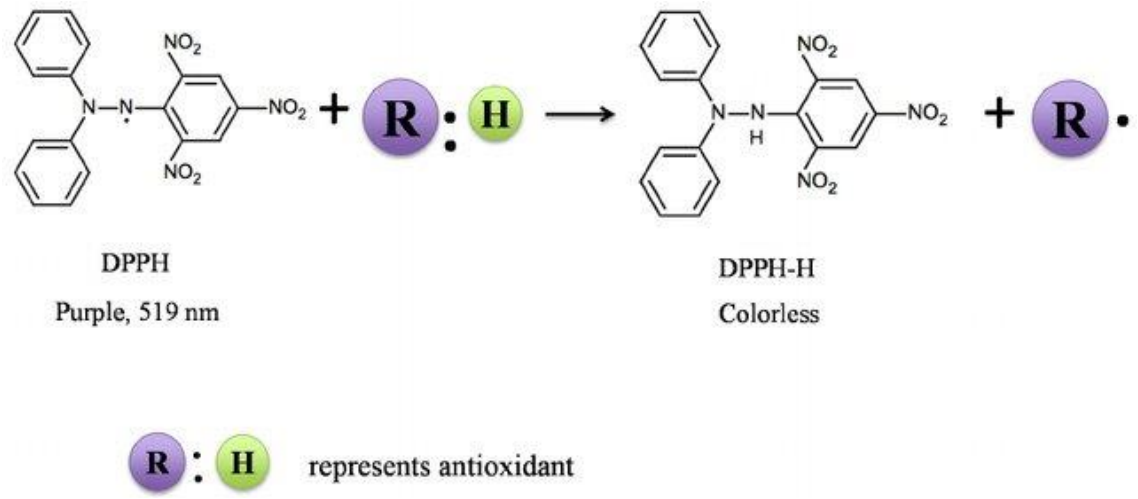


Figure 1.15. DPPH assay reaction[184]

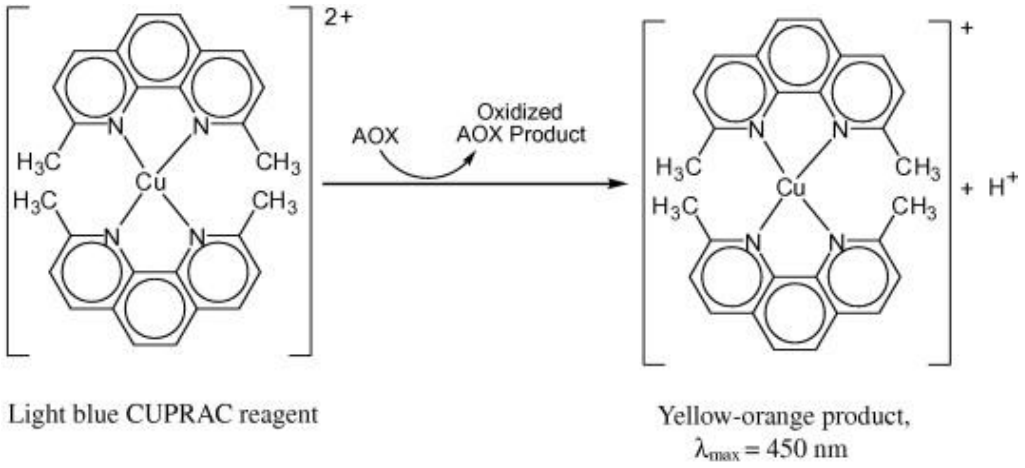


Figure 1.16. CUPRAC assay reaction[185]

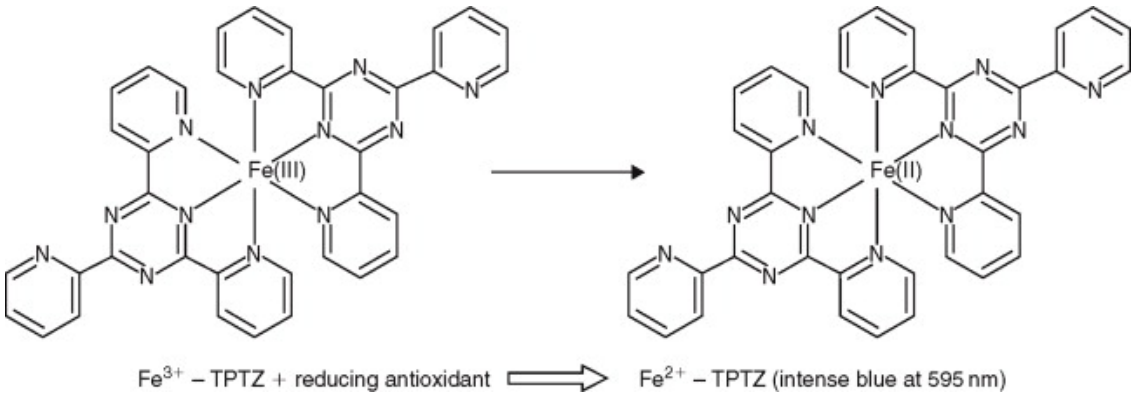


Figure 1.17. FRAP assay reaction[186]

Antioxidant capacity is usually measured relative to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, also known as Trolox, shown in **Figure 1.18**. As each method of testing is slightly different, the Trolox equivalent antioxidant capacity (TEAC) allows for the direct comparison of antioxidant activity. Some examples of reported TEAC values in honey are given in **Table 1.17**. Probably related to total

phenolic content, honeydew honeys have been observed to demonstrate higher antioxidant activity compared to floral varieties.[5]

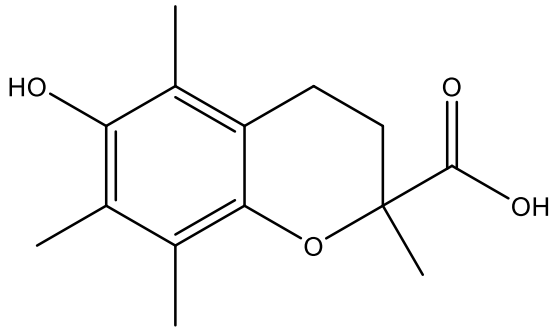


Figure 1.18. Structure of Trolox

Table 1.17. TEAC values for various assays of honey from different countries

Origin	Assay Type	TEAC (mg TE/kg)	Reference
Australia (Floral)	DPPH	0.05 - 0.18	[187]
	FRAP	0.17 – 0.94	
	CuPRAC	2.07 – 5.46	
Malaysia (Floral)	FRAP	5.24 – 8.25	[188]
New Zealand (Honeydew)	CuPRAC	15.77 – 16.36	[189]
	FRAP	4.74 – 5.38	
Romania (Honeydew)	DPPH	6.73 – 66.78	[190]

1.5 Research Aims

With the growth of apiculture on Rēkohu and the lack of knowledge surrounding the composition of honey sourced from the islands, this research aims to fill the gap and provide a preliminary but comprehensive analysis of the chemical and biological properties of Rēkohu honey. With 778 flowering plants found on Rēkohu, there is potential for unique properties. Using a variety of chromatographic, spectroscopic and bioassay techniques on a small subset of honeys, this research aims to determine if Rēkohu honey is different in its chemical composition to honey sourced from mainland New Zealand and if there are any potential beneficial properties.

Chapter 2 - Materials and Methods

2.1 Honey Samples

Twenty-seven samples were selected for the purposes of analysis and are shown in **Table 2.1**. An additional 12 samples could not be used due to the minimal amounts of sample available and visible signs of aging and reduced quality. These latter samples were unable to be replicated with an identical sample from the same batch in the same conditions. The samples used for analysis, covered a diverse range of locations, meeting the aim of this work. All samples were liquid honey stored at room temperature during analysis. The amount of sample provided varied.

In **Table 2.1**, all samples are indicated by the island origin, either Chatham Island or Pitt Island, and where the information was available a specific location is given. **Figure 2.1** is an aerial image, showing the relative size of Chatham Island and Pitt Island. **Figure 2.2** and **Figure 2.3** show the specific locations of samples from each island.

Table 2.1. Indicated location of origin for select Rēkohu honeys

Sample Number	Location	Sample Number	Location
1	Chatham Island	15	Kaingaroa, Chatham Island
2	Chatham Island	16	Chatham Island
3	Pitt Island	17	Chatham Island
4	Chatham Island	18	Go Wild Apiary, Chatham Island
5	Kaingaroa, Chatham Island	19	Go Wild Apiary, Chatham Island
6	Tupuarangi Orchard, Pitt Island	20	Go Wild Apiary, Chatham Island
7	Pitt Island	21	Go Wild Apiary, Chatham Island
8	Bluff House, Pitt Island	22	Go Wild Apiary, Chatham Island

Sample Number	Location	Sample Number	Location
9	Owenga, Chatham Island	23	Go Wild Apiary, Chatham Island
10	Pitt Island	24	Go Wild Apiary, Chatham Island
11	Henga, Chatham Island	25	Go Wild Apiary, Chatham Island
12	South Coast, Chatham Island	26	Pitt Island
13	Kaingaroa, Chatham Island	27	Kaingaroa, Chatham Island
14	Te Whanga Lagoon, Chatham Island		



Figure 2.1. Relative size of Chatham Island and Pitt Island labelled[191]



Figure 2.2. Chatham Island with sample origins labelled[191]

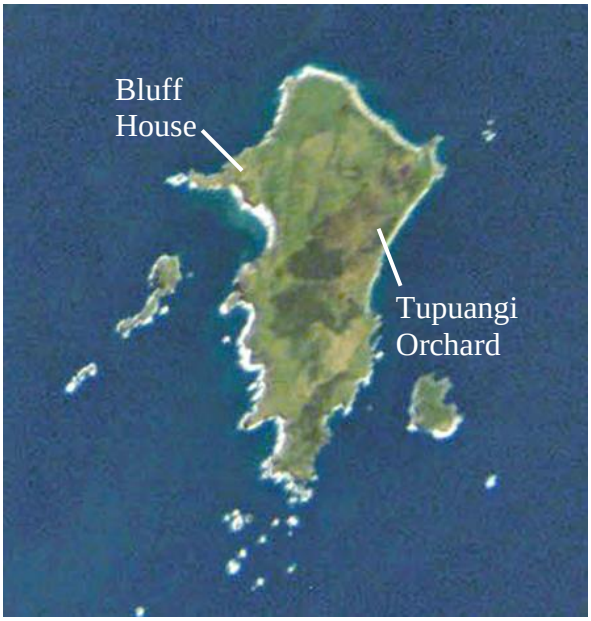


Figure 2.3. Pitt Island with sample origins labelled[191]

2.1.1 Comparative Samples

To compare the properties of Rēkohu honey to mainland New Zealand honey, honey samples from mainland New Zealand were analysed alongside Rēkohu honey. These are shown in **Table 2.2** together with the specific experiments in which they were used for comparison purposes.

Table 2.2. Comparative samples and the methods used to analyse these samples

Honey Type	Origin/Company	Methods
Mānuka (100 + MGO)	Zealandia Honey	3 in 1
Mānuka (900 + MGO)	Zealandia Honey	3 in 1, Antibacterial
Clover	Arataki Honey	Elemental Analysis, Antibacterial
Mānuka (100 + MGO)	Mānuka Health	Total Phenolics
Mānuka (300 + MGO)	Mānuka Health	Total Phenolics
Mānuka (600 + MGO)	Mānuka Health	Total Phenolics
Mānuka (900 + MGO)	Mānuka Health	Total Phenolics

2.2 Materials

2.2.1 Solvents

Acetonitrile used for preparing HPLC mobile phase was obtained from Merck. Methanol was obtained from Fischer Scientific. Ethanol was obtained from Merck. All water, unless otherwise specified, was Type-1, distilled and deionised using a Millipore Milli Q Reference water purification system (18.2 mΩ resistivity). From this point, Type-1 water is referred to as water.

2.2.2 Standards and reagents

O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (99%+) was obtained from Alfa Aesar. D-Glucose was obtained from BDH. Sucrose was obtained as Chelsea White Sugar. Nutrient agar and tryptic soy broth were acquired from Difco. The DPPH Antioxidant Assay kit was acquired from Dojindo. Methylglyoxal was obtained from Fluka. Melezitose was previously isolated and characterised by Nuclear Magnetic Resonance Spectroscopy at the University of Waikato. Analytical grade nitric acid (65%), sodium carbonate and sodium hydroxide were obtained from Merck. Oxacillin, Penicillin G and Streptomycin were acquired from Oxoid. Phenol standards (1% to 7%) were previously prepared at the University of Waikato. Hydroxyacetone, dihydroxyacetone, Folin-Ciocalteu reagent, citric acid, D-fructose, gallic acid, hydroxymethylfurfural, maltose, maltotriose and potassium chloride were obtained from Sigma-Aldrich. Analytical grade hydrogen peroxide (65%) was acquired from Univar (Ajax Finechem Pty Ltd).

2.2.3 Consumables

Deep well plates (96 well, 1.2 mL) were used with silicon lids for 3 in 1 analysis. Disposable microplates (96 well, 400 μL) were used for total phenolic analysis and antioxidant capacity assays. Syringe filters were Minisart polytetrafluoroethylene filters (0.45 μm). All glassware was class A. Round agar plates were standard laboratory petri dishes, with a diameter of 90 mm, sourced from Techno Plas. Square bioassay plates were Corning® 245 mm square bioassay dishes (Product No. 431272). 2 mL plain glass HPLC vials with caps and septa were used for sugar analysis.

2.3 Methods

Due to resourcing available at the University of Waikato, a number of methods were subcontracted to external laboratories for commercial analysis. These include 3 in 1 (samples 8-28), tutin (samples 1-28), glyphosate (samples 1-28), diastase (samples 1-28) and C4 (samples1-28) to Hill Lab[e]; and pollen (samples 1-28) to Massey University.

2.3.1 In house 3 in 1

This method is sourced from Bell (2021)[138].

2.3.1.1 Sample Preparation

Artificial honey was previously prepared in this laboratory by Bell (2021)[138]. The recipe consists of glucose (34.5 g ± 1 g), fructose (45.7 g ± 1 g), sucrose (1.5 g ± 1.1 g), dissolved in water (18.3 mL ± 1 mL). The artificial honey was stored in a freezer at −20 ^{o}C until analysis.

Honey (1 g ± 0.1 g) was homogenised and dissolved in water (4.400 mL). This makes a final solution volume of 5 mL. Each sample was prepared in triplicate. In addition to the samples, high (900+ MGO, Zealandia Honey) and low (100+ MGO, Zealandia Honey) quality control (QC) honeys were prepared in the same way. The masses of samples are given in **Appendix Table 1**. Artificial honey (3 g ± 0.1 g) was homogenised and diluted with water (3.200 mL). The artificial honey solution (0.05 mL) was further diluted with water (0.1 mL) and is referred to from this point as AHS.

Standard solutions of DHA, MGO and HMF were prepared. DHA (0.160 g ± 0.005 g) was added to a 20 mL volumetric flask and made up to the mark with water. The resulting DHA stock solution had a concentration of 8 mg/mL. MGO (0.300 g ± 0.030 g) was added to a 100 mL volumetric flask and made up to the mark with water. The resulting MGO stock solution had a concentration of 3 mg/mL. HMF (0.040 g ± 0.010 g) was added to a 25 mL volumetric flask and made up to the mark with water. The HMF solution (1.6 mg/mL, 1.250 mL) was diluted further with water (8.750 mL) to a concertation of 0.2 mg/mL. Hydroxyacetone (HA) was used as an internal standard. HA (0.5000 mL) was added to a 100 mL volumetric flask

[e] Previously known as Hill Laboratories

and made up to the mark with water. HA solution (0.5 %, 1 mL) was diluted with acetonitrile (ACN, 39.00 mL ± 0.20 mL).

Mixed standard (MS) solutions were prepared and the exact composition of each is given in **Table 2.3**.

Table 2.3.Volumes of DHA, MGO and HMF in mixed solutions

	MS 1	MS 2	MS 3	MS 4	MS 5	MS 6
	Volume of stock solution added (mL)					
DHA	3.000	2.000	1.500	0.750	0.100	0.020
MGO	1.500	1.000	0.600	0.300	0.150	0.015
HMF	5.000	3.750	2.500	1.250	0.125	0.0125

A clear 96-well plate (round bottomed, 1 mL) was prepared with the volumes of sample and reagents given in **Table 2.4**.

Table 2.4. Layout of 96-well plate

	1	2	3	4
A	Blank: 0.1500 mL water	Low QC: 0.1500 mL	Sample 3A: 0.1500 mL	Sample 5C: 0.1500 mL
B	Standard 1: 0.1000 mL MS1, 0.0500 mL AHS	High QC: 0.1500 mL	Sample 3B: 0.1500 mL	Sample 6A: 0.1500 mL
C	Standard 2: 0.1000 mL MS2, 0.0500 mL AHS	Sample 1A: 0.1500 mL	Sample 3C: 0.1500 mL	Sample 6B: 0.1500 mL
D	Standard 3: 0.1000 mL MS3, 0.0500 mL AHS	Sample 1B: 0.1500 mL	Sample 4A: 0.1500 mL	Sample 6C: 0.1500 mL
E	Standard 4: 0.1000 mL MS4, 0.0500 mL AHS	Sample 1C: 0.1500 mL	Sample 4B: 0.1500 mL	Sample 7A: 0.1500 mL
F	Standard 5: 0.1000 mL MS5, 0.0500 mL AHS	Sample 2A: 0.1500 mL	Sample 4C: 0.1500 mL	Sample 7B: 0.1500 mL
G	Standard 6: 0.1000 mL MS6, 0.0500 mL AHS	Sample 2B: 0.1500 mL	Sample 5A: 0.1500 mL	Sample 7C: 0.1500 mL
H	AHS blank: 0.1000 mL water, 0.0500 mL AHS	Sample 2C: 0.1500 mL	Sample 5B: 0.1500 mL	

To each well, HA:ACN solution (1:39 v/v, 0/7 mL) and PFBHA (0.2 mL) were added. The plate wells were fitted with a silicon lid and mixed by inverting. The plate was incubated (50 ^{o}C, 60 min ± 10 min). The plate was left to cool to room temperature (10 min) and analysis was carried out by HPLC.

2.3.1.2 Instrumentation

The HPLC system used for 3 in 1 analysis was a Waters Arc system with a quaternary solvent manager-R (with inbuilt degasser), Sample Manager FTN-R and 2998 PDA detector. The system was controlled using Empower™ 3 Chromatography Software.

Mobile phase A was 70% Water and 30% Acetonitrile. Mobile phase B was HPLC grade acetonitrile. Gradient conditions are shown in **Table 2.5**.

Table 2.5. Gradient conditions for 3 in 1 method

Time (min)	% Mobile phase A	% Mobile phase B
0	90	10
2.5	15	85
5.5	15	85
7.0	90	10
10.5	90	10

The flow rate was 0.8 mL min^{-1} with a total run time of 10.5 min per injection. The column used for separation was a Phenomenex Kinetex, 5 μm, C18 100Å, 150mm x 4.6mm column. The column temperature was 30 °C and the autosampler temperature was 20 °C. The injection volume was 10 μL. The 3D scanning range was 210-400 nm. Individual 2D channels were selected at 260 nm (DHA, HA and PFBHA), 273 nm (HMF) and 244 nm (MGO). The retention times of analytes is shown in **Table 2.6**.

Table 2.6. Retention times for 3 in 1 analytes

Analyte	Retention Time (min)
DHA	4.09
PFBHA	4.45
HA	4.80
HMF isomers	4.97, 5.06
MGO isomers	7.50 (MGO 1), 7.98 (MGO 2)

HMF and MGO both produce two isomers upon derivatisation with PFBHA. MGO quantification utilised the sum of both peak areas, whereas HMF was quantified with only the peak area at 4.97 min. The HMF peak at 5.06 min was not quantified due to interference.

2.3.1.3 Data Work Up

To calculate the concentrations of DHA, MGO and HMF in sample, a calibration curve of mass of analyte (mg) in standard against the corrected area ratio was constructed. The area ratio is calculated using **Equation 2.1**. The equation of the resulting calibration curve was rearranged to solve for the mass of analyte in sample, shown in **Equation 2.2**. The concentration of the analyte in sample was calculated using **Equation 2.3**.

$$Corrected\ area\ ratio = \frac{Peak\ area\ analyte_{Std}}{Peak\ area\ HA_{Std}} - \frac{Peak\ area\ analyte_{AHS\ Blank}}{Peak\ area\ HA_{AHS\ Blank}}$$ **Equation 2.1** [138]

$$Mass\ analyte\ (mg) = \frac{Corrected\ area\ ratio - intercept}{Slope}$$ **Equation 2.2** [138]

$$Concentration\ analyte\ \left(\frac{mg}{kg}\right) = \frac{Mass\ analyte\ (mg)}{Mass\ honey\ (g)} \times 1000\,\frac{g}{kg}$$ **Equation 2.3** [138]

2.3.1.4 Method Validation

This method was validated using the high and low QC samples, carried out in triplicate over 3 separate days.

2.3.2 Physicochemistry

2.3.2.1 Moisture

The moisture content of samples was analysed by a MISCO PA202 Palm Abbe Digital Handheld Refractometer. The instrument was zeroed with water. Raw honey was placed to cover the lens and the lid was shut. The sample was left to stabilise for 30 seconds before a reading was taken. Samples were measured in triplicate, cleaning with ethanol between readings and replacing the honey.

2.3.2.2 pH

The pH of samples was analysed by a Thermo Fischer Scientific Eutech pH700 pH meter. The instrument was calibrated at pH 7 and pH 4. Samples (1 g) were dissolved in water

(1.000 mL). Three measurements were taken from the same solution, cleaning with distilled water in between readings.

2.3.2.3 Conductivity

The conductivity of samples was analysed by a Thermo Fischer Scientific Eutech pH700 conductivity meter. The instrument was calibrated with a prepared solution of potassium chloride (1 M), with a conductivity of 1413 μS/cm. Samples (1 g) were dissolved in water (4.400 mL). Three measurements were taken from the same solution, cleaning with distilled water in between readings.

2.3.3 Total Phenolics

This method was adapted from Bobo-Garcia *et al.* (2015)[192]. It was a microplate method originally adapted from the Folin-Ciocalteu method[119] for total phenolic analysis.

2.3.3.1 Sample Preparation

For samples 1-5 and 4 mānuka samples, honey (500 mg ± 10 mg) was homogenised and dissolved in water (2.50 mL). For samples 6-27, honey (3 g ± 0.05 g) was homogenised and dissolved in water (15.00 mL). The masses of samples are given in **Appendix Table 2.** Folin-Ciocalteu (FC) reagent (10.00 mL) was added diluted with water (40.00 mL) to make 50 mL of 1:4 diluted FC reagent. Sodium carbonate (5 g) was added to water (50.00 mL) to make 50 mL of sodium carbonate (100 g/L).

Standard solutions of gallic acid (GA) were prepared. GA (200 mg) was added to a 200 mL volumetric flask and made up to the mark with methanol. From this 1000 mg/L stock solution, standards, were prepared to a volume of 10 mL ranging from 10 mg/L to 200 mg/L. Methanol was used as a 0 mg/L standard. The concentrations and volumes of solution are given in **Table 2.7**.

Table 2.7. Concentrations and volumes of GA in standard solutions

Volume of Stock Solution (mL)	Final Concentration (mg/L)	Volume of Methanol (mL)
2.00	200	8.00
1.75	175	8.25
1.50	150	8.50
1.25	125	8.75
1.00	100	9.00
0.75	75	9.25
0.50	50	9.50
0.25	25	9.75
0.10	10	9.90

A blank did not need to be prepared as the spectrometer, used for analysis, takes a blank reading automatically.

2.3.3.2 Assay Procedure

Each diluted extract (0.020 mL) blank and standard was added a separate well of a 96-well plate.

To each well, FC reagent (1:4 v/v, 0.100 mL) was added. The plate wells were mixed by shaking for 60 s. The plate was left for 4 min. Sodium carbonate (100 g/L, 0.075 mL) was added to each well. The plate was shaken for 60 s. The plate was left at room temperature for 2 hours. The absorbance was measured at 750 nm using a Thermo Fischer Scientific Multiskan GO microplate reader.

2.3.3.3 Data Work Up

To calculate the total phenolic content in samples, a standard calibration curve of gallic acid concentration (mg/L) versus absorbance at 750 nm was produced. The calibration curve was constrained through zero. The equation of the resulting calibration curve was rearranged to solve for the concentration of phenolics, as gallic acid equivalents, in the sample, shown in

Equation 2.4. The concentration of the analyte in the sample was calculated using **Equation 2.5**.

$$Concentration\ (\frac{mg}{L}\ GAE) = \frac{Absorbance}{Slope} \qquad \textbf{Equation 2.4}$$

$$Concentration\ analyte\ \left(\frac{mg}{kg}\ GAE\right) = \frac{Concentration\ \left(\frac{mg}{L}\ GAE\right)}{Concentration\ of\ honey\ extract\ (g/L)} \times 1000\ \frac{g}{kg} \qquad \textbf{Equation 2.5}$$

2.3.4 Elemental Analysis

Samples were prepared in-house and analysis was carried out by the University of Waikato as a commercial service. Sample preparation was based on Grainger *et al.* (2020)[131].

2.3.4.1 Sample Preparation

Honey (700 mg) was homogenised and dissolved in nitric acid (65%, 0.7 mL). The solution was left to pre-digest (1 hr). Water (0.7 mL) was added and heated (80 °C, 1 hour). The samples were cooled to room temperature (25 °C), and hydrogen peroxide was added (65%, 1.05 mL). The digestion procedure was repeated with hydrogen peroxide (65%, 1.05 mL). The samples were cooled to room temperature (25 °C) and diluted with water to a final volume of 20 mL. The samples were filtered (0.45 μm) before analysis by ICP-MS.

2.3.4.2 Instrumentation[f]

Elemental analysis was performed using an Agilent 8900 Triple Quadrupole Inductively Coupled Plasma Mass Spectrometer (Q-ICP-MS; Agilent Technologies, Santa Clara, California, USA). Sample introduction occurred via an SPS4 autosampler (Agilent Technologies, Santa Clara, California, USA) and PVC tubing (Pulse Instrumentation, Mequon, Wisconsin, USA). A 0.05-0.1 mL L^{-1} micromist U-Series nebuliser (Glass Expansion, Melbourne, Victoria, Australia) was attached to a quartz cyclonic spray chamber. A quartz torch with a 2.0 mm injector and nickel sampler and skimmer cones were used. A T-junction was used in-line for 1:20 dilution with internal standard (^{45}Sc, ^{103}Rh, ^{125}Te, ^{193}Ir). The ICP-MS was optimised to maximum sensitivity daily before analysis; oxides and doubly-charged species were monitored and considered acceptable if less than 2%. ICP-MS operating parameters are summarised in **Table 2.8**.

[f] Directly sourced from Grainger *et al.* (2020)[128]

Table 2.8. **Parameters and operating conditions used for determination of chemical elements by ICP-MS.**

Parameter	Value
Forward (reflected) power (watt) Ar Gas flows (L min^{-1}):	1570
Plasma	15
Auxiliary	0.90
Nebulizer (carrier gas)	0.95
Makeup/Dilution gas	0.10
Helium (mL min^{-1})	4.0
Sampling Depth (mm)	8.0
Detector mode	Pulse counting
Octoapole Bias (V)	−8.0
Energy discrimination	5.0
Number of replicates	3
Sweeps/replicate	40
Dwell time (s)	0.01-0.1

Data acquisition and processing were carried out by using MassHunter Workstation (version 4.5). A five-point calibration curve, consisting of concentrations between 0.1 and 500 μg kg^{-1} was prepared for all trace elements using stock standard IV-ICPMS-71-A. A separate calibration curve, consisting of concentrations between 100 and 10,000 μg kg^{-1} was prepared for major elements (Ca, Na, K, Fe) using single-element standards. The following isotopes were analysed using He mode: ^{10}B, ^{23}Na, ^{24}Mg, ^{27}Al, ^{39}K, ^{44}Ca, $^{31\text{-}47}P$, $^{34\text{-}50}S$, ^{51}V, ^{53}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{88}Sr, $^{75\text{-}91}As$, ^{111}Cd, ^{137}Ba, ^{205}Tl, ^{206}Pb, ^{238}U.

2.3.4.3 Data Work Up

The raw data was provided in parts per billion (ppb). The conversion to milligrams per kilogram (mg/kg) is shown in **Equation 2.6**.

$$Concentration\ \left(\frac{mg}{kg}\right) = \frac{Concentration\ (ppb) \times \frac{\mu g/L}{ppb}}{Concentration\ of\ honey\ extract\ (g/L)} \times \frac{1000\ g}{kg} \times \frac{mg}{1000\ \mu g}$$ **Equation 2.6**

2.3.5 Sugars

This method was adapted from Swears (2020)[193].

2.3.5.1 Sample Preparation

Honey (100 mg ± 10 mg) was homogenised and dissolved in water (5 mL). The masses of samples are given in **Appendix Table 3.** Standard solutions of fructose, glucose, sucrose, maltose, melizitose and maltotriose were prepared. The mass of sugar weighed out, volume of water added and resulting concentration are shown in **Table 2.9**. A blank of water was also prepared.

Table 2.9. Mass of sugar, volume of water added and resulting concentration.

Sugar	Mass (mg)	Water (mL)	Concentration (mg/mL)
Fructose 1	12.2	2	6.1
Fructose 2	36.8	3.5	10.5
Fructose 3	13.8	4.5	3.1
Fructose 4	4.3	4	1.1
Fructose 5	33.6	4	8.4
Glucose 1	13.2	2	6.6
Glucose 2	42.2	4	10.6
Glucose 3	15	5	3.0
Glucose 4	4.3	4	1.1
Glucose 5	33.7	4	8.4
Sucrose 1	13.1	2	6.6
Sucrose 2	26.9	2.5	10.8
Sucrose 3	14.3	4.5	3.2
Sucrose 4	5.3	4.5	1.2
Sucrose 5	33.5	4	8.4
Maltose 1	4.2	4	1.1
Maltose 2	13.1	5	2.6
Maltose 3	23.2	4.5	5.2
Maltose 4	30.1	4	7.5

Sugar	Mass (mg)	Water (mL)	Concentration (mg/mL)
Maltose 5	40.4	4	10.1
Melizitose 1	3.9	4	1.0
Melizitose 2	12.1	4	3.0
Melizitose 3	12.4	2	6.2
Melizitose 4	32.4	4	8.1
Melizitose 5	21.4	2	10.7
Maltotriose 1	4.2	4	1.1
Maltotriose 2	12.5	4	3.1
Maltotriose 3	13.5	2	6.8
Maltotriose 4	35.1	4	8.8
Maltotriose 5	22.6	2	11.3

2.3.5.2 Instrumentation

The HPLC system used for sugar analysis was a Waters Alliance system with a Waters 515 HPLC pump and 2414 Refractive index detector. The system was controlled using Empower™ 3 Chromatography Software.

Mobile phase was water eluted isocratically at 0.8 mL min^{-1} with a total run time of 30 min per injection. The column used for separation was a Shodex KS-801, 6 μm, 8mm x 300mm column in series with a Shodex KS-802, 6 μm, 8mm x 300mm and a KS-G guard column. The column temperature was 80 °C and the autosampler temperature was 4 °C. The injection volume was 20 μL. The retention times of analytes is shown in
Table 2.10.

Table 2.10. Retention times for sugar analytes

Analyte	Retention Time (min)
Fructose	23.0
Glucose	21.8
Disaccharides	19.1
Trisaccharides	17.5

2.3.5.3 Data Work Up

To calculate the carbohydrate content in each sample, standard calibration curves of each carbohydrate were constructed plotting concentration (mg/L) in the standard versus HPLC peak area. The calibration curves were constrained through zero. The equation of the resulting calibration curve was rearranged to solve for the concentration of each carbohydrate in the sample, shown in **Equation 2.7**. The concentration of the analyte in the sample was calculated using **Equation 2.8**.

$$Concentration\ (\frac{mg}{L}) = \frac{Absorbance}{Slope} \qquad \textbf{Equation 2.7}$$

$$Concentration\ analyte\ \left(\frac{mg}{kg}\right) = \frac{Concentration\ \left(\frac{mg}{L}\right)}{Concentration\ of\ honey\ extract\ (g/L)} \times 1000\frac{g}{kg} \qquad \textbf{Equation 2.8}$$

2.3.6 Antibacterial Activity

This method is sourced from Allen et al. (1991)[194].

Standard solutions of phenol at 1%, 2%, 3%, 4%, 5%, 6%, and 7% concentrations were previously prepared in the laboratory.

2.3.6.1 Bacterial Growth

The bacterial strain used for this analysis was *S. aureus*, strain MRSA4549 NZ isolate MRS05/115, AK3 MRSA. This Methicillin-resistant strain was provided from the −80 °C inventory at the University of Waikato. This strain is a Risk Group 2 organism and was handled directly in a Heraeus Herasafe Safety Cabinet Class 2 (Model HS12) in a Physical Containment Level 2 facility at the University of Waikato.

For round plates, nutrient agar powder (11.5 g) was dissolved in double distilled water (500 mL). For bioassay plates, nutrient agar powder (3.25 g) was dissolved in double distilled water (150 mL). Following autoclaving, agar was stored at 55 °C until pouring. Bacteria were streaked from the frozen stock onto a sterile round agar plate and incubated inverted overnight (37 °C). Bacteria were subcloned from the initial streak onto a second nutrient agar plate and incubated overnight (37 °C).

Tryptic soy broth powder (15 g) was added to double distilled water (500 mL) and autoclaved. A single colony was selected from the subcloned plate using an inoculation hoop and transferred to a culture tube containing tryptic soy broth (10 mL). A second culture tube of tryptic soy broth (10 mL) was used as a control. Both the inoculum and control were incubated and shaken at overnight (37 ^{o}C, 180 rpm).

The absorbance of the overnight inoculum was measured at 540 nm using a Helios Gamma UV-Visible spectrophotometer. The inoculum was diluted to an absorbance of 0.5. Diluted inoculum (100 µL) was added to nutrient agar (150 mL) and mixed. The inoculated agar was poured into a sterile bioassay plate and left to set overnight (2-5 ^{o}C). Similarly, diluted inoculum (100 µL) was added to nutrient agar (500 mL) and mixed. The inoculated agar was poured into round agar plates and left to set overnight (2-5 ^{o}C).

2.3.6.2 Bioassay Procedure

A screening bioassay was carried out using pure honey together with a 7% phenol standard to determine if any antibacterial activity was present in the honey samples. This was followed by a bioassay with serial dilutions of each honey sample were run alongside a range of phenol standards to determine minimum inhibitory concentrations and phenol equivalent antibacterial activity at various concentrations.

Five wells (6 mm in diameter) were equidistantly made into an inoculated round agar plate using a flame sterilised cork borer. The five wells consisted of a pure honey sample in duplicate alongside a 7% phenol standard. This was repeated for all samples. The plates were incubated overnight (18 hr, 37 ^{o}C). The zones of inhibition were observed and recorded as present or not present.

Honey (2 g) was dissolved in water (2 mL). A serial dilution was carried out by taking 1 mL of the previous dilution and diluting further with water (1 mL).

Sixty-four wells (6 mm in diameter) were equidistantly made into the bioassay plate using a flame sterilised cork borer. Seven wells were filled with 1-7% phenol standards, respectively. One well was filled with tryptic soy broth as a negative control. Five wells were filled with each serial dilution of a honey sample, in duplicate, respectively. This was completed in duplicate for five samples per plate. An antibiotic disc was added to show antibiotic

resistance. Due to the availability of the antibiotic discs, Streptomycin, Oxacillin and Penicillin G were used interchangeably. Two wells were filled with water. The samples were not randomly distributed on the plate. The plates were completed in duplicate so samples were analysed in quadruplicate. The plates were incubated overnight (18 hr, 37 ^{o}C). The zones of inhibition were observed and measured once per zone using digital calipers. The point of measurement was taken where there was uniform inhibition around the well.

2.3.6.3 Data Work Up

To calculate the antibacterial activity in sample, a calibration curve of the radius of zone of inhibition (mm) against phenol concentration (%v/v) was made. The general equation of the phenol standard curve was rearranged to solve for the antibacterial activity, as phenol equivalents, in the sample, shown in **Equation 2.9**.

$$Phenol\ Equivalence\ Antibacterial\ Activity = \frac{Zone\ of\ inhibition\ radius}{Slope} \qquad \textbf{Equation 2.9}$$

2.3.7 Antioxidant Capacity

This analysis was carried out using a Dojindo DPPH Antioxidant Assay kit based on Shimamura *et al.* (2014)[195].

2.3.7.1 Sample Preparation

For samples 1-5, honey (500 mg ± 10 mg) was homogenised and dissolved in water (2.50 mL). For samples 6-27, honey (3 g ± 0.05 g) was homogenised and dissolved in water (15.00 mL). The mass of samples is given in **Appendix Table 2.** 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was prepared by dissolving a provided amount of DPPH in ethanol (1 mL). Dissolution was assisted by sonication (60 s). The DPPH solution was made up to 10 mL with ethanol. Standard solutions of Trolox were prepared. Ethanol was used as a 0 mg/L standard. Trolox (1 mg) was dissolved in ethanol (1 mL) and vortexed. The Trolox solution was made up to 10 mL with ethanol. From this 100 µg/mL stock solution, standards were prepared to a volume of 1 mL ranging from 40 µg/mL to 80 µg/mL. The concentrations and volumes of solution are given in **Table 2.11**. Blanks of ethanol and water were also prepared.

Table 2.11. Concentrations and volumes of Trolox in standard solutions

Volume of Stock Solution (µL)	Final Concentration (µg/mL)	Volume of Ethanol (µL)
800	80	200
600	60	400
400	40	600

2.3.7.2 Assay Procedure

Each diluted honey extract and standard (20 µL) was added a separate well of a 96-well plate. Three blanks were added. For blank 1, water (20 µL) was added. For blank 2, water (20 µL) and ethanol (100 µL) were added. For blank 3, ethanol (120 µL) was added.

A provided assay buffer (80 µL) was added to every well. DPPH solution (100 µL) was added to every well except blanks 2 and 3. The plate was incubated in the dark (30 min, 25 ^{o}C). The absorbance was measured at 517 nm using a Thermo Fischer Scientific Multiskan GO microplate reader.

2.3.7.3 Data Work Up

To calculate the antioxidant capacity in sample, the DPPH inhibition ratio was calculated for the Trolox standards, shown in **Equation 2.10**. A calibration curve of DPPH inhibition (%) against Trolox concentration (µg/mL) was made. The DPPH inhibition ratio was calculated for the samples, shown in **Equation 2.11**. The equation of the Trolox standard curve was rearranged to solve for the antioxidant capacity as Trolox equivalents in sample, shown in **Equation 2.12**. The antioxidant capacity was converted to mgTE/kg in sample using **Equation 2.13.**

$$DPPH\ Inhibition\ ratio_{Trolox} = \frac{(Absorbance_{0\ \mu g/mL} - Absorbance_{Blank\ 3}) - (Absorbance_{standard} - Absorbance_{Blank\ 3})}{(Absorbance_{0\ \mu g/mL} - Absorbance_{Blank\ 3})} \times 100$$ **Equation 2.10** [195]

$$DPPH\ Inhibition\ ratio_{sample} = \frac{(Absorbance_{Blank\ 1} - Absorbance_{Blank\ 2}) - (Absorbance_{sample} - Absorbance_{Blank\ 2})}{(Absorbance_{Blank\ 1} - Absorbance_{Blank\ 2})} \times 100$$ **Equation 2.11** [195]

$$Trolox\ Equivalance\ Antioxidant\ Capacity = \frac{Inhibition\ ratio}{Slope}$$ **Equation 2.12** [195]

$$Antioxidant\ Capacity\ \left(\frac{mgTE}{kg}\right) = \frac{Antioxidant\ Capacity\ (\mu gTE/mL)}{Concentration\ of\ honey\ extract\ (g/mL)} \times \frac{mg}{1000\mu g} \times 1000\frac{g}{kg}$$ **Equation 2.13**

2.3.8 Subcontracted Methods

2.3.8.1 3 in 1

3 in 1 analyses were carried out by Hill Labs, using an internally developed method. The method description, which is provided with each set of customer results, is outlined below.

MGO, DHA and HMF were extracted from the honey matrix using aqueous extraction. The extract was derivatised and analysis was carried out using uHPLC/UV-Vis. The detection limit for HMF was 1.0 mg/kg. The detection limit for MGO and DHA was calculated based on the sample weight and was given as a range 10.0 – 11.0 mg/kg.

2.3.8.2 Diastase

Diastase analysis was carried out by Hill Labs, using an internally modified Phadebas® method. The official Phadebas® method, as described by the Harmonised Methods of the International Honey Commission[97], is outlined below.

Honey (1.00 g) was dissolved in a sodium acetate buffer (100 mL, 0.1 M, pH 5.2) and heated (40 ^{o}C, 5 min). A blank of sodium acetate buffer was also prepared. A Phadebas® tablet was added to each sample, mixed until dissolution and heated (40 ^{o}C, 30 min). Sodium hydroxide (0.5 M, 1 mL) was added and mixed. Samples were filtered before analysis by UV-Vis spectroscopy at 620 nm. The detection limit of this method is 2.0 DN.

2.3.8.3 C4 Detection

C-4 sugars analysis was carried out by Hill Labs, using an internally modified version of the AOAC 998.12 method.[149] This version is a screen analysis only and is not intended to meet export regulations. The official AOAC 998.12 method[149] is summarised below.

Protein is extracted from the honey. The stable carbon isotope ratios of the protein and the honey itself are measured using Isotope Ratio Mass Spectrometry. The difference between these values measures the C4 sugar content.

2.3.8.4 Tutin

Tutin analysis was carried out by Hill Labs, using an internally developed method under RLP Official Test 8.42. As samples were subcontracted in two batches, the method used for each analysis is slightly different. The method description provided with each set of customer results are outlined below.

For samples 1 – 15, tutin was extracted from the honey matrix using solvent extraction. The extract was cleaned up using solid phase extraction. Analysis was carried out using LC-MS/MS. The detection limit for this test was 0.010 mg/kg.

For samples 16 – 27, tutin was extracted from the honey matrix using solvent extraction. The extract was diluted, and analysis was carried out using LC-MS/MS. The detection limit for this test was 0.010 mg/kg.

2.3.8.5 Glyphosate

Glyphosate analysis was carried out by Hill Labs, using an internally developed method under RLP Official Test 8.47.1. The method description provided with customer results is outlined below.

Glyphosate and other pesticide residues were extracted from the honey matrix using aqueous extraction. Analysis was carried out using LC-MS/MS. The detection limit for this test was 0.010 mg/kg.

2.3.8.6 Pollen

Pollen analysis was carried out by the Massey University Palynology Laboratory. The method description provided with customer results is summarised below.

Pollen was extracted from honey. A *Lycopodium clavatum* standard was added and dissolved. Glycerine and safranin stain were added and mounted to a microscope slide for counting. Samples were counted using a light microscope until more than 500 grains of total nectar plant pollen had been counted. The pollen concentration was extrapolated from the ratio between the nectar plant pollen count and the pollen count of the standard.

2.3.9 Statistical Analysis

Statistical analysis was carried out using Microsoft® Excel® for Microsoft 365 MSO (Version 2307 Build 16.0.16626.20170) 64-bit. All graphs were produced in the same software. Correlation analysis was performed using the built in Analysis ToolPak. For triplicate measurements of pH, a sample standard deviation was calculated using **Equation 2.14**.

$$s = \sqrt{\frac{(\sum(x - \bar{x})^2}{n - 1}} \qquad \textbf{Equation 2.14}$$

A t-test was carried out to compare data between bioassay plates, using **Equation 2.15.**

$$t = \frac{(x_1 - x_2)}{\sqrt{\frac{(s_1)^2}{n_1} + \frac{(s_2)^2}{n_2}}} \qquad \textbf{Equation 2.15}$$

A p value less than 0.05 was considered statistically significant.

Chapter 3 - Results and Discussion

3.1 Pollen Analysis

3.1.1 Results

Samples were analysed externally by the Palynology Laboratory at Massey University. The pollen grains per 10 g of honey, proportion of pollen from endemic species, top five most common pollen from nectar-bearing species, non-nectar bearing pollen detected and pollen classification for Rēkohu honeys are given in **Table 3.1.**

Table 3.1. Pollen grains per 10 g of honey, proportion of pollen from species endemic to Rēkohu, top five most common pollen types from nectar-bearing species, non-nectar bearing pollen detected and pollen classification for Rēkohu honeys

Sample	Pollen grains per 10 g of honey and proportion of total pollen from endemic species	Relative proportions of top five nectar-bearing species	Non-nectar-bearing pollen present	Classification
1	Total: 35756 Endemic: 2.66%	• Clover (70.07%) • Trefoil (15.05%) • Gorse (8.06%) • Pouteretere (1.43%) • Brassica (1.43%)	• Grass	Clover Monofloral
2	Total: 26398 Endemic: 14.42%	• Gorse (38.72%) • Clover (23.67%) • Trefoil (14.86%) • Tarahina (14.50%) • Catsear (3.30%)	• Macrocarpa • Grass	Multifloral
3	Total: 102003 Endemic: 0%	• Clover (63.05%) • Trefoil (31.53%) • Catsear (1.53%) • Kawakawa (1.02%) • Pohuehue (0.85%)	• Grass • English Plantain	Clover Monofloral
4	Total: 31720 Endemic: 0.39%	• Clover (49.90%) • Trefoil (40.59%) • Gorse (4.95%) • Catsear (2.57%) • Tarahina (0.40%)	• Grass • Coprosma	Clover Monofloral

Sample	Pollen grains per 10 g of honey and proportion of total pollen from endemic species	Relative proportions of top five nectar-bearing species	Non-nectar-bearing pollen present	Classification
5	Total: 3616 Endemic: 15.55%	• Clover (26.51%) • Kopi (17.92%) • Brassica (16.09%) • Tarahina (15.72%) • Gorse (8.41%)	• Tutu • English Plantain • Macrocarpa	Multifloral
6	Total: 142888 Endemic: 0%	• Clover (65.18%) • Pohuehue (7.83%) • Trefoil (7.67%) • Catsear (4.95%) • Buttercup (3.99%)	• Grass • English Plantain	Clover Monofloral
7	Total: 74494 Endemic: 2.05%	• Clover (41.12%) • Trefoil (25.12%) • Pohuehue (7.36%) • Kopi (6.88%) • Gorse (4.32%)	• Grass • Tutu • English Plantain	Multifloral
8	Total: 89039 Endemic: 0.80%	• Clover (37.36%) • Trefoil (27.88%) • Pohuehue (10.21%) • Kopi (5.51%) • Gorse (4.54%)	• Tutu • Sedge • Grass	Multifloral
9	Total: 15118 Endemic: 1.57%	• Clover (68.62%) • Trefoil (11.93%) • Pohuehue (2.94%) • Catsear (2.75%) • Blackberry (2.57%)	• Tutu • Grass • Sedge	Clover Monofloral
10	Total: 137926 Endemic: 0%	• Clover (64.44%) • Trefoil (9.53%) • Pohuehue (5.83%) • Catsear (3.56%) • Buttercup (3.13%)	• Grass • English Plantain	Clover Monofloral
11	Total: 27992 Endemic: 5.33%	• Clover (67.95%) • Trefoil (16.31%) • Tarahina (4.99%) • Gorse (3.45%) • Catsear (1.73%)	• Grass • English Plantain • Tutu	Clover Monofloral

Sample	Pollen grains per 10 g of honey and proportion of total pollen from endemic species	Relative proportions of top five nectar-bearing species	Non-nectar-bearing pollen present	Classification
12	Total: 32249 Endemic: 0.48%	• Clover (52.03%) • Trefoil (38.21%) • Gorse (4.07%) • Catsear (2.44%) • Myrtle (0.65%)	• Grass • Macrocarpa • Coprosoma	Clover Monofloral
13	Total: 22163 Endemic: 11.09%	• Clover (70.27%) • Trefoil (14.20%) • Tarahina (10.98%) • Gorse (2.46%) • Flax (0.57%)	• Grass • English Plantain • Macrocarpa	Clover Monofloral
14	Total: 58435 Endemic: 0%	• Trefoil (55.22%) • Clover (37.54%) • Gorse (1.68%) • Brassica (1.68%) • Thistle (0.67%)	• Grass	Clover Monofloral
15	Total: 27060 Endemic: 12.43%	• Clover (65.09%) • Trefoil (16.04%) • Tarahina (12.08%) • Gorse (2.64%) • Flax (1.32%)	• Grass	Clover Monofloral
16	Total: 67390 Endemic: 0%	• Trefoil (68.13%) • Clover (26.10%) • Blackberry (3.34%) • Catsear (1.37%) • Buttercup (0.30%)	-	Multifloral
17	Total: 13554 Endemic: 21.34%	• Clover (31.04%) • Gorse (30.86%) • Pouteretere (20.99%) • Trefoil (10.76%) • Kopi (2.82%)	-	Clover Monofloral
18	Total: 12813 Endemic: 0.19%	• Clover (85.45%) • Trefoil (8.02%) • Catsear (3.92%) • Gorse (1.12%) • Flax (0.37%)	-	Clover Monofloral

Sample	Pollen grains per 10 g of honey and proportion of total pollen from endemic species	Relative proportions of top five nectar-bearing species	Non-nectar-bearing pollen present	Classification
19	Total: 49232 Endemic: 0.36%	• Clover (85.15%) • Trefoil (12.16%) • Catsear (1.43%) • Myrtle (0.36%) • Tarahina/ Pouteretere (unclassified) (0.36%)	• English Plantain	Clover Monofloral
20	Total: 32878 Endemic: 37.13%	• Clover (48.94%) • Pouteretere (37.32%) • Trefoil (6.87%) • Gorse (3.87%) • Catsear (1.41%)	• Grass • Pine	Clover Monofloral
21	Total: 134108 Endemic: 0%	• Clover (87.58%) • Trefoil (5.39%) • Catsear (4.90%) • Thistle (1.63%) • Gorse (0.33%)	• Grass • English Plantain	Clover Monofloral
22	Total: 18269 Endemic: 0.19%	• Clover (77.40%) • Trefoil (19.21%) • Gorse (1.32%) • Blackberry (1.13%) • Tarahina/ Pouteretere (unclassified) (0.19%)	• English Plantain	Clover Monofloral
23	Total: 47680 Endemic: 0.51%	• Clover (65.99%) • Trefoil (28.91%) • Catsear (2.38%) • Blackberry (1.19%) • Tarahina/ Pouteretere (unclassified) (0.51%)	• English Plantain	Clover Monofloral
24	Total: 66430 Endemic: 0%	• Clover (69.09%) • Trefoil (27.35%) • Blackberry (2.49%) • Myrtle (0.89%) • Catsear (0.18%)	-	Clover Monofloral
25	Total: 299984 Endemic: 0%	• Clover (54.89%) • Trefoil (43.78%) • Blackberry (0.83%) • Catsear (0.33%) • Gorse (0.17%)	-	Clover Monofloral

Sample	Pollen grains per 10 g of honey and proportion of total pollen from endemic species	Relative proportions of top five nectar-bearing species	Non-nectar-bearing pollen present	Classification
26	Total: 57697 Endemic: 24.81%	• Clover (49.07%) • Hoho (24.81%) • Trefoil (8.96%) • Blackberry (7.65%) • Catsear (7.28%)	-	Clover Monofloral
27	Total: 7383 Endemic: 6.88%	• Clover (33.14%) • Brassica (16.37%) • Kopi (15.78%) • Gorse (8.09%) • Poutereterе (5.33%)	• Tutu • Macrocarpa • Pine • English Plantain	Multifloral

3.1.2 Discussion

Apiculture New Zealand (2008)[196] introduced a classification system for honeys based on the pollen count and variety of pollen types present. A honey is classified as monofloral clover when 45% or more of the pollen count is clover (*Trifolium repens*). As shown in **Table 3.1**, twenty-one samples were considered monofloral clover. The rest were classified as multifloral as they contained a higher proportion of pollen from floral sources other than clover.

In all cases, the dominant pollen sources were the introduced species, clover and trefoil (*Lotus repens*), however, nineteen samples contained detectable levels of pollen from endemic species only found on Rēkohu; the highest level of endemic species pollen observed was 37.13% in sample 20. The most prevalent endemic species were tarahina (*Dracophyllum arboretum*) and poutereterе (*Leptecophylla robusta*); 15.72% in sample 5 and 37.32 in sample 20, respectively.

In a pollen analysis of 119 samples of New Zealand honey, Moar (1985) reported that most honeys from mainland New Zealand contains between 20,000 and 100,000 pollen grains per 10 g of honey. Varieties such as thyme honey contain less than 20,000 grains per 10 g and are considered under-represented, whereas those that contain more than 100,000 grains per 10 g, such as mānuka honey, are considered over-represented. Of the honey samples from Rēkohu, 5 samples contained more than 100,000 grains per 10 g, with sample 25 demonstrating the highest pollen count with 299,984 grains per 10 g. All these over-represented samples were

classified as monofloral clover honeys. Six samples contained less than 20,000 grains per 10 g, with sample 5 providing the lowest pollen count with 3,616 grains per 10 g. The two under-represented honeys with the lowest pollen counts were classified as multifloral. The rest were clover monofloral.

The pollen counts of Rēkohu honeys were generally lower than that observed in mainland NZ honeys. Introduced species such as clover, trefoil (*Lotus corniculatus*) and gorse (*Ulex europeaus*) produce pollen abundantly so honeys deriving from these plants usually have high pollen counts. In the Rēkohu honey samples analysed in this work, there were no significant correlations between the proportions of clover, trefoil, gorse, total introduced pollen and total endemic pollen and total pollen grains per 10 g of honey. This is shown in **Table 3.2**. Straining and filtering following harvest can reduce the pollen count. Pollen grains typically range from 10 - 60 µm and filtering can remove particles as small as 1 µm. Straining removes much larger impurities from the honey, but pollen remains in the honey. It is not known what straining/filtration systems are in place for Rēkohu honey as samples were sourced from many different beekeepers. Pollen degradation may also play a role in low pollen count in Rēkohu honey. Samples 5 and 27 had high levels of HMF, indicating long term storage, and low pollen counts. These samples also had the largest proportions of unknown/unidentifiable pollen which consists of degraded pollen and species for which pollen has not been catalogued.

Table 3.2. Correlation matrix of proportions of clover, trefoil, gorse and endemic pollen and total pollen grains per 10 g of honey in Rēkohu honey[g]

	Percentage of Pollen				
	Endemic Species	Clover	Gorse	Trefoil	Introduced Species
Total Pollen	-0.322	0.119	-0.301	0.268	0.235

[g] For all correlation matrices, the Pearson correlation coefficient was calculated, ranging from -1 to 1. Negative correlations are coloured red and positive correlations are coloured green, with the strength of the correlation visually shown by the intensity of the colour.

Rēkohu honey also contains pollen from non-nectar-bearing species. Samples 5 and 27 contained high levels of tutu pollen, indicating the presence of tutu near the hives from which they originate. This suggests an increased risk of tutin contamination in the honey.

Kopi (*Corynocarpus laevigatus*) nectar is known to be toxic to honeybees.[197] Samples 5 and 27 had significant levels of kopi pollen (17.92% and 15.78%, respectively). These samples were sourced from a site in Kaingaroa on Chatham Island, located near a large kopi forest. Samples 7 and 8 contained moderate levels of kopi pollen (6.88%, and 5.51%, respectively) and were sourced from Pitt Island, where kopi is known to grow. Minor amounts of kopi were detected in Sample 17 (2.82%). This sample originated from Chatham Island, but a specific location is not known, however the presence of kopi spread across the island would account for small levels of kopi in some samples. Detection of kopi pollen suggests that bees are visiting kopi for nectar collection, which may have significant impact on hive health due to the toxicity of its nectar. It is not known whether this impact is visible in hives on Rēkohu but levels of kopi pollen should be monitored to ensure bees are not adversely affected by the placement of their hives near kopi. Alternatively, bees may be visiting kopi specifically for its pollen but not its nectar. Kopi is known to contain nitropropanoyl glucopyranoses and nitropropanoic acid, which are both toxic to animals.[198] Detection of these compounds in the honey may determine if kopi poses a risk for bees on Rēkohu.

Native New Zealand plants did not evolve to rely on European honeybee pollination and the structure of the flower allows bees to collect nectar without collecting pollen. This means that pollen analysis of New Zealand honey does not give the full picture of the honey's origin. The detection of floral marker compounds may prove a more reliable method for originating honey, as seen with mānuka honey, which has four mandated marker compounds. (MPI). No work has been carried out to date to determine floral indicators of the endemic species found on Rēkohu.

3.2 3 in 1 assay for MGO, DHA and HMF

3.2.1 Results

The chromatograms of MS 1 are shown in **Figure 3.1** as an example. Chromatograms for MS 2-6 are given in **Appendix Figures 1-5**. The retention time and peak areas of standards and samples are given in **Appendix Tables 4** and **5**.

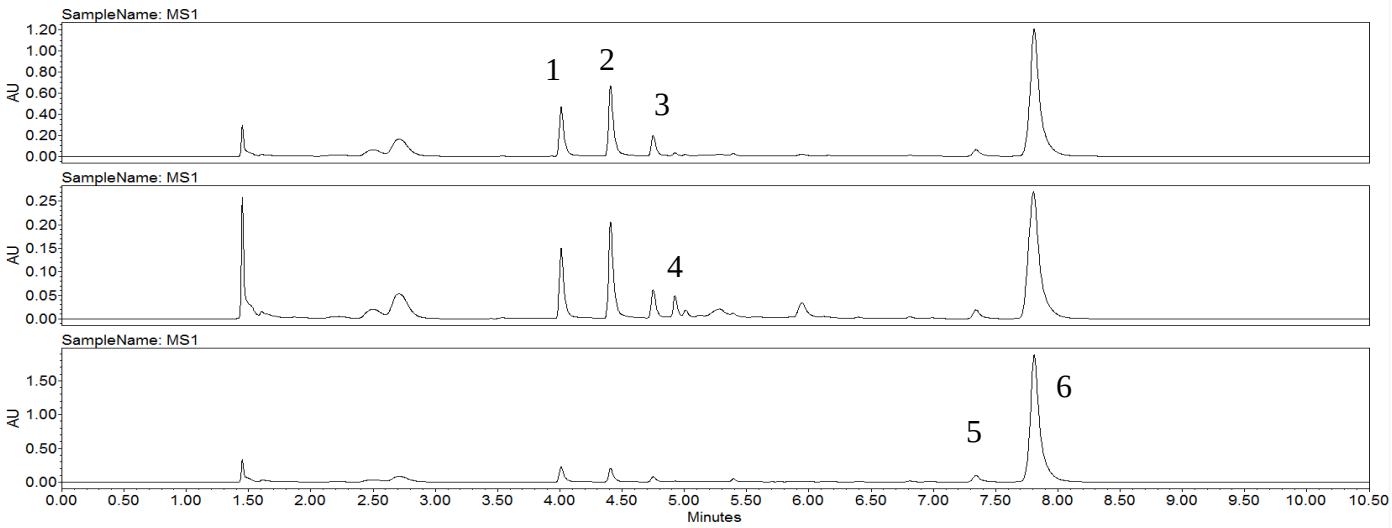


Figure 3.1. HPLC chromatograms of MS1 (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Peak 1 = DHA, retention time: 4.011 min; Peak 2 = PFBHA, retention time: 4.406 min; Peak 3 = HA, retention time: 4.749 min; Peak 4 = HMF, retention time: 4.924 min; Peak 5 = MGO 1, retention time: 7.341 min; Peak 6 = MGO 2, retention time: 7.810 min

The peak area of peaks 1,3-6 were quantified for all six mixed standards, and the corrected area ratio was calculated using **Equation 2.1** to produce standard curves for DHA, MGO and HMF, shown in **Figure 3.2 - Figure 3.4,** respectively.

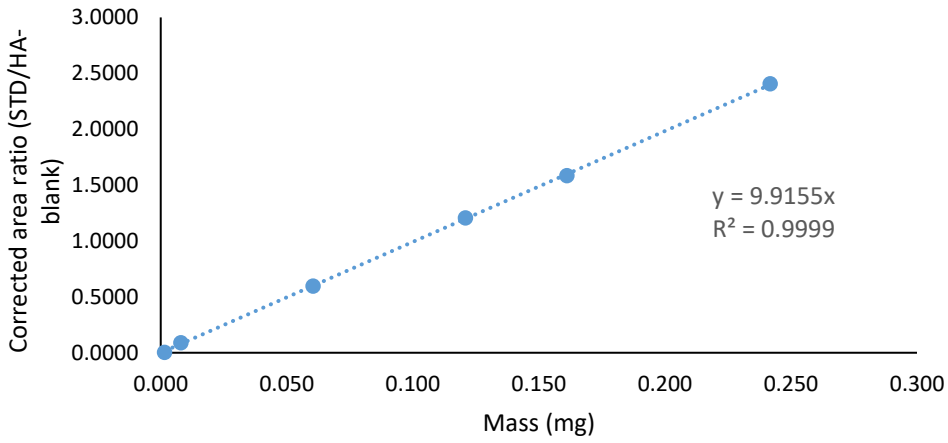


Figure 3.2. Calibration curve for DHA

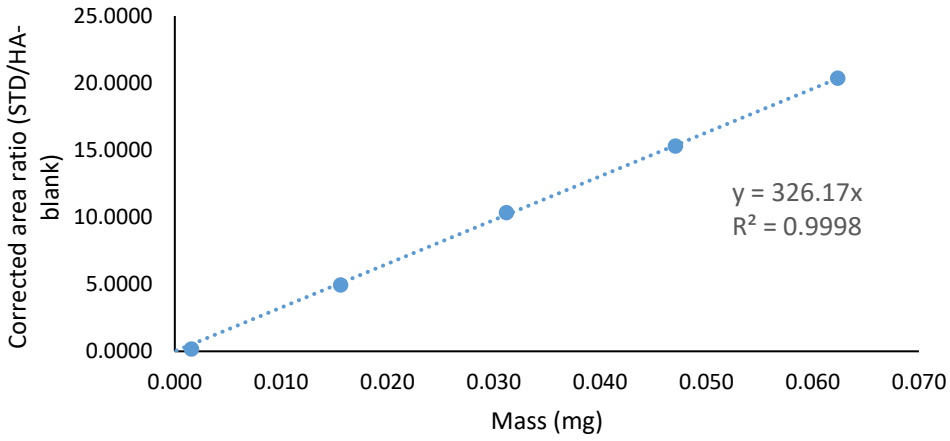


Figure 3.3. Calibration curve for MGO (Peaks 5 & 6 combined)

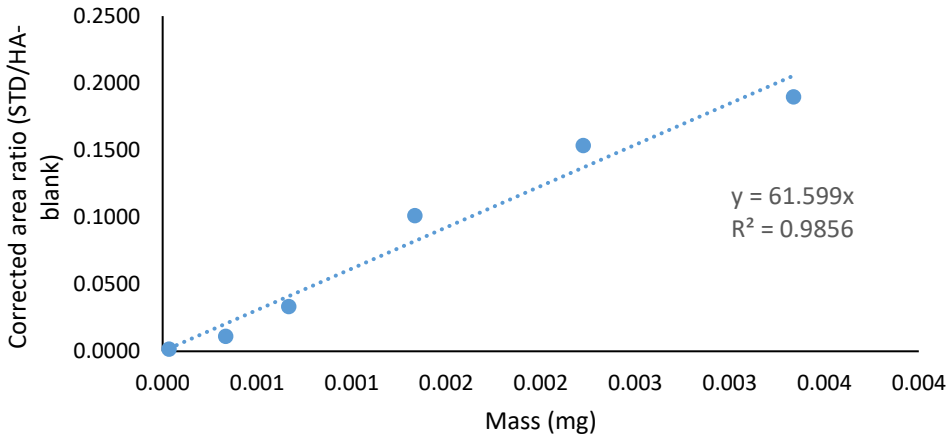


Figure 3.4. Calibration curve for HMF

The chromatograms of Sample 1A are shown in **Figure 3.5** as an example. The chromatograms of sample 1B, 1C and the triplicate assays of samples 2-27 are given in **Appendix Figures 6 - 25**. The retention time and peak areas of standards and samples are given in **Appendix Tables 4** and **5**.

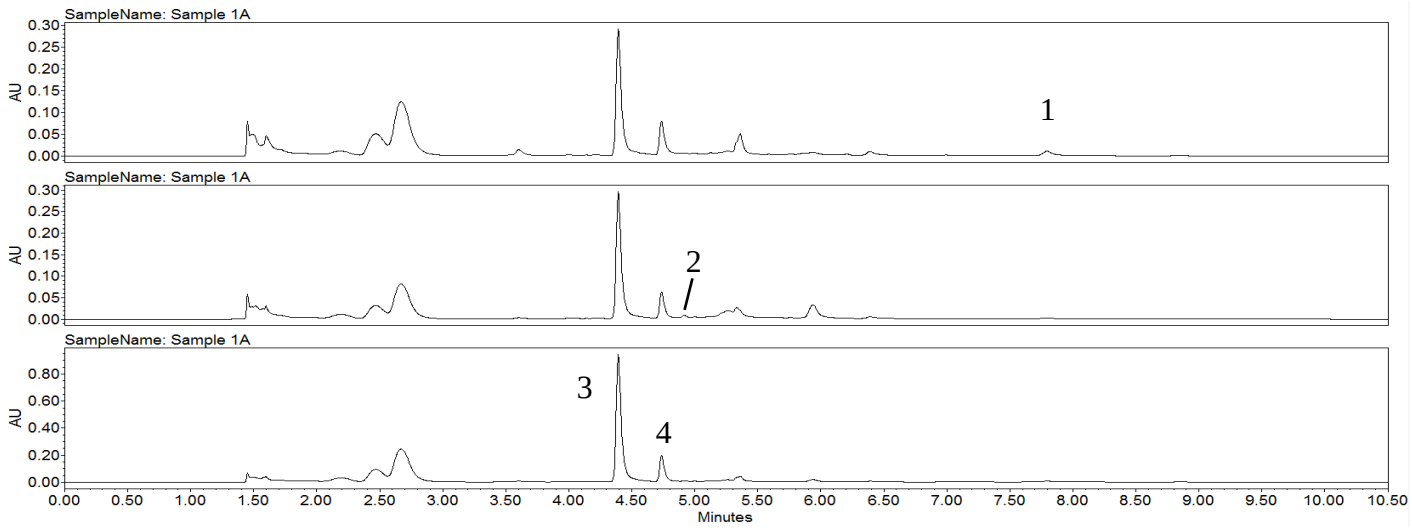


Figure 3.5. HPLC Chromatograms of sample 1A (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 244 nm (top), 273 nm (middle) and 260 nm (bottom)). Peak 1 = MGO 2, retention time: 7.796 min; Peak 2 = HMF, retention time: 4.915 min; Peak 3 = PFBHA, retention time: 4.391 min; Peak 4 = HA, retention time: 4.736 min

The peak area of peaks 1-4 were quantified, together with data for Sample 1B, 1C and the triplicate assays of Samples 1-7 and the corrected area ratio was calculated using **Equation 2.1**. Using the equation of the standard curves of DHA MGO and HMF, rearranged as shown in **Equation 2.2**. The concentrations of DHA, MGO and HMF in samples 1-7 of Rēkohu honey were calculated using **Equation 2.3**, and are given in **Table 3.3**, together with the results for samples 8-27, which were analysed commercially by Hill Labs.

For samples 8 to 27, which were analysed by Hill Labs, the non-peroxide activity was calculated as part of commercial analysis. This used a curve based on work by Adams *et al.* (2008)[89] comparing MGO concentration to non-peroxide activity in phenol equivalents. For samples 1-7, the curve used by Adams *et al.* (2008) was sourced directly from Merilyn Manley-Harris (Personal Communication)[199], shown in **Figure 3.6**.

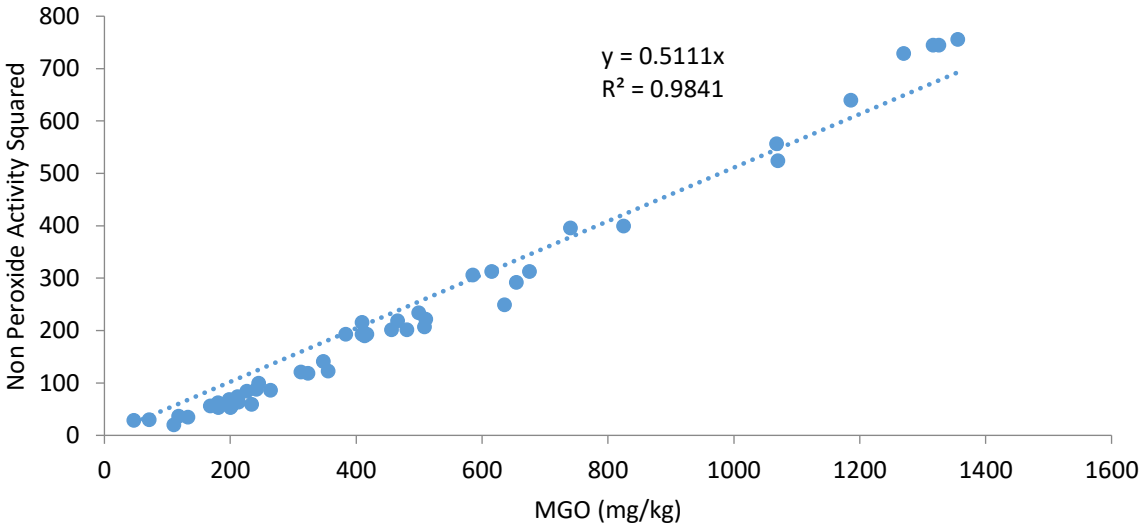


Figure 3.6. Non-peroxide activity squared for concentrations of MGO[89, 199]

Using the equation of the curve in **Figure 3.6**, the non-peroxide activity was calculated for samples 1-7 and shown in **Table 3.3**, together with the values for samples 8-27 supplied by Hill Labs. For samples 1-7, using the AHS blank, the LOD was determined to be 10.80 mg/kg for DHA, 1.85 mg/kg for HMF and 2.38 mg/kg.

Table 3.3. DHA, MGO and HMF concentrations and corresponding non-peroxide activity in 27 samples of Rēkohu honey, a low mānuka QC (100+ MGO) and a high mānuka QC (900+ MGO). Samples 1-7 and QC samples were assayed in-house; the remaining samples were assayed by Hill Labs.

Sample Number	DHA (mg/kg)	MGO (mg/kg)	HMF (mg/kg)	Non-Peroxide Activity Squared (% phenol equivalents^2)
1	12.9 ± 4.4	3.4 ± 0.6	7.4 ± 0.1	1.8 ± 0.3
2	30.7 ± 0.7	7.7 ± 0.5	<LOD	3.9 ± 0.3
3	11.4 ± 13.5	3.1 ± 0.7	8.5 ± 1.6	1.6 ± 0.4
4	13.4 ± 2.3	2.6 ± 0.7	3.7 ± 0.1	1.3 ± 0.4

Sample Number	DHA (mg/kg)	MGO (mg/kg)	HMF (mg/kg)	Non-Peroxide Activity Squared (% phenol equivalents^2)
5	<LOD	4.1 ± 0.2	30.9 ± 1.1	2.1 ± 0.1
6	<LOD	<LOD	<LOD	<LOD
7	<LOD	<LOD	<LOD	<LOD
8	<10	<5	13.5	<1.0
9	34	<5	20.1	<1.0
10	20	<5	7.0	<1.0
11	14	<6	17.2	<1.0
12	18	<6	19.3	<1.0
13	<10	<5	22.2	<1.0
14	<11	<6	9.5	<1.0
15	<11	<6	25.1	<1.0
16	<10	<5	3.2	<1.0
17	<10	16	3.7	3.6
18	15	<6	8.5	<1.0
19	<11	<6	3.2	<1.0
20	10	15	2.2	3.2
21	<11	<6	1.3	<1.0
22	<11	<6	1.3	<1.0
23	<11	<6	3.4	<1.0
24	<10	<5	<1.0	<1.0

Sample Number	DHA (mg/kg)	MGO (mg/kg)	HMF (mg/kg)	Non-Peroxide Activity Squared (% phenol equivalents[2])
25	<11	<6	1.3	<1.0
26	<11	<6	4.4	<1.0
27	<11	6	31.6	1.2
Low Mānuka QC	<LOD	89.8	41.9	45.9
High Mānuka QC	2552.3	930.3	31.8	475.5

[k] Samples 1-7 and QC samples were assayed in-house; the remaining samples were assayed by Hill Labs.

3.2.2 Discussion

Neither DHA nor MGO are present in Rēkohu honey in the high quantities observed in mānuka honey. Ten samples had concentrations of DHA above the LOD and eight had concentrations of MGO above the LOD. The highest DHA concentration was 34 mg/kg in sample 9 and the highest MGO concentration was 16 mg/kg in sample 17. In contrast to this, the known low mānuka QC sample had an MGO concentration of 89.8 mg/kg, suggesting that anything less than this is not significant. The observation of low MGO and DHA can be explained by the lack of mānuka trees present on the islands. No mānuka pollen was detected in any samples and there is no record of mānuka being present on the islands, native or naturalised.[3] Adams *et al.* (2008)[89] observed a correlation between MGO to the non-peroxide antibacterial activity of mānuka honey. Alongside low MGO values, Rēkohu honey demonstrated low non-peroxide activity. As this is calculated based on the correlation between MGO and non-peroxide activity, this only suggests that any non-peroxide activity of Rēkohu honey is not attributable to MGO. There is a possibility that some of the antibacterial activity, discussed in **Section 3.11**, is non-peroxide in origin.

The quality of honey is indicated by the levels of HMF present in the samples. Twenty-six samples demonstrated HMF levels above the LOD. Eight samples contained HMF levels greater than 10 mg/kg with the highest HMF concentration being 31.6 mg/kg in sample 27.

While no samples exceeded the Codex recommended limit of 40 mg/kg of HMF[10], samples 5 and 27 were noticeably high in HMF. These samples are collected from the same location and are thought to have been harvested some time ago, although a harvest date was not provided with the samples. As HMF accumulates naturally over time, it is assumed that the high levels of HMF in these samples is the result of ageing. Samples 13 and 15 were from the same location and both exhibited high HMF. Samples 18 – 25 were collected in Feb 2023 as part of this study, and so these samples had levels of HMF below 10 mg/kg. It is clear that the range of harvest times for samples in this study impacted the apparent quality of Rēkohu honey. Storage practices on Rēkohu should be monitored to ensure honey is fresh with low levels of HMF. As apiculture grows on the island and honey processing is implemented, heating processes should be monitored to ensure that HMF levels do not inadvertently increase during processing.

3.3 Physicochemistry

3.3.1 Moisture Results

The moisture contents of Rēkohu honeys are given in **Figure 3.7**.

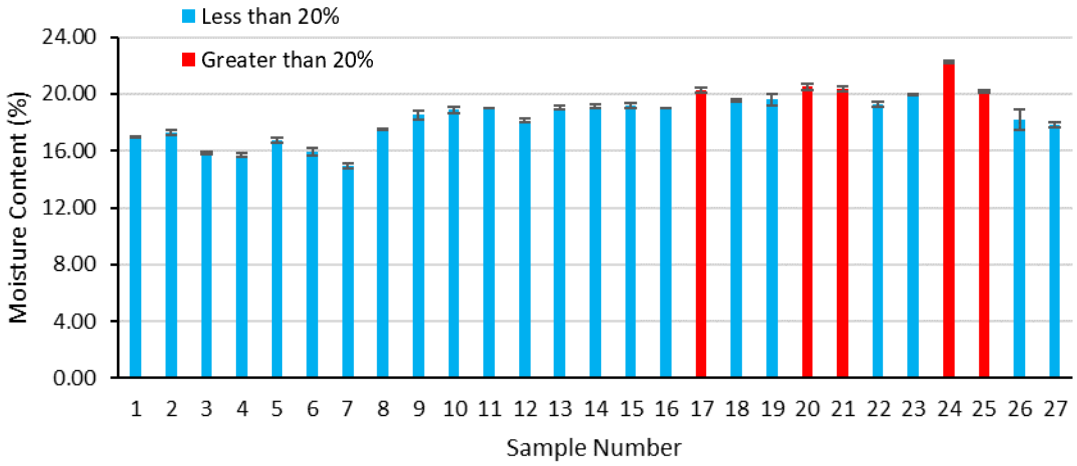


Figure 3.7. Moisture contents of 27 samples of Rēkohu honey

3.3.2 Discussion

Moisture levels in Rēkohu honey ranged from 14.9% to 22.3%, with 5 samples demonstrating moisture levels above the recommended 20% limit set by the Codex Alimentarius.[10] These samples are classed as at risk of fermentation and would not be fit for sale. It is unknown whether these honeys had begun to ferment. Moisture content can be reduced by drying the

honey immediately following harvest. This process involves heating the honey and while it reduces the moisture content, it risks increasing HMF content and destroying the diastase enzyme, thus reducing the quality of the honey. Alternatively, the use of dehumidifiers removes moisture from the air, drawing moisture out of the honey without heat.[200]

Langford *et al.* (2012)[14] reported that for 9 varieties of New Zealand honey, moisture ranged from 15.3% to 17.4%. For Rēkohu honey, 20 samples had moisture levels above this range, with sample 24 having the highest at 22.27%. The origin of these honeys varies greatly so the increase in moisture is the result of an external factor. Most commonly, high moisture content can be linked to prematurely harvesting honey. In a beehive frame, honey cells are capped by the bees when a suitable moisture content is reached. If honey is collected before this process occurs, its moisture content may be greater than expected as it is still an uncured nectar.[201] In addition to this, honey is hygroscopic and will absorb water from the atmosphere if collected and stored incorrectly. All of these factors are important to monitor to ensure moisture content does not reach the 20% limit at which fermentation can begin to take place.

3.3.3 pH Results

The pH measurements of Rēkohu honeys are given in **Figure 3.8**.

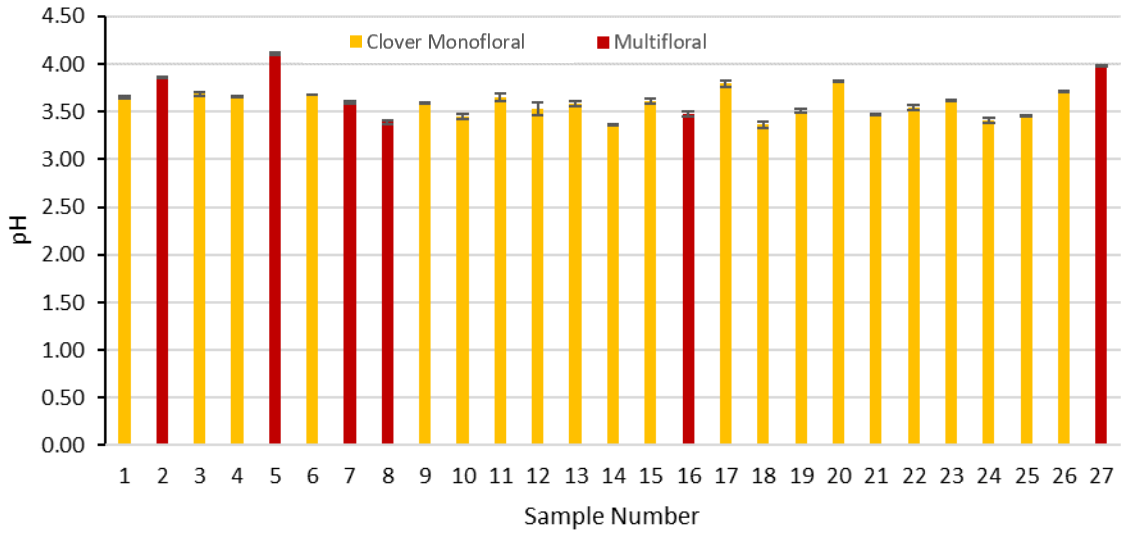


Figure 3.8. pH of 27 samples of Rēkohu honey

3.3.4 Discussion

pH levels in Rēkohu honey ranged from 3.36 to 4.11. There is no strict pH regulation for honey but mainland New Zealand monofloral honeys have been shown to fall between 3.57 for viper's bugloss honeys and 4.78 for kamahi honeys. Monofloral clover honey from mainland New Zealand was reported to have a pH of 3.67[11], compared to the pH of Rēkohu monofloral clover honey which ranged from 3.36 to 3.82 and averaged 3.58. The pH of Rēkohu multifloral honey ranged from 3.39 to 4.11 and averaged 3.74. Over time, pH increases and depending on floral origin, the pH of honey can be an indicator of its quality. Samples 5 and 27 exhibited the highest pH values of 4.11 and 3.99, respectively These samples also contained the highest levels of HMF (30.9 and 31.6, respectively). Excluding these samples which demonstrate degradation over time, the pH range of Rēkohu multifloral honey was 3.39 to 3.86 with an average of 3.58, which is very similar to monofloral clover on Rēkohu.

3.3.5 Conductivity Results

The conductivities of Rēkohu honeys are given in **Figure 3.9**.

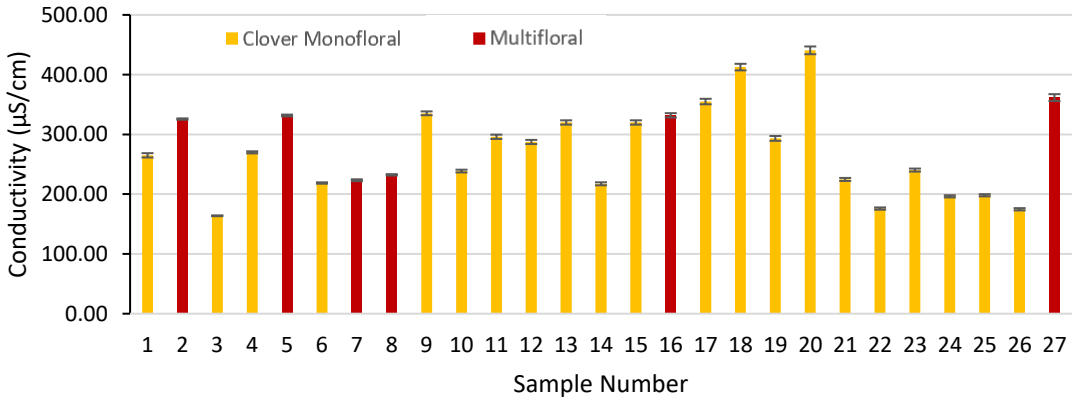


Figure 3.9. Conductivities of 27 samples of Rēkohu honey

3.3.6 Discussion

Conductivity in Rēkohu honey ranged from 164 µS/cm to 441 µS/cm. The Codex Alimentarius states that conductivity in floral honeys should be below 800 µS/cm. Mainland New Zealand honeys have been shown to fall between 170 µS/cm for viper's bugloss honey and 680 µS/cm for kamahi honeys. Mainland monofloral clover honey was reported to have a

conductivity of 260 µS/cm[11], compared to the conductivity of Rēkohu monofloral clover honey which ranged from 164 µS/cm to 440 µS/cm and averaged 269 µS/cm. The conductivity of Rēkohu multifloral honeys ranged from 223 µS/cm to 362 µS/cm and averaged 301 µS/cm.

Balos *et al.* (2018)[202] indicated that electrical conductivity in honey stems from minerals, organic acids, and proteins. Total phenolic content includes phenolic acids, which are also organic acids, so it is expected that there will be a correlation between total phenolics and conductivity. The correlation matrix of pH, total element concentration, diastase, total phenolics and colour for conductivity in Rēkohu honey is shown in **Table 3.4**. There was no significant correlation between diastase and conductivity. There was a moderate positive correlation between pH and conductivity, shown in **Figure 3.10** and total element concentration and conductivity, shown in **Figure 3.11**. However, there was a high positive correlation between total phenolics and conductivity, shown in **Figure 3.12** and colour and conductivity, shown in **Figure 3.13**. Colour in honey stems from total phenolic content and total element concentration so it is expected that colour is correlated with conductivity. The absence of an overwhelming correlation and presence of outliers suggests that these multiple factors contribute to the total conductivity of honey.

Table 3.4. Correlation matrix of pH, total element concentration, diastase, total phenolics, colour and conductivity in Rēkohu honey

	pH	Total Elements (mg/kg)	Diastase (DN)	Total Phenolics (mgGAE/kg)	Colour (mmPfund)
Conductivity (µS/cm)	0.360	0.421	0.047	0.706	0.714

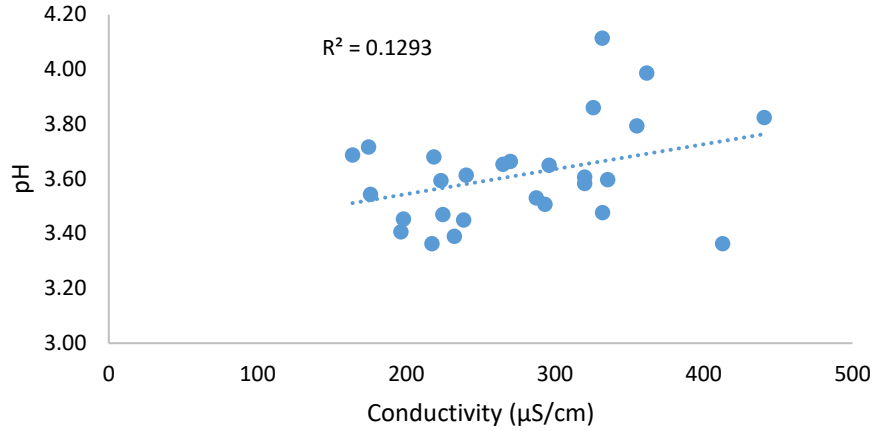


Figure 3.10. Correlation between pH and conductivity in Rēkohu honey

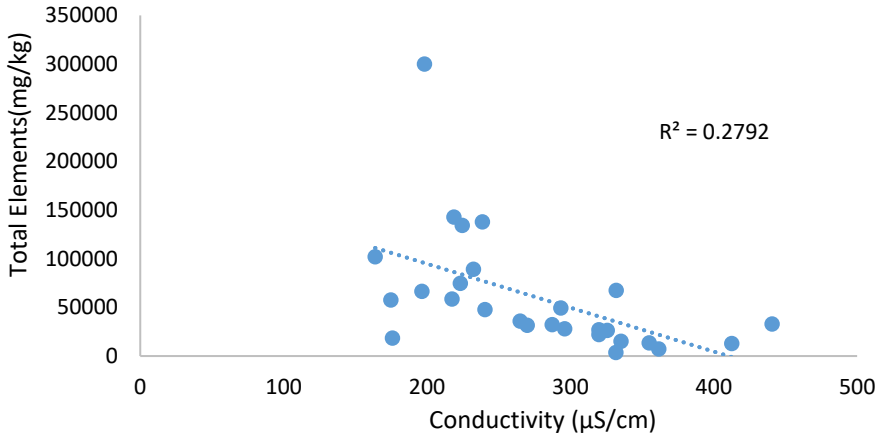


Figure 3.11. Correlation between total element concentration and conductivity in Rēkohu honey

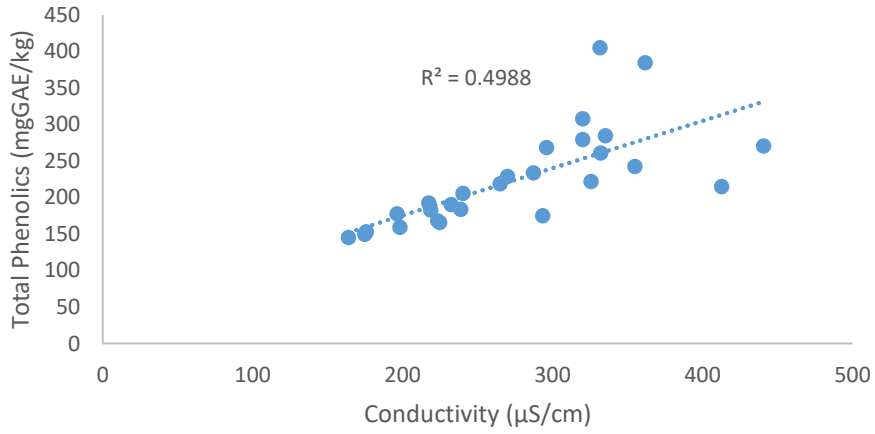


Figure 3.12. Correlation between total phenolic content and conductivity in Rēkohu honey

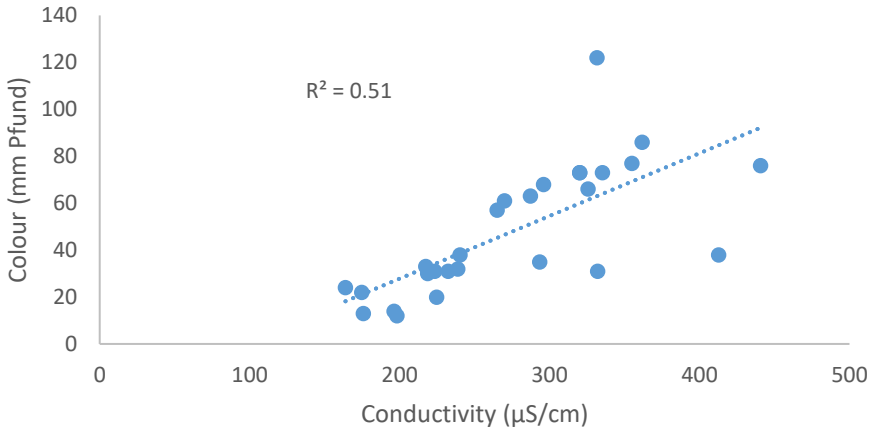


Figure 3.13. Correlation between colour and conductivity in Rēkohu honey

3.3.7 Colour Results

The colour measurements of Rēkohu honeys are given in **Figure 3.14**.

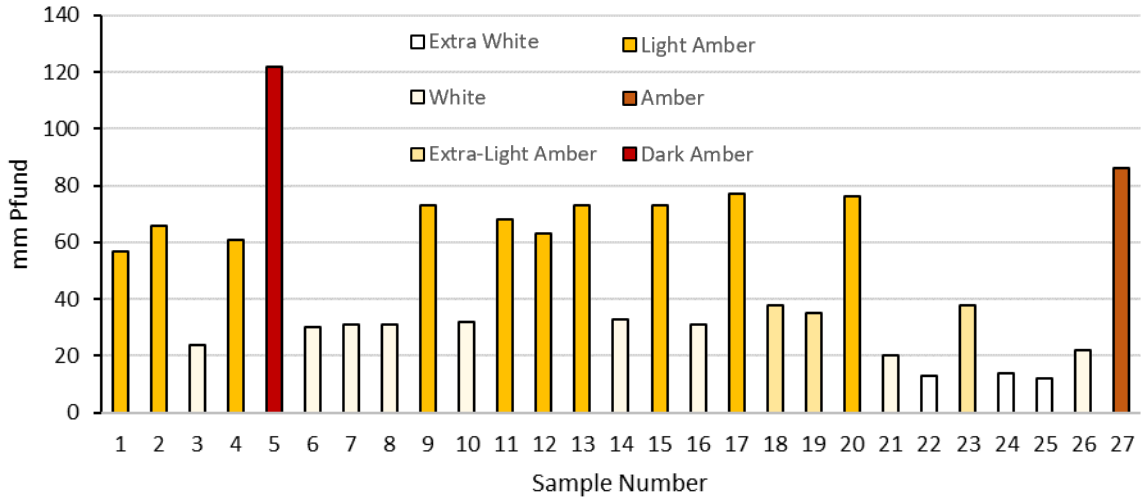


Figure 3.14. Colour of 27 samples of Rēkohu honey, classified by colour classification on the Pfund scale

3.3.8 Discussion

Colour analysis measurements of Rēkohu honey ranged between 12 mm Pfund (Extra White) and 122 mm Pfund (Dark Amber). Three samples were classified as extra white. These three samples were monofloral clover sourced from Go Wild Apiary on Chatham Island and their colour is similar to mainland thistle honey.[130] Nine samples were classified as white, similar to mainland monofloral clover honeys.[14, 130] Three of these samples were classified as

multifloral but were dominated by clover and trefoil pollen, resulting in a lighter colour typical of clover honeys.[14, 130] Three samples were classified as extra light amber and all were monofloral clover sourced from Go Wild Apiary on Chatham Island and their colour is similar to kamahi honey. [14, 130] The most common colour was Light Amber, with ten samples in this category. All but one of these samples were monofloral clover. All were sourced from Chatham Island and their colour is similar to thyme honey. [14, 130] Sample 27 was classified as amber, similar to mainland mānuka honey and sample 5 was classified as dark amber, similar to Spanish chestnut and honeydew honeys.[203] For the six samples from Pitt Island, their colour was only classified into extra white and white.

Al Farsi *et al.* (2018)[204] reported that colour is correlated with a number of factors including ageing indicators such as HMF, phenolic content and antioxidant capacity. Alqarni *et al.* (2014)[205] found that the total element content also impacts the colour of honey. The correlation matrix of HMF, total phenolic content, and total element content for colour in Rēkohu honey is shown in **Table 3.5**. There was a moderate positive correlation between HMF concentration and colour, shown in **Figure 3.15**; and total element content and colour, shown in **Figure 3.16**. There was a high positive correlation between the total phenolic content and colour, shown in **Figure 3.17**. These observations indicate a direct impact on colour by constituents in the honey.

Table 3.5. Correlation matrix of HMF concentration, total phenolic content, total element concentration and colour in Rēkohu honey

	HMF (mg/kg)	Total Elements (mg/kg)	Total Phenolics (mgGAE/kg)
Colour (mm Pfund)	0.711	0.710	0.903

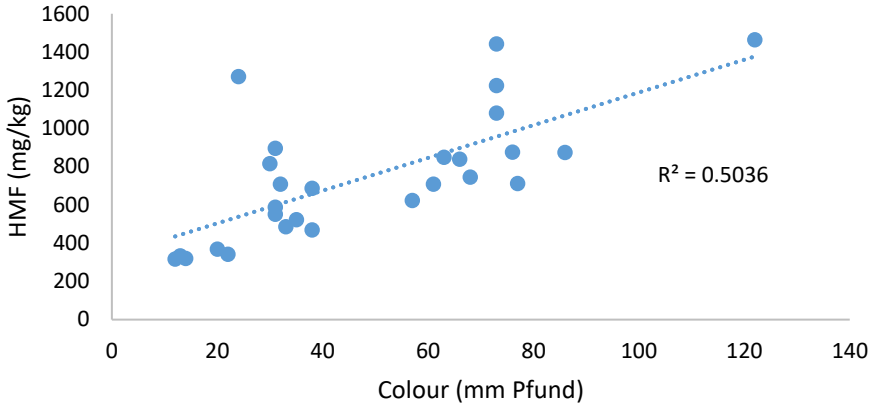


Figure 3.15. Correlation between HMF concentration and colour in Rēkohu honey

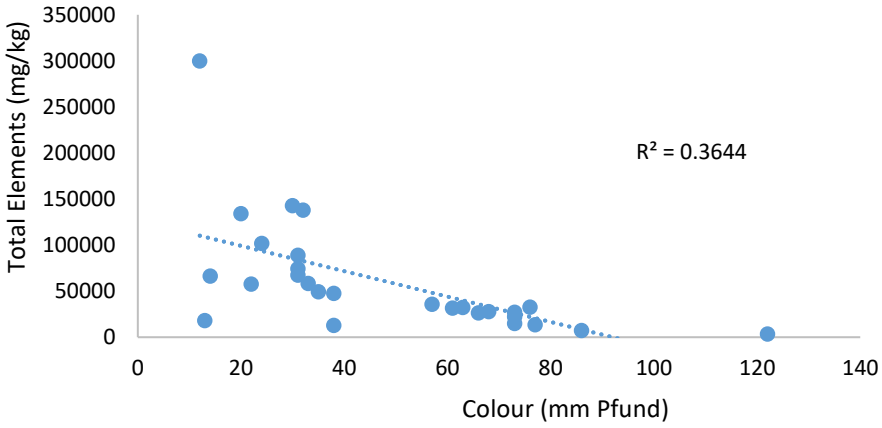


Figure 3.16. Correlation between total elements concentration and colour in Rēkohu honey

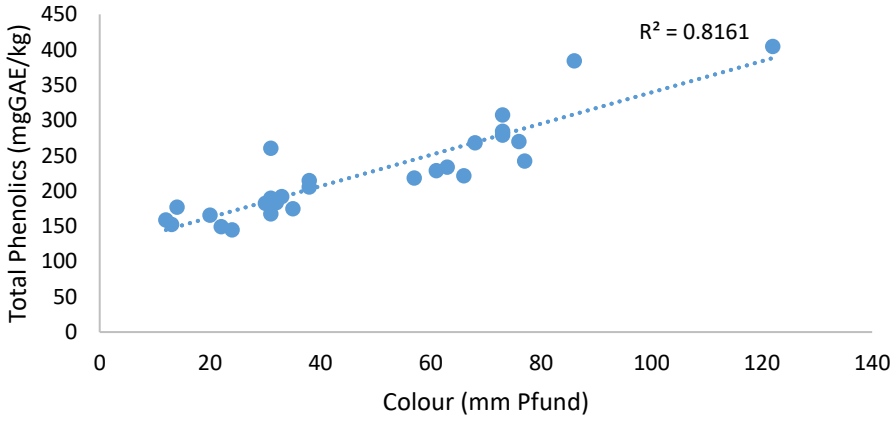


Figure 3.17. Correlation between total phenolic content and colour in Rēkohu honey

It was also observed that correlations occurred with other factors that are not directly contributing to colour. The correlation matrix of pH, antioxidant capacity, conductivity, and

colour in Rēkohu honey is shown in **Table 3.6**. There was a moderate positive correlation between pH concentration and colour, shown in **Figure 3.18**; antioxidant capacity and colour, shown in **Figure 3.19**; and conductivity and colour, shown in **Figure 3.13**. An increased pH is the side effect of an aged honey, which darkens due to increased levels of Maillard products. Antioxidant capacity is correlated with the total phenolic content, and conductivity is influenced by total element concentration.

Table 3.6. Correlation matrix of pH, antioxidant capacity, conductivity and colour in Rēkohu honey

	pH	Antioxidant Capacity (mg TE/kg)	Conductivity (µS/cm)
Colour (mm Pfund)	0.734	0.760	0.714

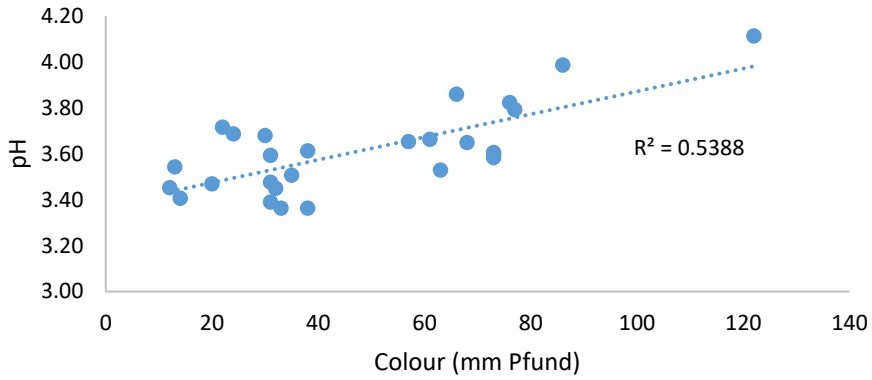


Figure 3.18. Correlation between pH and colour in Rēkohu honey

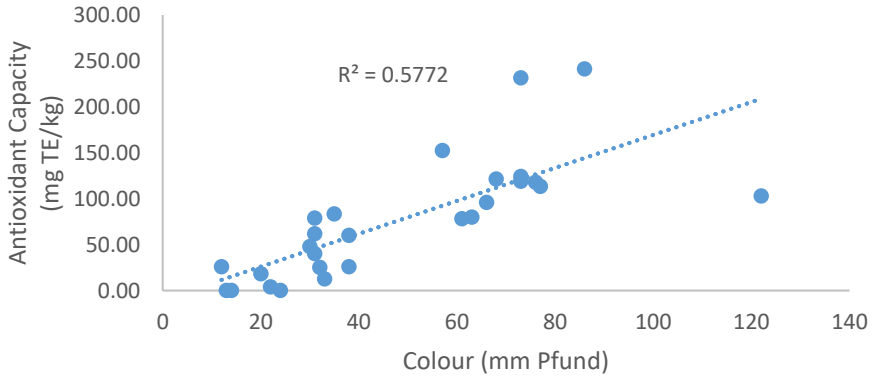


Figure 3.19. Correlation between antioxidant capacity and colour in Rēkohu honey

3.4 Total Phenolics

3.4.1 Results

The absorbance of gallic acid reacted with Folin-Ciolcalteu reagent was measured and a standard curve was created for the total phenolic content of gallic acid solutions, shown in **Figure 3.20**. The average absorbance for each sample, standard and blank are given in **Appendix Table 6.**

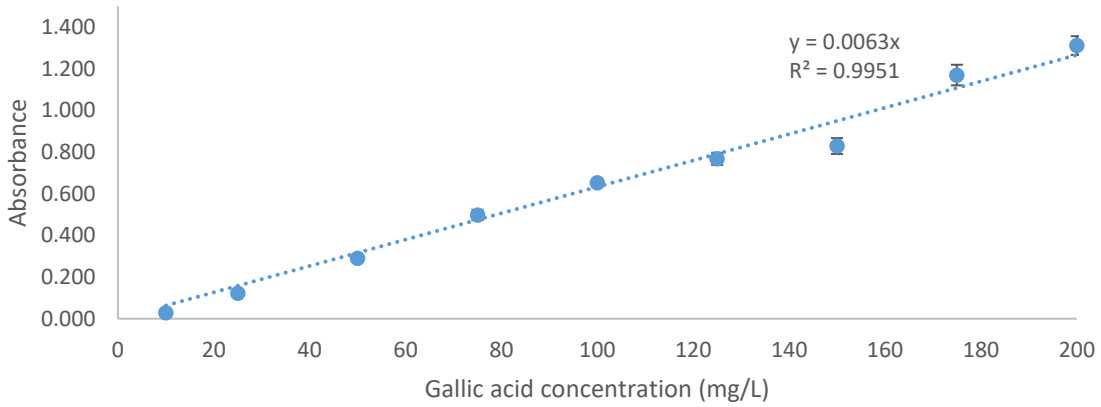


Figure 3.20. Calibration curve of total phenolic content in gallic acid

The total phenolic contents of Rēkohu honeys was calculated using **Equation 2.4** and **Equation 2.5** and are shown in **Figure 3.21**. The total phenolic contents of mānuka honey samples are included for comparison.

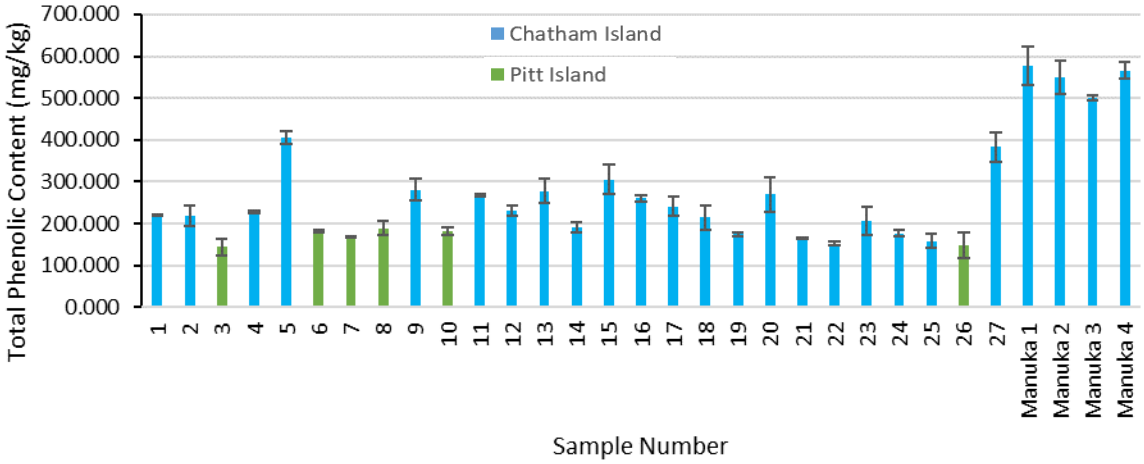


Figure 3.21. Total phenolic contents for 27 samples of Rēkohu honey and 4 mānuka honeys. Mānuka 1 is a 100+ MGO honey; Mānuka 2 is a 300+ MGO honey; Mānuka 3 is a 600+ MGO honey; Mānuka 4 is a 900+ MGO honey.

3.4.2 Discussion

The total phenolic content of Rēkohu honey ranged from 145 mg GAE/kg to 414 mg GAE/kg. This is lower than mainland mānuka honey, which ranged from 506 mg GAE/kg to 579 mg GAE/kg. Samples 5 and 27 demonstrated the highest phenolic contents of the Rēkohu honeys, with 405 mg GAE/kg and 384 mg GAE/kg, respectively. These two samples differed from other samples as they contained significant proportions of kopi pollen, and it is possible that this high phenolic content stems from the kopi tree. As kopi pollen is toxic to bees, mainland beekeepers place their hives away from kopi trees[197] and there have been no reports of kopi honey nor its phenolic content. In addition to this, there were moderate positive correlations between total phenolics and the percentage of pollen from the indigenous species; cabbage tree, kopi and sand daphne, shown in **Table 3.7.**

Table 3.7. Correlation matrix of percentage of cabbage tree pollen, percentage of kopi pollen, percentage of sand daphne pollen and total phenolic content in Rēkohu honey

	% Pollen		
	Cabbage Tree	Kopi	Sand Daphne
Total Phenolics (mgGAE/kg)	0.543	0.647	0.739

To identify possible floral sources of phenolic compounds, the correlation matrix of percentage of introduced pollen, percentage of endemic species pollen, percentage of New Zealand indigenous species pollen, total pollen and total phenolic content in Rēkohu honey is shown in **Table 3.8**.

Table 3.8. Correlation matrix of percentage of introduced pollen, percentage of endemic pollen, percentage of indigenous pollen and total phenolic content in Rēkohu honey

	% Pollen			Total Pollen (grains per 100 g of honey)
	Introduced Species	Endemic Species	Indigenous Species	
Total Phenolics (mgGAE/kg)	-0.572	0.355	0.394	-0.528

There was a moderate positive correlation between the percentage of endemic species pollen and total phenolics, shown in **Figure 3.22** and percentage of New Zealand indigenous species pollen and total phenolics, shown in **Figure 3.23**. There was also a moderate negative correlation between the percentage of introduced species pollen and total phenolics, shown in **Figure 3.24** and total pollen and total phenolics, shown in **Figure 3.25.** As introduced species such as clover produce more pollen than endemic and New Zealand indigenous species, the total pollen count is also representative of the presence of introduced species. A negative correlation to introduced pollen and a positive correlation to endemic and indigenous pollen indicates that the phenolic content of Rēkohu honey is most likely derived from endemic or New Zealand indigenous species. Of the five endemic species, there was a moderate positive correlation between total phenolics and percentage of tarahina pollen, with a correlation coefficient of 0.608, shown in **Figure 3.26.**

Supporting this observation, the total phenolic content of six samples from Pitt Island ranged from 145 mg GAE/kg to 190 mg GAE/kg, which is very similar to the phenolic content of mainland New Zealand monofloral clover honeys, shown to have a phenolic content of 186 mg GAE/kg. The pollen analysis of these samples showed that the endemic pollen percentage ranged from 0% to 2.05%. The exception was sample 26 with 24.81%. This sample contained hoho (*Pseudopanax chathamicus*) as the only endemic species. This sample demonstrated a total phenolic content of 149 mg GAE/kg, which is lower than mainland monofloral clover. This suggests that hoho does not provide a significant contribution to the total phenolic content and the higher phenolic content seen in samples with high endemic pollen must stem from another species.

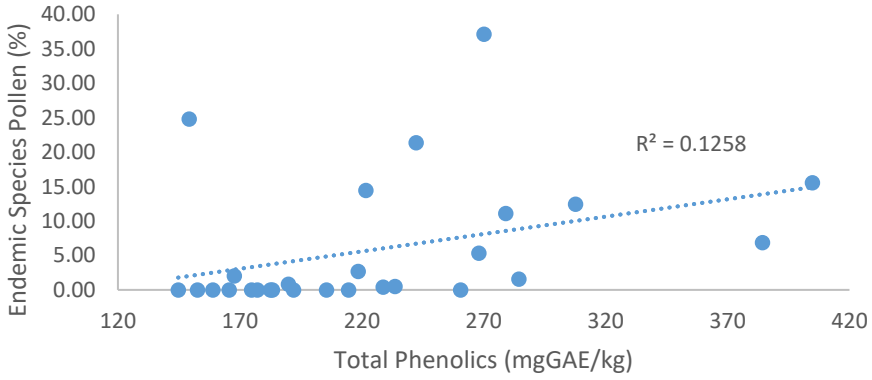


Figure 3.22. Correlation between percentage of endemic pollen and total phenolic content in Rēkohu honey

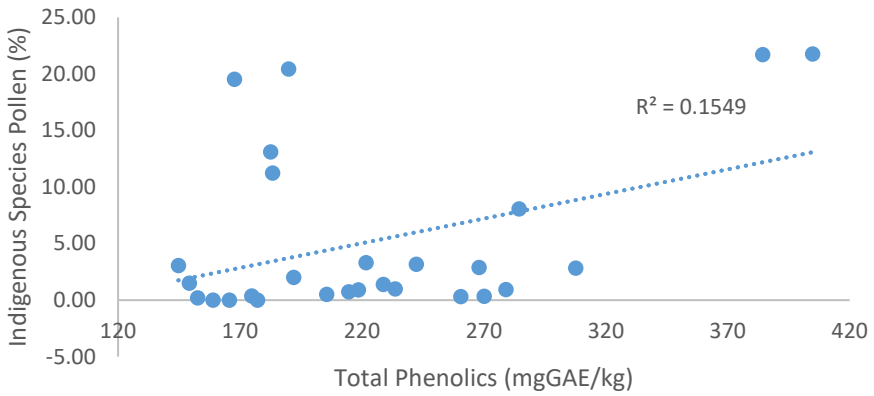


Figure 3.23. Correlation between percentage of indigenous pollen and total phenolic content in Rēkohu honey

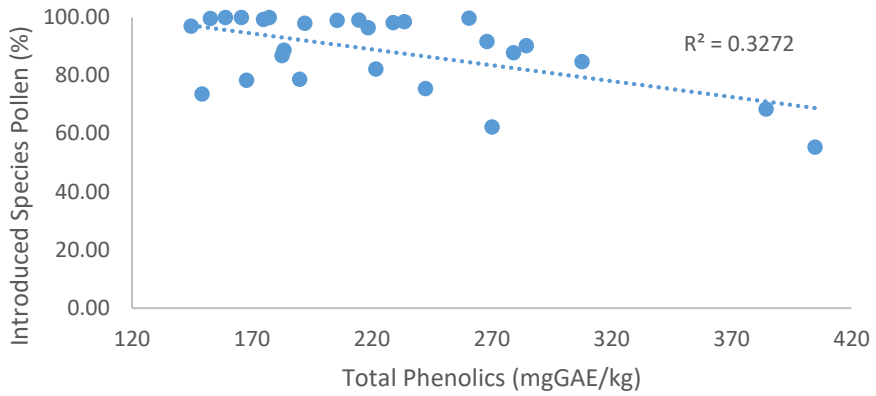


Figure 3.24. Correlation between percentage of introduced pollen and total phenolic content in Rēkohu honey

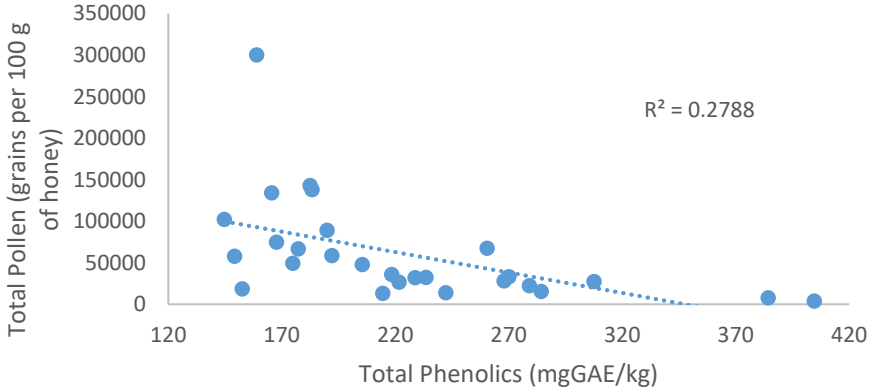


Figure 3.25. Correlation between total pollen and total phenolic content in Rēkohu honey

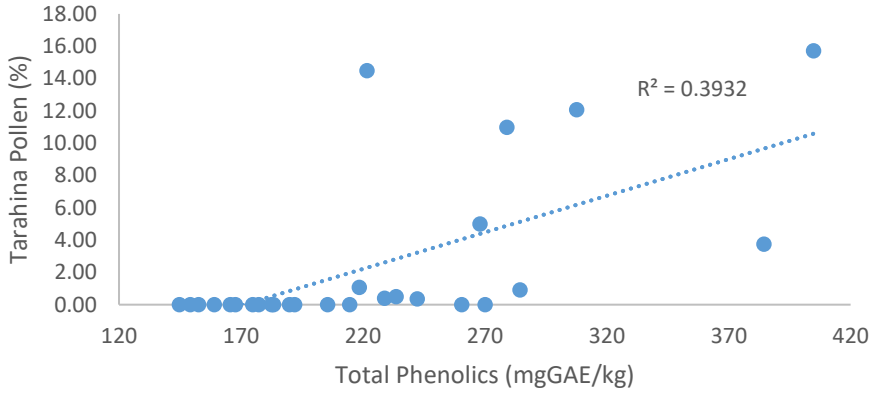


Figure 3.26. Correlation between percentage of tarahina pollen and total phenolic content in Rēkohu honey

3.5 Elemental Analysis

3.5.1 Results

The raw elemental data of Rēkohu honeys was converted to mg/kg using **Equation 2.6** and the summary statistics of elemental data is given in **Table 3.9.**

Table 3.9. Summary statistics for elemental concentrations within 27 samples of Rēkohu honey

Element	<LoD	LoD	Average (% of Total Elements)	Standard Deviation	Minimum	Q1	Median	Q3	Maximum	Range of New Zealand Honey[11, 129]
						mg/kg				
Na	0	0.286	99.119 (13.4%)	29.960	59.173	76.309	98.480	113.967	186.480	9.900 – 488.700
Mg	0	0.286	18.384 (2.5%)	5.440	11.966	13.492	17.441	21.763	32.353	10.200 – 77.300
Al	0	0.286	0.656 (0.1%)	0.252	0.301	0.478	0.607	0.866	1.233	0.320 – 19.320
K	0	2.857	369.964 (50.1%)	177.211	137.789	240.353	324.540	510.115	790.915	230.000 – 2030.000
Ca	0	2.857	158.017 (21.4%)	185.109	29.858	48.426	67.319	213.647	703.276	23.900 – 173.100
P	0	0.029	61.542 (8.3%)	16.723	36.514	48.087	59.160	70.993	93.694	29.500 – 53.600
S	6	14.286	23.811 (3.2%)	11.688	10.169	16.536	20.325	27.708	63.629	13.400 – 93.900
V	19	0.003	0.003 (< 0.1%)	0.002	0.000	0.002	0.002	0.003	0.009	n.d.
Cr	0	0.003	0.026 (< 0.1%)	0.019	0.004	0.008	0.026	0.041	0.065	0.009 – 0.127

Element	<LoD	LoD	Average (% of Total Elements)	Standard Deviation	Minimum	Q1	Median	Q3	Maximum	Range of New Zealand Honey[11, 129]
						mg/kg				
Mn	0	0.003	1.437 (0.2%)	1.360	0.218	0.470	0.948	1.699	4.762	0.310 – 18.900
Co	0	0.003	0.020 (< 0.1%)	0.033	0.004	0.004	0.005	0.014	0.126	0.006 – 0.022
Ni	0	0.003	0.595 (0.1)	0.442	0.107	0.111	0.938	0.982	1.046	0.002 – 0.793
Cu	0	0.003	0.345 (< 0.1%)	0.243	0.062	0.085	0.450	0.552	0.727	0.130 – 1.760
Zn	0	0.029	9.760 (1.3%)	22.501	0.663	1.037	1.687	8.836	102.351	0.350 – 5.970
Sr	0	0.003	0.268 (< 0.1%)	0.198	0.136	0.160	0.191	0.290	0.940	0.040 – 0.610
As	19	0.009	0.009 (< 0.1%)	0.009	0.003	0.005	0.007	0.011	0.050	0.040 – 0.170
Cd	13	0.003	0.005 (< 0.1%)	0.004	0.000	0.001	0.007	0.008	0.010	0.006 – 0.052
Ba	0	0.003	0.171 (< 0.1%)	0.341	0.036	0.059	0.098	0.140	1.848	0.050 – 1.100
Tl	24	0.003	<0.003 (< 0.1%)	0.001	0.000	0.000	0.001	0.001	0.007	0.006 – 0.153
Pb	9	0.003	0.117 (< 0.1%)	0.100	0.001	0.002	0.189	0.200	0.243	0.010 – 0.077
U	27	0.003	<0.003 (< 0.1%)	0.000	0.000	0.000	0.000	0.000	0.001	n.d.
Total	-	-	738.772	324.386	315.603	512.200	708.078	873.999	1463.098	126.000 – 4060.000

3.5.2 Discussion

Twenty-one elements were detected in Rēkohu honeys, comprising seven major elements (greater than 1% of total elements) and fourteen minor elements (less than 1% of total elements). The total elemental concentration ranged from 315.603 mg/kg to 1463.098 mg/kg, which is in the lower end of the range of mainland New Zealand honeys.[11] The most abundant element was potassium, ranging from 137.789 mg/kg to 790.915 mg/kg. This is typical of New Zealand honeys, which are shown to be high in potassium.[129] The second most abundant element was calcium, ranging from 29.858 mg/kg to 703.276, greater than the concentrations seen in New Zealand honeys previously. This is likely due to Rēkohu soil containing high levels of limestone (calcium carbonate). This is the result of prehistoric seafloor sedimentation which now makes up a large portion of Rēkohu's landmass.[42] Sodium is also abundant in Rēkohu honey at typical concentrations seen in mainland honey.[129] This due to the coastal exposure in many known hive sites across the islands. Phosphorus was present in larger quantities than typical ranges of New Zealand honey.[11] Soil analysis by Wright (1959)[206] suggested low natural levels of phosphorus. In the same study, leaf litter around forest abundant in kopi and kawakawa showed high levels of phosphorus. However, the elemental composition of the nectar and pollen from these species is unknown. Sulphur was present in amounts similar to mainland honeys with some samples displaying higher levels which are typical of mainland rewarewa honey.[11] Magnesium was also present in typical quantities seen in mainland honey.[129] Zinc levels were unusual, ranging from 0.663 mg/kg to 102.351 mg/kg. Eight samples contained zinc quantities higher than mainland honeys.[129] Of these, the three highest (16.439 – 102.351 mg/kg) were sourced from Pitt Island. The rest were from locations scattered around Chatham Island, but interestingly, no samples from Go Wild Apiary presented abnormal zinc levels. Zinc in honey is usually linked with industrial activities such as steel manufacturing. However, these do not occur much on Rēkohu. The use of galvanised iron roofing on hives may explain the elevated zinc levels. Honey from Pitt Island demonstrated the highest levels of zinc but is and has been very sparsely populated so high zinc levels may, in fact, be naturally occurring in Rēkohu honey. It should be noted that these amounts of zinc are not high enough to cause concern.

All minor element concentrations in Rēkohu honey were similar in range to typical mainland honeys. Copper levels were low indicating that copper-based pesticides are not used in places of bee forage on Rēkohu. Phosphate fertilisers made from phosphate rock contain small

amounts of cadmium. When these are used near hives, cadmium levels in honey can increase. However, cadmium is only present in trace amounts in Rēkohu honey. Levels of lead were also higher than mainland honeys. Lead in honey is typically caused by residual lead in the environment from the era of leaded fuels. With the only ways to Rēkohu being air and sea, lead contamination can also occur from airplanes and ships. Even though lead in petrol and diesel has been banned, it still remains as a contaminant in soil. Another source of environmental lead contamination is the disposal of lead-acid batteries. In mainland New Zealand, lead-acid batteries are considered 90-99% recyclable[207], however, limited access to recycling services on Rēkohu may lead to informal disposal and environmental pollution.

3.6 Diastase

3.6.1 Results

The diastase activities of Rēkohu honeys are given in **Figure 3.27**.

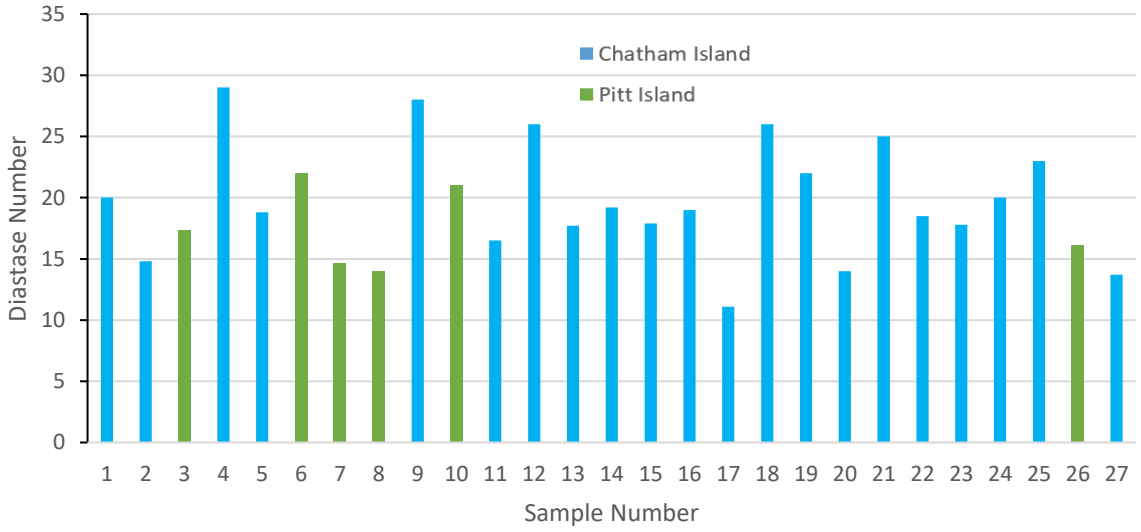


Figure 3.27. Diastase numbers for 27 samples of Rēkohu honey

3.6.2 Discussion

The Codex Alimentarius and the International Honey Standards both state that honey must contain a diastase number of 8 or greater. The diastase numbers of Rēkohu honey were all greater than 8, ranging from 11.1 to 29.0. Bell[138] demonstrated that mainland clover honeys ranged from 11.6 to 16.8. Nineteen samples of Rēkohu honey exhibited diastase numbers

above this range. Diastase predominantly originates from the honeybee itself so its naturally occurring concentration in honey varies.[138]

As they are both quality indicators, it would be expected that HMF and diastase would be negatively correlated. In the case of Rēkohu honeys, HMF and diastase are not correlated, with a correlation coefficient of 0.005, shown in **Figure 3.28**. HMF forms at a slow rate at room temperature, whereas diastase remains stable up to 45 ^{o}C. Therefore, HMF can increase while diastase activity remains high.

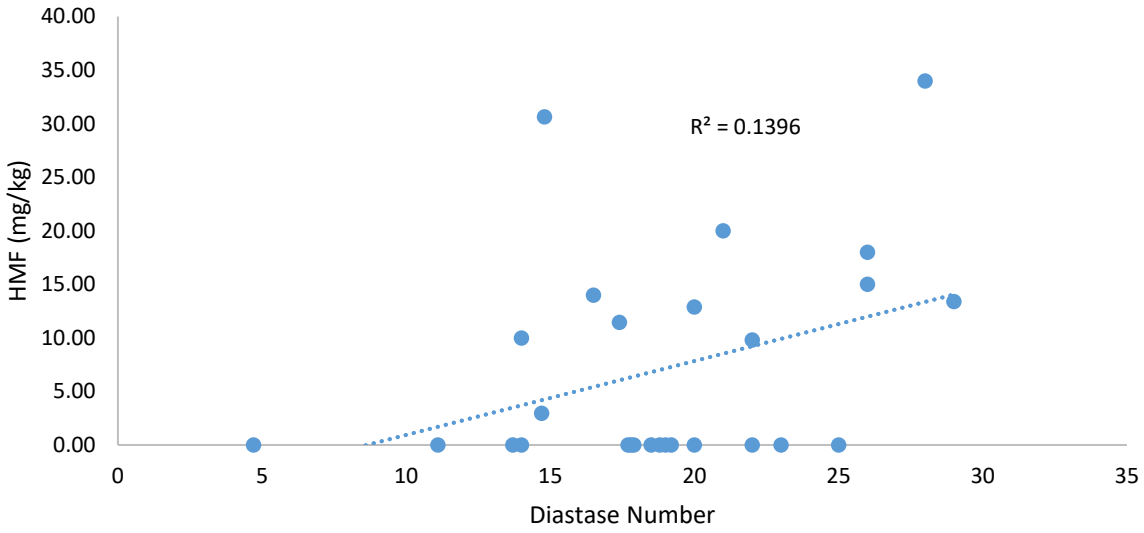


Figure 3.28. Correlation between HMF concentration and diastase number in Rēkohu honey

Bell[138] reported that MGO may inhibit diastase in mānuka honey. In the case of Rēkohu honeys, MGO and diastase are moderately negatively correlated, with a correlation coefficient of -0.393. However, when considering only the eight samples in which MGO was detected, the correlation coefficient increases to -0.704, suggesting a high negative correlation, between diastase and MGO. As MGO is not present in significant amounts in Rēkohu honeys, reduced diastase activity is more likely to be the result of heating and storage effects, but it is interesting that even in trace amounts, MGO is still correlated with diastase in honey.

3.7 Sugars

3.7.1 Results

The chromatograms of 10 mg/mL sugar standards are shown in **Figure 3.29** as an example. The chromatograms of all other sugar standards are given in **Appendix Figures 26 - 31**. The retention times and peak areas of each standard are given in **Appendix Table 7**.

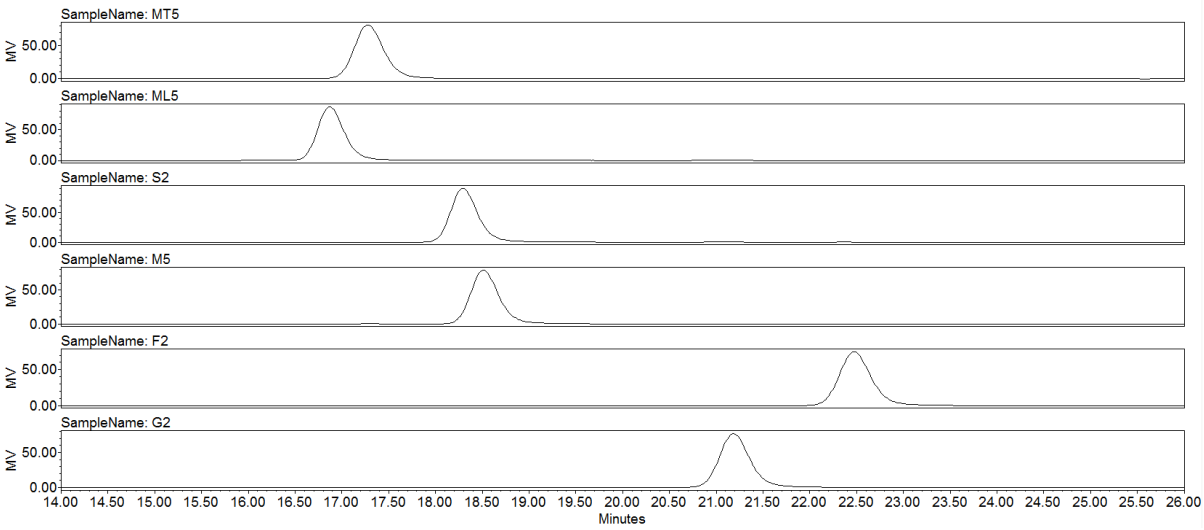


Figure 3.29. HPLC chromatograms (zoomed to 14 min – 26 min) of 10 mg/mL sugar standards for glucose (G2), fructose (F2), maltose (M5), sucrose (S2), melizitose (ML5), and maltotriose (MT5). (Shodex KS-G, KS-801 and KS-802 columns in series eluted with water at 0.8 mL/min at 80ºC, with refractive index detection. Maltotriose retention time: 17.301 min; Melizitose retention time: 16.893 min; Sucrose retention time: 18.227 min; Maltose retention time: 18.484 min; Glucose retention time: 21.087 min; Fructose retention time: 22.436 min.

Standard curves were created for peak area versus concentrations of glucose, fructose, sucrose, maltose, melizitose and maltotriose, **Figure 3.30 - Figure 3.35,** respectively.

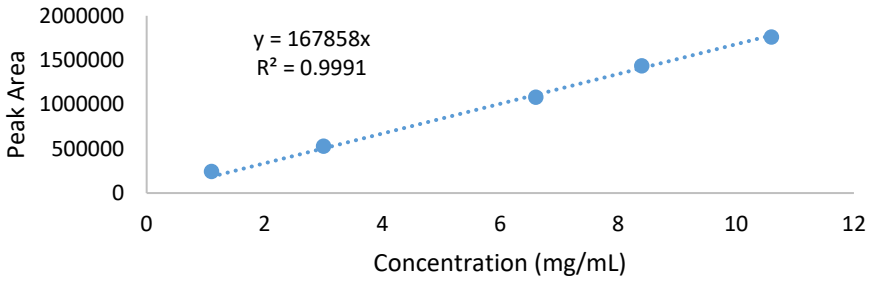


Figure 3.30. Standard curve for glucose

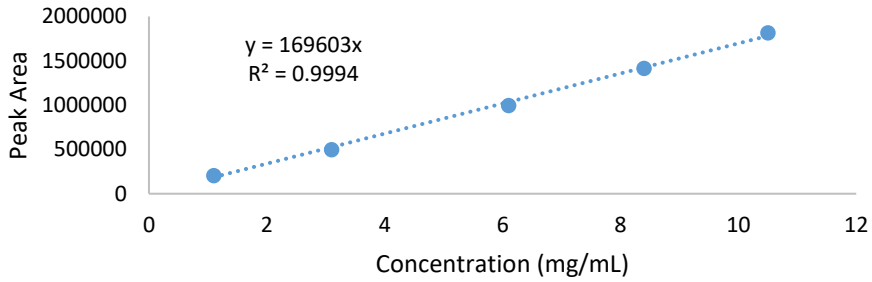


Figure 3.31. Standard curve for fructose

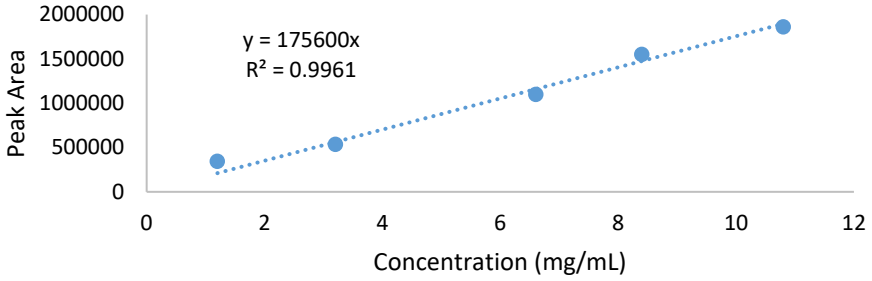


Figure 3.32. Standard curve for sucrose

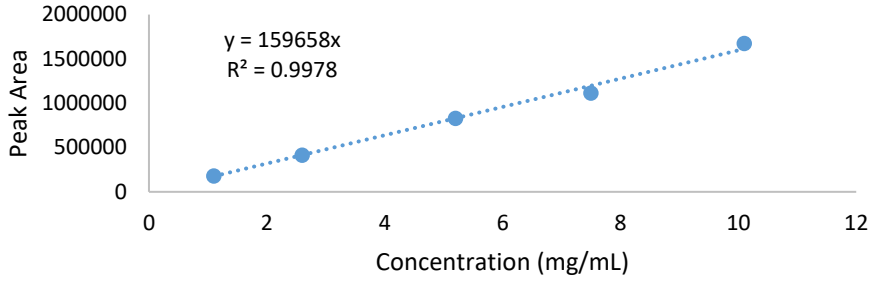


Figure 3.33. Standard curve for maltose

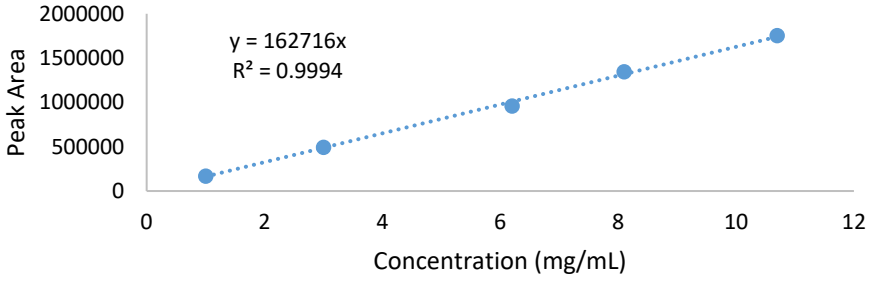


Figure 3.34. Standard curve for melizitose

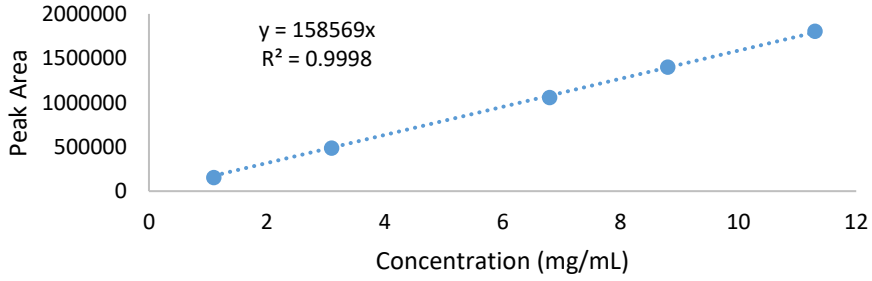


Figure 3.35. Standard curve for maltotriose

The chromatogram of sample 1 is shown in **Figure 3.36** as an example. The chromatograms of samples 2-27 are given in **Appendix Figures 32 - 36.** The retention times and peak areas of each sample are given in **Appendix Table 8**.

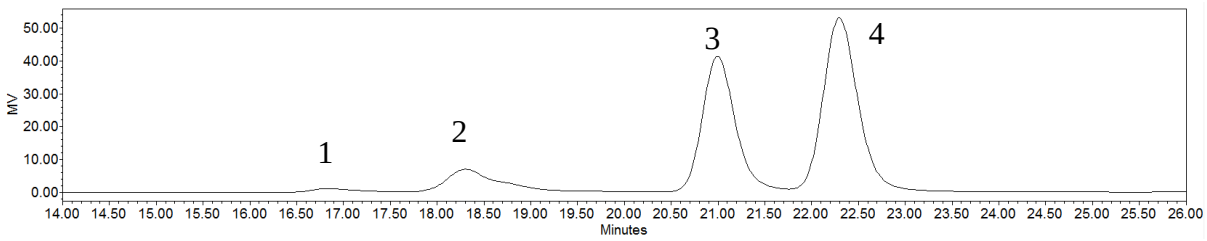


Figure 3.36. HPLC chromatogram (zoomed to 14 min – 26 min) of Sample 1. Shodex KS-G, KS-801 and KS-802 columns in series eluted in water at 0.8 mL/min at 80ºC, with refractive index detection. Peak 1 = trisaccharides, retention time: 16.831 min; Peak 2 = disaccharides, retention time: 18.302 min; Peak 3 = glucose, retention time: 20.995 min; Peak 4 = fructose, retention time: 22.296 min.

The peak area of peaks 3 and 4 were quantified and the concentrations of glucose and fructose in Rēkohu honey were calculated using the standard curves for glucose and fructose, **Equation 2.7** and **Equation 2.8**, and are given in **Figure 3.37** and **Figure 3.38**, respectively. The fructose:glucose ratio was calculated and is shown in **Figure 3.39**.

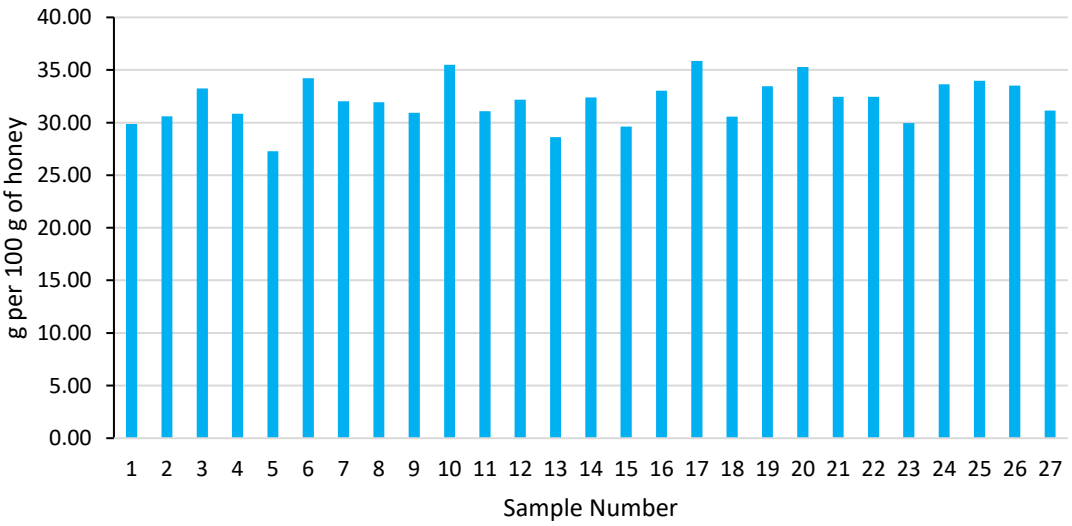


Figure 3.37. Concentrations of glucose in 27 samples of Rēkohu honey

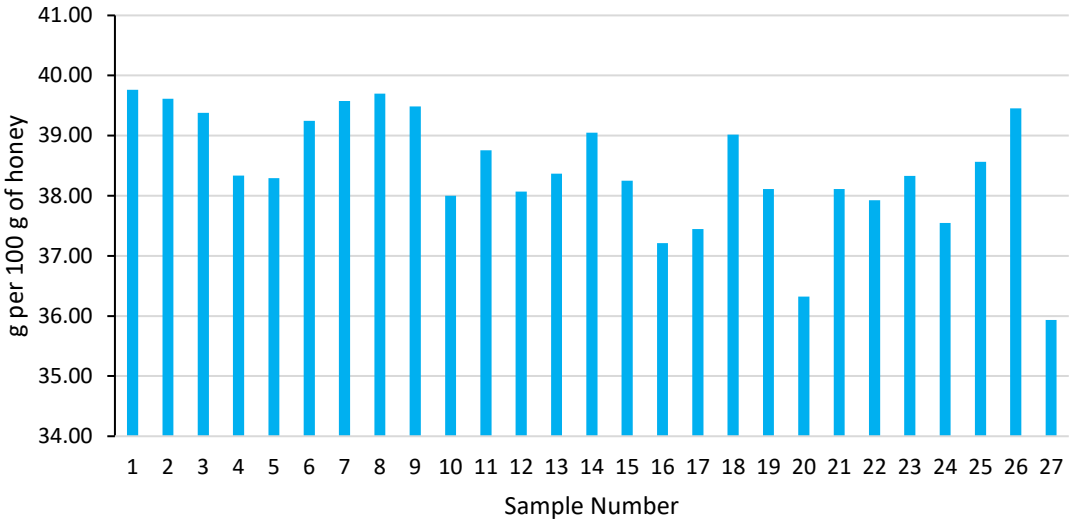


Figure 3.38. Concentrations of fructose in 27 samples of Rēkohu honey

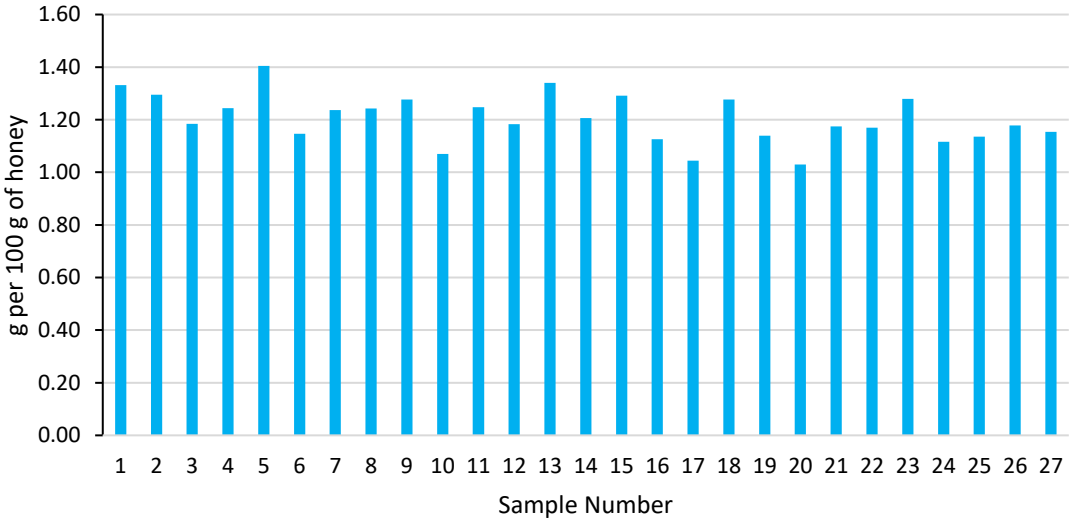


Figure 3.39. Fructose:glucose ratio in 27 samples of Rēkohu honey

Peak 2 was quantified and the concentration of disaccharides was approximated using the standard curves of sucrose and maltose and **Equation 2.7** and **Equation 2.8**. The average of these approximations gives the approximate concentration of disaccharides, given in **Figure 3.40**.

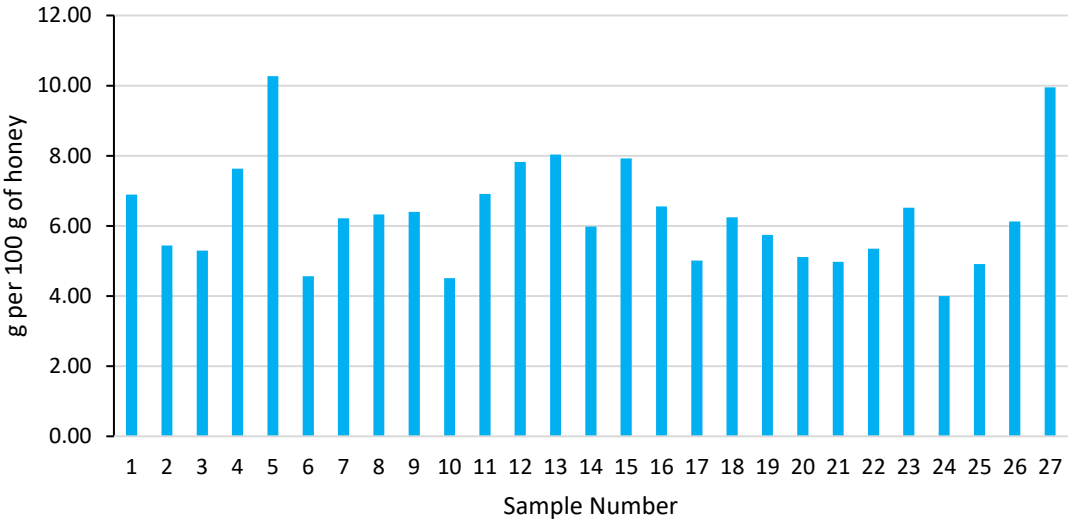


Figure 3.40. Approximate concentrations of disaccharides in 27 samples of Rēkohu honey

Peak 1 was quantified and the concentration of trisaccharides was approximated using the standard curves of melizitose and maltotriose and **Equation 2.7** and **Equation 2.8**. The average of these approximations gives the approximate concentration of trisaccharides, given in **Figure 3.41**.

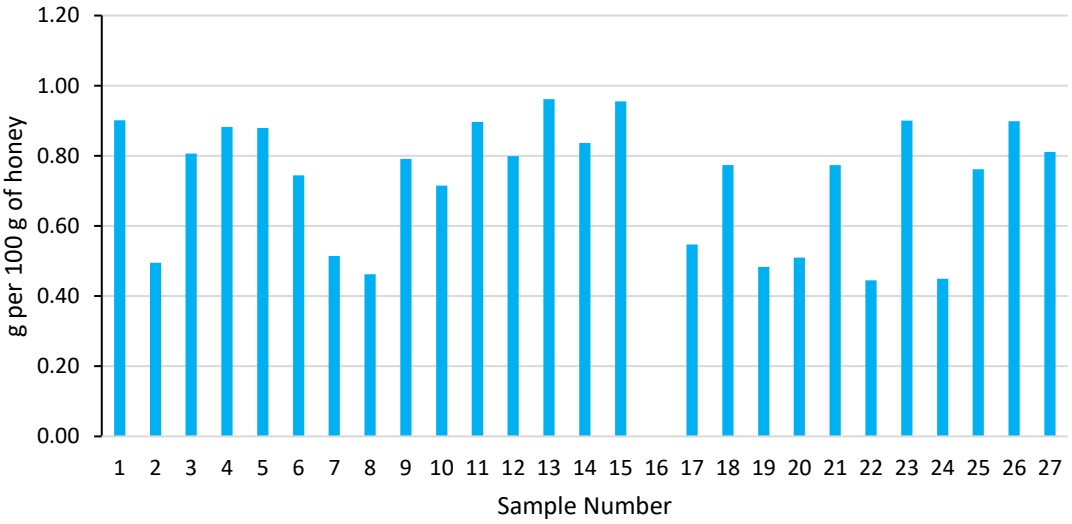


Figure 3.41. Approximate concentrations of trisaccharides in 27 samples of Rēkohu honey

3.7.2 Discussion

The range of carbohydrates in Rēkohu honey, compared to the typical range of carbohydrates in honey[29, 145, 189, 193], is given in **Table 3.10.**

Table 3.10. Range of carbohydrate concentrations in 27 samples of Rēkohu honey compared to the typical range of carbohydrate concentrations in honey.

	Range in Rēkohu Honey	Typical Range in Floral Honey[145]	Typical Range in Honeydew Honey[29, 189, 193]
	g per 100 g of honey		
Glucose	27.27 – 35.86	22.7 – 36.5	19.4 – 22.3
Fructose	35.93 – 39.76	30.0 – 45.6	35.8 – 44.6
F/G Ratio	1.03 – 1.40	1.1 – 1.6	1.8 – 2.0
Disaccharides	4.00 – 10.27	6.8 – 19.5	8.2 – 14.6
Trisaccharides	0 – 0.96	0.4 – 3.0	2.3 – 6.2

Concentrations of glucose and fructose and the fructose:glucose ratio in Rēkohu honeys were similar to typical floral honeys. The Codex Alimentarius states that the fructose and glucose content must be greater than 60 g per 100 g of honey. This was true for all samples of Rēkohu honey. The approximation of disaccharides was in the typical range of disaccharides in floral honey, with samples 5 and 27 having higher levels of disaccharides compared to the other samples. Trisaccharides in Rēkohu honey were in the range of typical floral honeys. It should be noted that sample 17 had no detectable trisaccharide peak. Higher concentrations of di- and trisaccharides are indicative of honeydew contributions to the honey[5], although some floral varieties such as eucalyptus honeys have demonstrated high disaccharide concentrations.[145] For Rēkohu honeys, di- and trisaccharides were low in concentration so it is difficult to confirm if any Rēkohu honeys originate from honeydew. The detection of tutin suggests small contributions from honeydew and will be discussed further in **Section 3.9**. To see if any particular species was influencing the sugar concentrations, the correlation matrix for fructose, glucose, di- and trisaccharides, pouteretere pollen, tarahina pollen, sand daphne pollen and kopi pollen is shown in **Table 3.11.**

Table 3.11. Correlation matrix of fructose, glucose, di and tri-saccharides, pouteretere pollen, tarahina pollen, sand daphne pollen and kopi pollen in Rēkohu honey

	% of pollen			
	Pouteretere	Tarahina	Sand Daphne	Kopi
Fructose (g per 100 g of honey)	-0.631	0.160	-0.205	-0.039
Glucose (g per 100 g of honey)	0.238	-0.666	-0.340	-0.318
Disaccharides (g per 100 g of honey)	-0.122	0.560	0.697	0.664
Trisaccharides (g per 100 g of honey)	-0.476	0.297	0.271	0.016

There were moderate negative correlations between pouteretere pollen and fructose; pouteretere pollen and trisaccharides; and tarahina pollen and glucose. Disaccharides were moderately positively correlated with levels of pollen from tarahina, sand daphne and kopi. With no significant positive correlations between any particular species pollen and trisaccharides, it is possible that these are sourced from small amounts of honeydew.

3.8 C4 Detection

3.8.1 Results

The C4 sugar contents of Rēkohu honeys and their likelihood of passing the AOAC method for C4 sugars are given in **Table 3.12.**

Table 3.12. C4 sugar content in 27 samples of Rēkohu honey

Sample Number	C4 Sugar Content (%)	Likely to pass AOAC test	Sample Number	C4 Sugar Content (%)	Likely to pass AOAC test
1	<0.0	Yes	15	<0.0	Yes
2	1.8	Yes	16	<0.0	Yes
3	2.5	Yes	17	1.3	Yes

Sample Number	C4 Sugar Content (%)	Likely to pass AOAC test	Sample Number	C4 Sugar Content (%)	Likely to pass AOAC test
4	<0.0	Yes	18	<0.0	Yes
5	0.1	Yes	19	0.2	Yes
6	<0.0	Yes	20	<0.0	Yes
7	<0.0	Yes	21	<0.0	Yes
8	<0.0	Yes	22	<0.0	Yes
9	<0.0	Yes	23	0.1	Yes
10	<0.0	Yes	24	<0.0	Yes
11	<0.0	Yes	25	2.6	Yes
12	<0.0	Yes	26	0.2	Yes
13	<0.0	Yes	27	1.6	Yes
14	<0.0	Yes			

3.8.2 Discussion

C4 sugars were detectable in nine samples. The levels of C4 sugars ranged from 0.1% to 2.6%. The Codex Alimentarius indicates that C4 sugars must be below 7% to be considered unadulterated and fit for sale. All samples of Rēkohu honey were below this limit. As part of analysis, samples were analysed using a screen method which provides a faster result for clients, however, the results are not suitably accredited for export purposes. Therefore, an indication of whether the samples would pass the fully accredited AOAC test is given.

Of the samples that had detectable levels of C4, this may have been the result of feeding practices. If bees are to be fed by beekeepers, it is recommended that a sugar syrup, derived from cane sugar to mimic the high sucrose content of nectar, is used, although sometimes, where they are easily available, corn syrups are used which have high levels of fructose or glucose. Syrups may also be added to fraudulently extend the honey.[150] Deliberate or inadvertent adulteration may be detected in the honey by the direct detection of higher levels

of sucrose or these monosaccharides but usually the C4 assay is used, which detects sugars from C4 plants such as sugar cane or maize/corn.

In New Zealand, sucrose is more readily available than fructose or glucose syrups. In Rēkohu honeys where C4 sugars were detected, there is a moderate negative correlation between disaccharides and C4 content, with a correlation coefficient of -0.359, shown in

Figure 3.42.

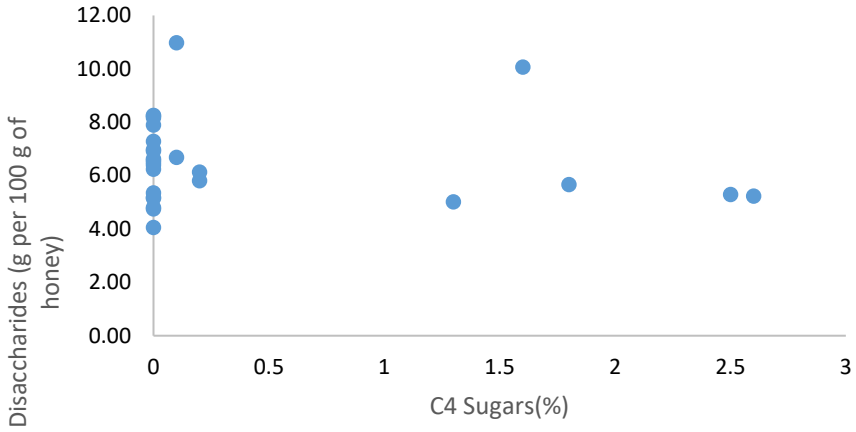


Figure 3.42. Correlation between disaccharide concentration and C4 sugar content in Rēkohu honey

The disaccharide concentration does not exclusively represent sucrose so the negative correlation with C4 is likely attributed to the naturally variable levels of disaccharides in honey. Direct quantification of sucrose may yield additional information, however, from the C4 results, it is evident that adulteration is not a significant issue in Rēkohu honey.

3.9 Tutin

3.9.1 Results

The tutin concentrations in Rēkohu honey are given in **Table 3.13.**

Table 3.13. Tutin concentrations in 27 samples of Rēkohu honey

Sample Number	Tutin Concentration (mg/kg)	Sample Number	Tutin Concentration (mg/kg)
1	<0.010	15	<0.010
2	<0.010	16	<0.010
3	<0.010	17	<0.010
4	<0.010	18	<0.010
5	0.068	19	<0.010
6	<0.010	20	<0.010
7	<0.010	21	<0.010
8	<0.010	22	<0.010
9	0.026	23	<0.010
10	<0.010	24	<0.010
11	<0.010	25	<0.010
12	<0.010	26	<0.010
13	<0.010	27	0.06
14	<0.010		

3.9.2 Discussion

Tutin was detected above the LOD in three samples of Rēkohu honey. The levels of tutin in these samples ranged from 0.026 mg/kg to 0.068 mg/kg. This is significantly lower than the regulation limit of 0.7 mg/kg[163] so these samples are safe for consumption in regards to tutin contamination. The three samples in which tutin was detectable, also exhibited noticeable levels of tutu pollen, suggesting bees were foraging near the tutu plant. However, in order for tutin to end up in honey, the bee must collect the honeydew of the passion vine hopper. There is no record of the passion vine hopper on Rēkohu, however, this is not to say it is not present. The presence of tutin in some samples of Rēkohu honey strongly suggest that the passion vine hopper inhabits Rēkohu. This is supported by the sugar analysis where samples 5 & 27,

which had the highest levels of tutin, also contained the highest levels of disaccharides and trisaccharides, indicators of potential honeydew contribution. There is a moderate positive correlation between disaccharides and tutin, with a correlation coefficient of 0.695, and a low positive correlation between trisaccharides and tutin, with a correlation coefficient of 0.209. When considering only the three samples with detectable tutin levels, the correlation coefficient for disaccharides and tutin is 1.00; and the correlation coefficient for trisaccharides is 0.73The low levels of pollen found in these samples also suggest that the nectar source is not significantly pollen-bearing. This can be either low pollen yield from endemic plants or potential honeydew. A larger proportion of samples would be needed to confirm these observations.

3.10 Glyphosate

3.10.1 Results

All samples of Rēkohu honey demonstrated levels of AMPA, glufosinate and glyphosate below the LOD of 0.010 mg/kg.

3.10.2 Discussion

This is a good indication that, currently, no issue of contamination needs to be addressed. It is unknown whether these common pesticides are being used on Rēkohu, but these results suggest that, currently, the bees are foraging in pesticide-free environments. On mainland New Zealand, glyphosate is used to control populations of the pest plant, gorse,[208] however on Rēkohu, gorse grows freely with, currently, no intention of culling its population. It is recommended that caution be taken surrounding the future use of pesticides on farmland and domestic garden areas known for bee forage, to ensure levels do not increase in future honey samples.

3.11 Antibacterial Activity

3.11.1 Results

The results of the antibacterial activity screen of pure honey against *S. aureus* for Rēkohu honeys are given in **Table 3.14**.

Table 3.14. Antibacterial activity against *S. aureus* for 27 samples of Rēkohu honey

Sample Number	Observable Bacterial Inhibition by Pure Honey (Y/N)	Sample Number	Observable Bacterial Inhibition by Pure Honey (Y/N)
1	N	15	Y
2	N	16	Y
3	N	17	Y
4	N	18	Y
5	N	19	Y
6	Y	20	Y
7	Y	21	Y
8	Y	22	Y
9	Y	23	Y
10	Y	24	Y
11	Y	25	Y
12	Y	26	Y
13	Y	27	Y
14	Y		

Samples 6-27 displayed noticeable bacterial inhibition. Samples 1-5 did not show any inhibition so were not used for a full bioassay. Samples 1-5 were the earliest provided samples and contained low concentrations of MGO.

A set of phenol standards from 1% to 7% were run per plate. Each plate was run in duplicate and the zones of inhibition (ZOI) from both plates were plotted in a standard curve. An example for duplicates plates 1 and 2 are shown in **Figure 3.43**. The standard curves of the 4 remaining pairs of plates are shown in **Appendix Figures 37-40**.

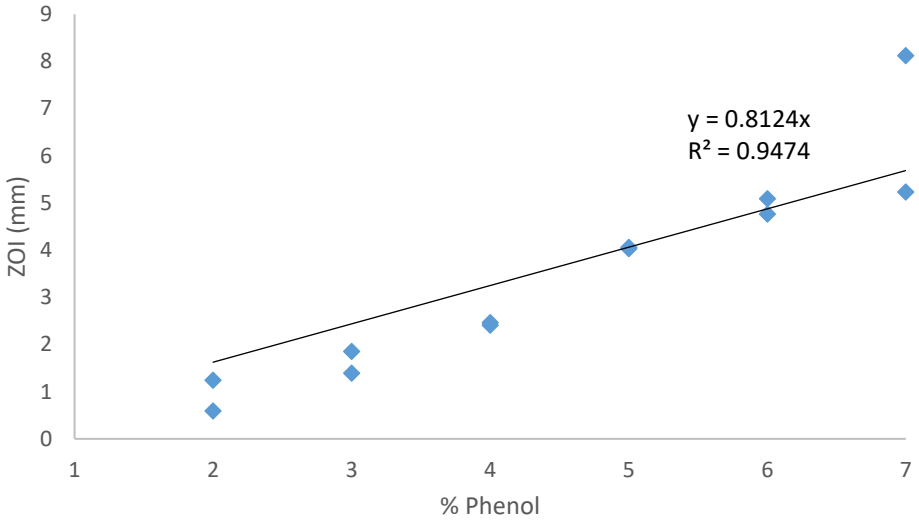


Figure 3.43. Standard curve of the antibacterial activity of phenol for duplicate plates 1 and 2

Samples of 900+ MGO mānuka and clover honey were also analysed for comparison. A series of honey dilutions were run in duplicate per plate and each plate was duplicated. The four zones of inhibition were measured, averaged, and squared. The results are given for each dilution of honey in **Table 3.15**.

Table 3.15. Antibacterial inhibition of *S. aureus* for 27 samples of Rēkohu honey, and single samples of clover honey and 900+ MGO mānuka honey

Sample Number	1000 mg/mL	500 mg/mL	250 mg/mL	125 mg/mL
	Zone of Inhibition radius (mm ± SD)			
6	-	-	-	-
7	2.85 ± 0.25	0.55 ± 0.00	-	-
8	3.04 ± 0.01	0.42 ± 0.00	-	-
9	2.14 ± 0.07	-	-	-
10	-	-	-	-
11	2.50 ± 0.21	1.57 ± 0.22	-	-
12	2.33 ± 0.39	1.25 ± 0.20	-	-
13	2.47 ± 0.23	1.82 ± 0.50	-	-
14	1.23 ± 0.26	-	-	-

Sample Number	1000 mg/mL	500 mg/mL	250 mg/mL	125 mg/mL
	Zone of Inhibition radius (mm ± SD)			
15	2.56 ± 0.28	1.36 ± 0.22	-	-
16	5.40 ± 0.05	2.30 ± 0.23	-	-
17	0.97 ± 0.04	-	-	-
18	1.18 ± 0.06	-	-	-
19	0.33 ± 0.14	-	-	-
20	2.25 ± 0.18	0.39 ± 0.10	-	-
21	5.60 ± 0.16	4.18 ± 0.20	-	-
22	5.55 ± 0.80	3.91 ± 0.45	-	-
23	1.00 ± 0.31	-	-	-
24	6.53 ± 0.29	4.89 ± 0.56	1.54 ± 0.53	-
25	7.51 ± 0.46	5.98 ± 0.58	3.60 ± 0.30	-
26	2.35 ± 0.80	-	-	-
27	-	-	-	-
Mānuka	8.35 ± 0.40	6.51 ± 0.47	4.45 ± 0.28	2.47 ± 0.08
Clover	-	-	-	-

The equation of the standard curve is shown in **Figure 3.43**. It was rearranged to **Equation 2.9** and used to calculate the phenol equivalent antibacterial strength of honey against *S. aureus*, given in **Table 3.16.**

Table 3.16. Phenol equivalent antibacterial strength against *S. aureus* at 1000 mg/mL, 500 mg/mL, 250 mg/mL and 125 mg/mL for 27 samples of Rēkohu honey, and single samples of clover honey and 900+ MGO mānuka honey

Sample Number	% phenol equivalent ± SD			
	1000 mg/mL	500 mg/mL	250 mg/mL	125 mg/mL
6	-	-	-	
7	3.50 ± 0.31	0.68 ± 0.00	-	
8	3.74 ± 0.01	1.03 ± 0.00	-	
9	2.64 ± 0.09	-	-	
10	-	-	-	
11	2.97 ± 0.25	1.87 ± 0.26	-	
12	2.76 ± 0.47	1.48 ± 0.23	-	
13	2.93 ± 0.28	2.16 ± 0.59	-	
14	1.46 ± 0.31	-	-	
15	3.03 ± 0.33	1.61 ± 0.26	-	
16	8.18 ± 0.07	3.49 ± 0.35	-	
17	1.46 ± 0.05	-	-	
18	1.79 ± 0.08	-	-	
19	0.50 ± 0.21	-	-	
20	3.41 ± 0.27	0.58 ± 0.15	-	
21	7.19 ± 0.20	5.36 ± 0.26	-	
22	7.12 ± 1.03	5.02 ± 0.57	-	
23	1.28 ± 0.39	-	-	
24	8.38 ± 0.37	6.28 ± 0.71	1.97 ± 0.68	
25	9.65 ± 0.59	7.67 ± 0.75	4.62 ± 0.38	
26	3.22 ± 1.09	-	-	-
27	-	-	-	-
Mānuka	11.45 ± 0.55	8.93 ± 0.65	6.10 ± 0.38	3.39 ± 0.10
Clover	-	-	-	-

3.11.2 Discussion

Samples 6, 10 and 27 displayed no antibacterial activity at any of the four concentrations investigated in this study. This indicates that the effect seen in the pure honey was diluted to a point where it was no longer observed. The clover honey also showed no bacterial inhibition, once diluted. As the age of the clover honey was unknown, there is a possibility that the sample's inhibitive effect had degraded.

The minimum inhibitory concentration (MIC) was calculated as the minimum concentration at which antibacterial activity was displayed. Seven samples of Rēkohu honey had a MIC of 1000 mg/mL, and sample 25 showing the highest strength of 9.65% phenol equivalent at this concentration. Ten samples of Rēkohu honey had a MIC of 500 mg/mL, and sample 25 also showed the highest strength of 7.67% phenol equivalent at this concentration. Two samples of Rēkohu honey had a MIC of 250 mg/mL, with sample 25 also having the strongest potency of 4.62% phenol. No sample of Rēkohu honey was stronger than the mānuka honey, which exhibited a MIC of 125 mg/mL. At all concentrations, the potency of mānuka honey was greater than Rēkohu honey. Since catalase was not used in this experiment, the assay is measuring total antibacterial activity not non-peroxide activity. The antibacterial activity of manuka honey is primarily linked to the MGO concentration as it has been shown that MGO inhibits glucose oxidase, reducing natural hydrogen peroxide production.[209] As MGO levels were low in Rekohu honeys, the bacterial inhibition observed can probably be attributed to hydrogen peroxide. The fact that some honeys exhibited no inhibition upon dilution may be attributed to age or effects of processing.[210] Hydrogen peroxide levels were not measured and repeating the assay with the addition of catalase to destroy hydrogen peroxide would yield an answer.

Other antibacterial factors in honey include low pH, high sugar content, total phenolic content and antioxidant capacity. Moisture demonstrated a moderate positive correlation with antibacterial activity, with a correlation coefficient of 0.624. This is unusual as higher moisture encourages microbial growth. There was a moderate negative correlation with pH, with a correlation coefficient of -0.469, which was expected. There was no correlation with high sugar content. Total phenolic content and antioxidant capacity both demonstrated slight negative correlations with correlation coefficients of -0.216 and -0.317 respectively. This is unexpected as a positive correlation would be expected.

Interestingly, samples 24 and 25, which had the lowest MICs contained no endemic pollen, were classified as extra white in colour, had non-remarkable phenolic contents and no measurable antioxidant capacity.

A t-test, using **Equation 2.15**, was carried out for all measurements with a standard deviation to ensure the differences in measurements across duplicate plates were not statistically significant. The results of the t-test are given in **Appendix Table 9**. Samples that had no observable zones of inhibition could not be analysed by the t-test. The 500 mg/mL dilution of sample 7 and the 500 mg/mL dilution of sample 8 had no standard deviation between measurements so could not be analysed by the t-test.

The differences between the measurements of the 1000 mg/mL dilution of sample 8 were considered statistically significant ($p = 0.03$) so the antibacterial result for this should be used with caution. As the 500 mg/mL dilution of sample 7 and the 500 mg/mL dilution of sample 8 were not able to be analysed by a t-test, these results should also be used with caution.

3.12 Antioxidant Capacity

3.12.1 Results

The absorbance of Trolox inhibiting DPPH was measured and the DPPH inhibition ratio of Trolox was calculated using **Equation 2.10**. A standard curve was created for the DPPH inhibition ratio of Trolox, shown in **Figure 3.44**. The average absorbance of each sample, blank and standard is given in **Appendix Table 10.**

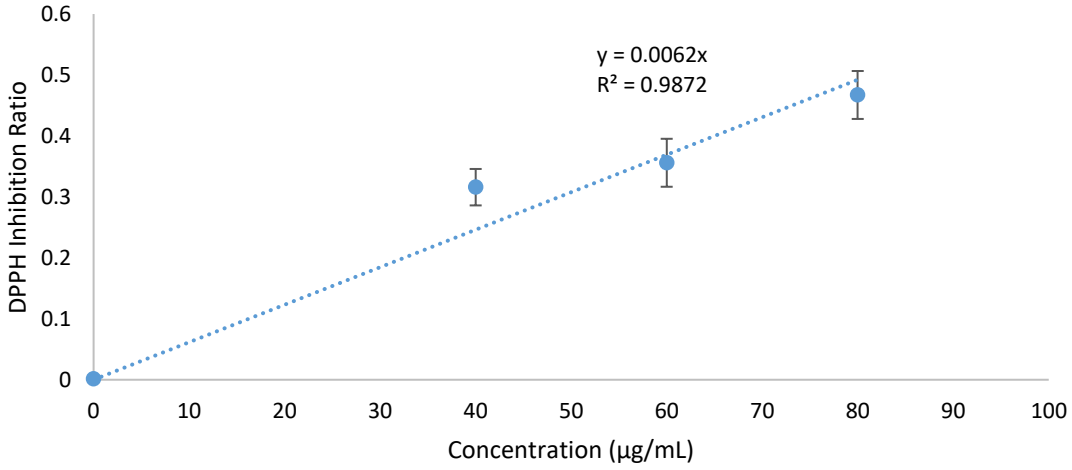


Figure 3.44. Standard curve for Trolox standards

The absorbance of DPPH inhibited by Rēkohu honey was measured and the DPPH inhibition ratio of the sample was calculated using **Equation 2.11**. These were converted to a Trolox equivalent antioxidant capacity (TEAC) using the rearranged standard curve equation, shown in **Equation 2.12**. The TEAC values were converted to mgTE/kg using **Equation 2.13** and are shown in **Figure 3.45**.

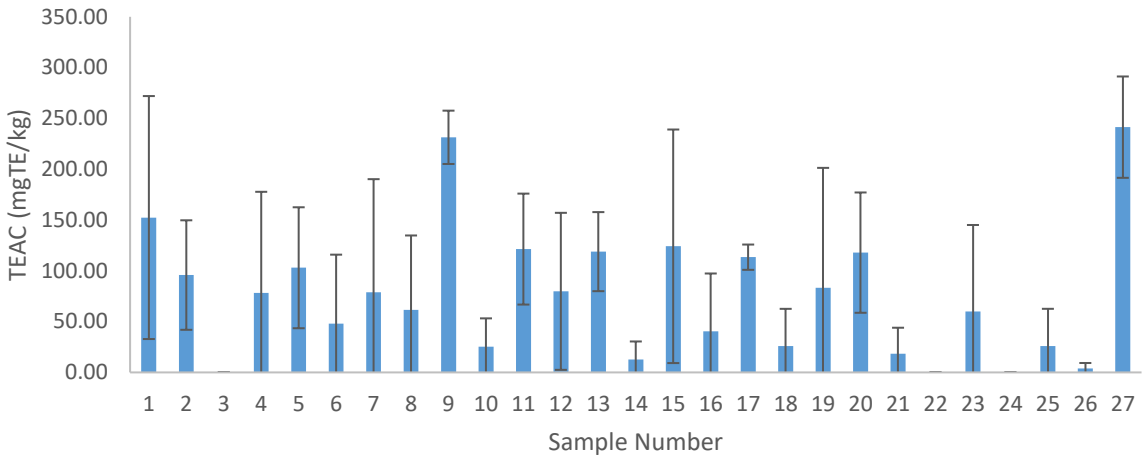


Figure 3.45. TEAC values for 27 samples of Rēkohu honey

3.12.2 Discussion

A TEAC value could not be calculated for three samples. This does not mean they do not have antioxidant capacity as measurement of antioxidant capacity using a FRAP or CuPRAC assay may yield different results.

The TEAC value of Rēkohu honey ranged from 3.84 mgTE/kg ± 5.43 mgTE/kg to 241.39 mgTE/kg ± 49.88 mgTE/kg. Samples 27 displayed the highest TEAC value and also demonstrated the highest total phenolic content. There is high correlation between total phenolics and antioxidant capacity, shown in **Figure 3.46**. As phenolic compounds are natural antioxidants, their concentration in honey is linked to the antioxidant capacity of the honey. It was also observed that antioxidant capacity is moderately positively correlated with pH, conductivity and colour, shown in **Figure 3.47**, and **Figure 3.48**. These factors are linked to phenolic content rather than directly influencing antioxidant capacity. The correlation matrix for antioxidant capacity against total phenolics, pH, conductivity and colour is shown in **Table 3.17**.

Table 3.17. Correlation matrix of total phenolic content, pH, conductivity, colour and antioxidant capacity in Rēkohu honey

	Total Phenolics (mgGAE/kg)	pH	Conductivity (µS/cm)	Colour (mm Pfund)
Antioxidant Capacity (mgTE/kg)	0.742	0.525	0.620	0.760

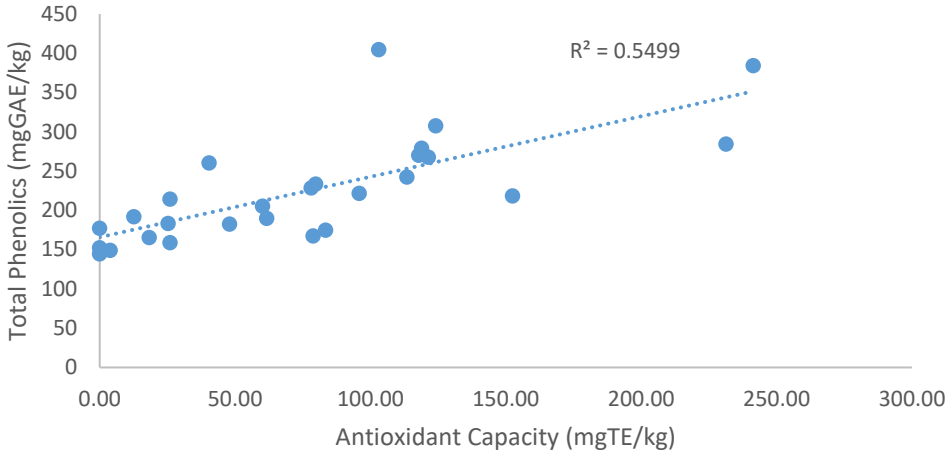


Figure 3.46. Correlation between total phenolic content and antioxidant capacity in Rēkohu honey

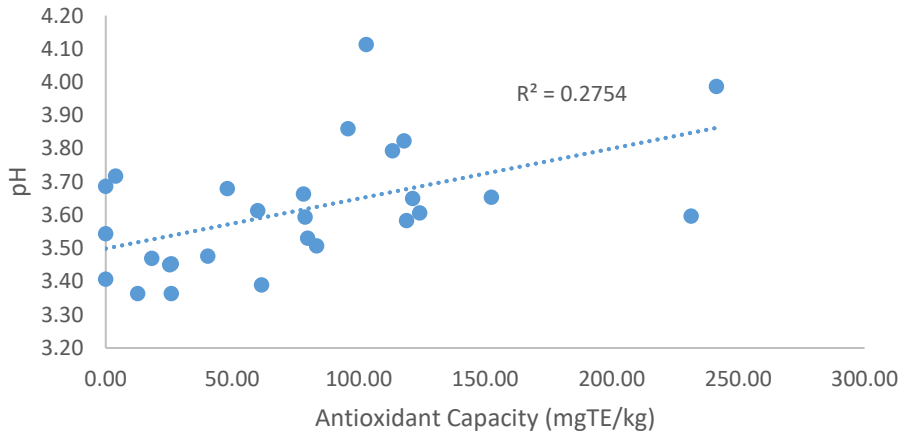


Figure 3.47. Correlation between pH and antioxidant capacity in Rēkohu honey

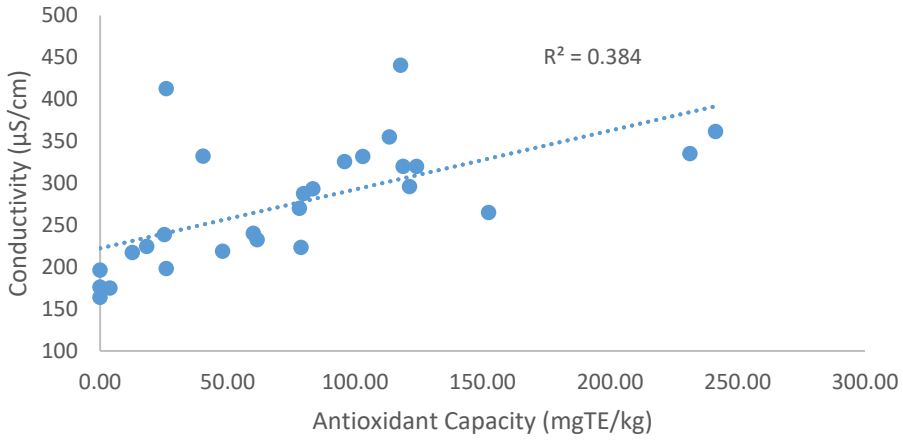


Figure 3.48. Correlation between conductivity and antioxidant capacity in Rēkohu honey

Goslinkski *et al.* (2020)[13] reported that the antioxidant capacities, measured by the DPPH inhibition assay, of 250+ MGO mānuka honey and 400 + MGO mānuka honey were 353 mgTE/kg and 400 mgTE/kg, respectively. All Rēkohu honey samples demonstrated antioxidant capacities lower than mānuka honey. Ciappini *et al.* (2014)[211] reported the DPPH inhibition of clover and eucalyptus honeys from Argentina. Clover honeys ranged from 200 mgTE/kg to 350 mgTE/kg and eucalyptus honeys ranged from 300 mgTE/kg to 1000 mgTE/kg. All Rēkohu honey samples displayed antioxidant capacities below that of eucalyptus honeys and only 2 samples were in range of reported clover honeys. The remaining samples had low antioxidant capacities presumably linked to low phenolic contents.

Chapter 4 – Conclusions and Recommendations

This research aimed to fill the gap in knowledge surrounding Rēkohu honeys and provide a preliminary but comprehensive analysis of its chemical and biological properties. This research also aimed to determine if Rēkohu honey is different in its chemical composition to honey sourced from the mainland and if there are any beneficial properties stemming from the endemic species found only on Rēkohu.

In most cases, Rēkohu honeys were similar to mainland honeys, with some samples displaying greater levels of endemic pollen than others, up to 37.13%. Twenty-one samples were classified as monofloral clover honeys and six were multifloral. Notable points of interest where honeys differed from typical mainland honeys included higher levels of calcium, phosphorus and zinc; and higher total phenolic contents, up to 414 mgGAE/kg. Element differences are likely due to environmental differences, whereas high phenolic content appeared to be linked with the native species: kopi, cabbage tree and sand daphne; and the endemic species, tarahina. Compared to mainland clover, Rekohu honey demonstrated higher levels of disaccharides, however these were lower than mainland honeydew honey. This can be attributed to access to honeydew on the tutu vine, indicated by levels of tutu pollen and tutin in some sample. There were also moderate correlations between disaccharides and the species: kopi, sand daphne and tarahina. These observations suggest that the presence of native and endemic species contribute to the unique properties seen in Rēkohu honey.

Colour, conductivity and pH were in the typical range for mainland honeys. Moisture was generally within range of typical honeys but five demonstrated levels above the limit of 20%. MGO and DHA were present in eight and twelve samples, respect. The detected levels of MGO were less than mainland mānuka honey. This is not unexpected as mānuka does not grow on Rēkohu and endemic species do not produce these compounds in large amounts. No pesticide residues, problematic levels of tutin or significant adulteration were detected. All requirements were met for regulations on diastase, HMF, tutin and glyphosate. Some samples demonstrated antibacterial activity comparable to lower grade mānuka honeys, up to MICs of 250 mg/mL. Others demonstrated higher antioxidant capacities, up to 241.94 mgTE/kg. Compared to other mainland honeys including clover and mānuka, contributions from

endemic species do not appear to provide any additional bioactive properties in Rēkohu honeys greater than those of mainland varieties. However, this work is preliminary and further research may reveal these properties.

It is recommended that future work repeat the antibacterial assays using catalase to determine if the antibacterial activity stems from hydrogen peroxide. If this proves not to be the case, a closer investigation of the source of the activity would be justified. The application of other antioxidant capacity assays would also determine a more representative antioxidant capacity and indicate if any bioactivity stems from antioxidant compounds. Additional analysis of the specific phenolics found in Rēkohu honey would be useful for determining any chemical markers for endemic species. A more in-depth analysis of the sugars, using a method capable of separating larger carbohydrates, would provide a greater understanding of whether there is a honeydew contribution. A larger scale tutin analysis would also assist in determining if the passion vine hopper inhabits the island and whether this poses a threat for tutin contamination in honey. To differentiate Rēkohu honey from mainland honey, a wider survey would be required to use elemental analysis to obtain a unique fingerprint. Preliminary analysis in this research shows that Rēkohu honey has a different elemental fingerprint to mainland honey and may be unique.

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Appendix

Appendix Table 1. Mass of samples 1-7, high QC and low QC used for 3 in 1 analysis

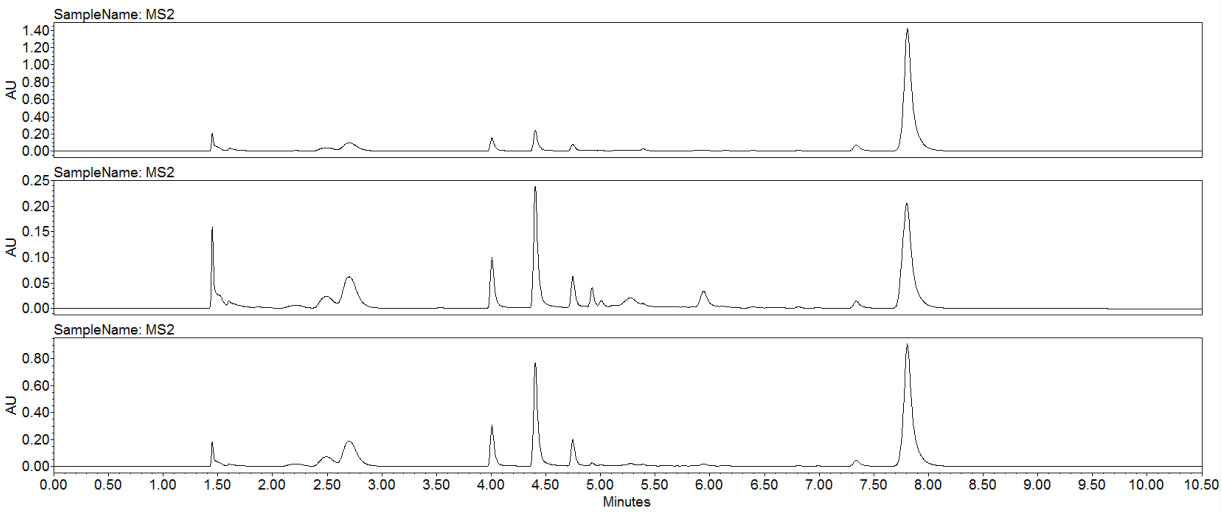
Sample	Mass (g)			Sample	Mass (g)		
	A	B	C		A	B	C
1	1.0693	1.0990	1.0453	6	1.0540	1.0778	1.0245
2	1.0107	1.0814	1.0753	7	1.0688	1.0050	1.0428
3	1.0648	1.0905	1.0036	High QC		1.0043	
4	1.0866	1.0748	1.0515	Low QC		1.0387	
5	1.0046	1.0119	1.0516				

Appendix Table 2. Mass of 27 samples of Rēkohu honey used for total phenolic analysis and determination of antioxidant capacity. Mānuka samples 1-4 were only used for total phenolic analysis.

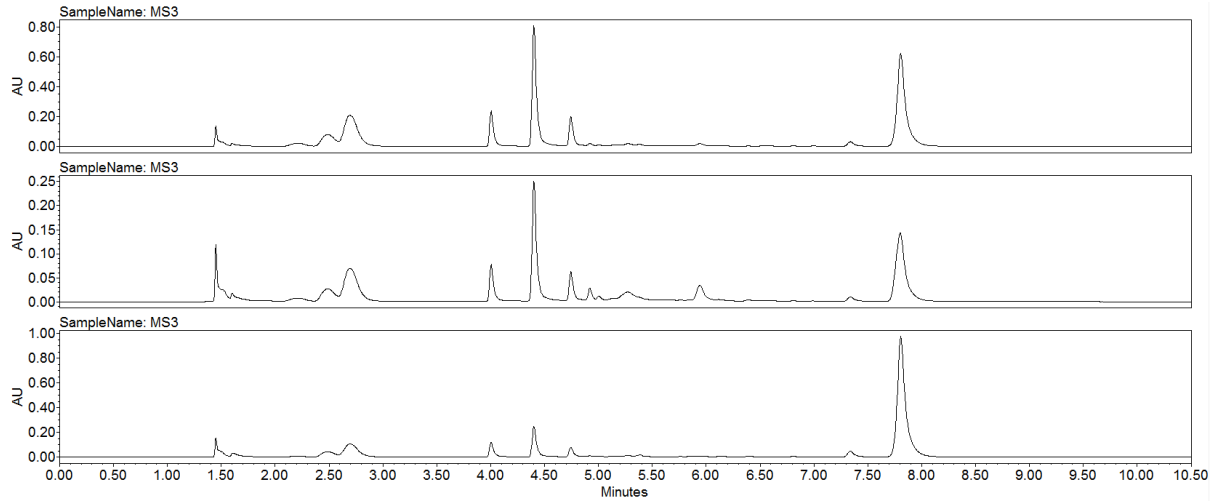
Sample	Mass (g)	Sample	Mass (g)
1	0.4965	17	3.0104
2	0.5073	18	3.0053
3	0.4998	19	3.0009
4	0.5019	20	3.0083
5	0.4987	21	3.0022
6	0.5005	22	3.0003
7	0.5013	23	3.0056
8	3.0119	24	3.0004
9	3.0365	25	3.0041
10	3.0199	26	3.0228
11	3.0039	27	3.0068
12	3.0325	Mānuka 1	0.5013
13	3.0115	Mānuka 2	0.5006
14	3.0218	Mānuka 3	0.505
15	3.0174	Mānuka 4	0.4999
16	2.9996		

Appendix Table 3. Mass of 27 samples of Rēkohu honey used for sugar analysis

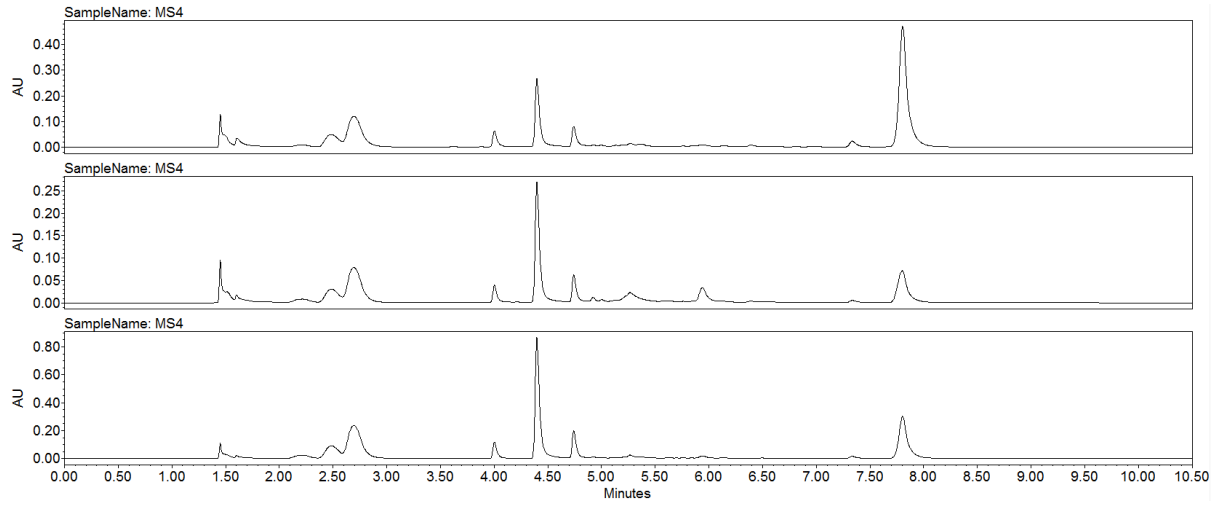
Sample	Mass (mg)	Sample	Mass (mg)
1	100.4	15	104.2
2	104.0	16	106.4
3	100.0	17	100.2
4	103.6	18	102.5
5	106.9	19	101.1
6	104.2	20	100.9
7	105.7	21	104.1
8	104.8	22	100.1
9	101.5	23	102.7
10	106.5	24	101.4
11	105.4	25	106.6
12	104.5	26	100.3
13	102.7	27	101.1
14	104.4		



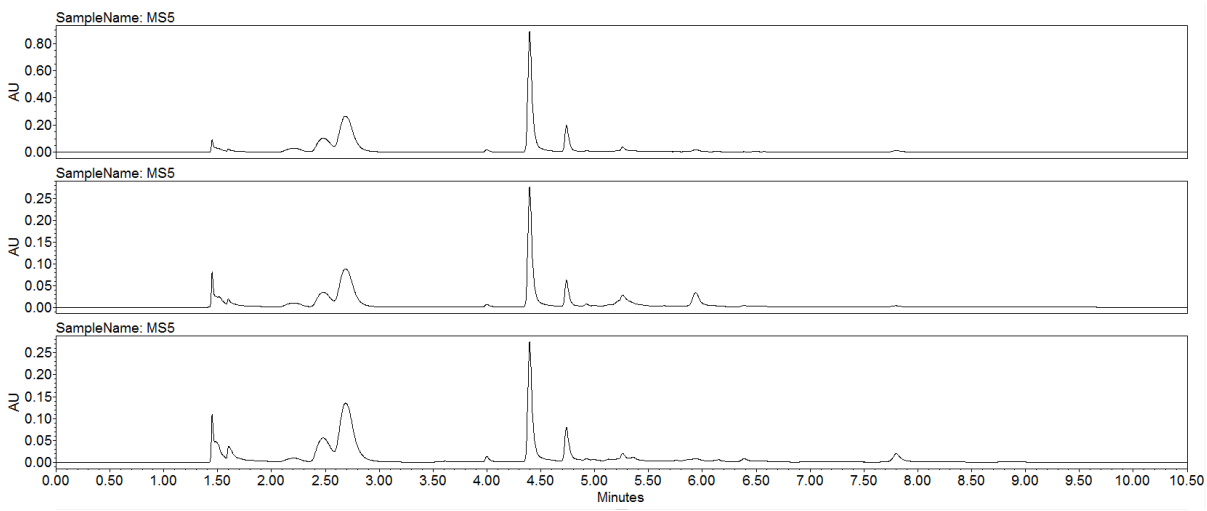
Appendix Figure 1. HPLC chromatograms of standard MS2 (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection at 244 nm (top), 273 nm (middle) and 260 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



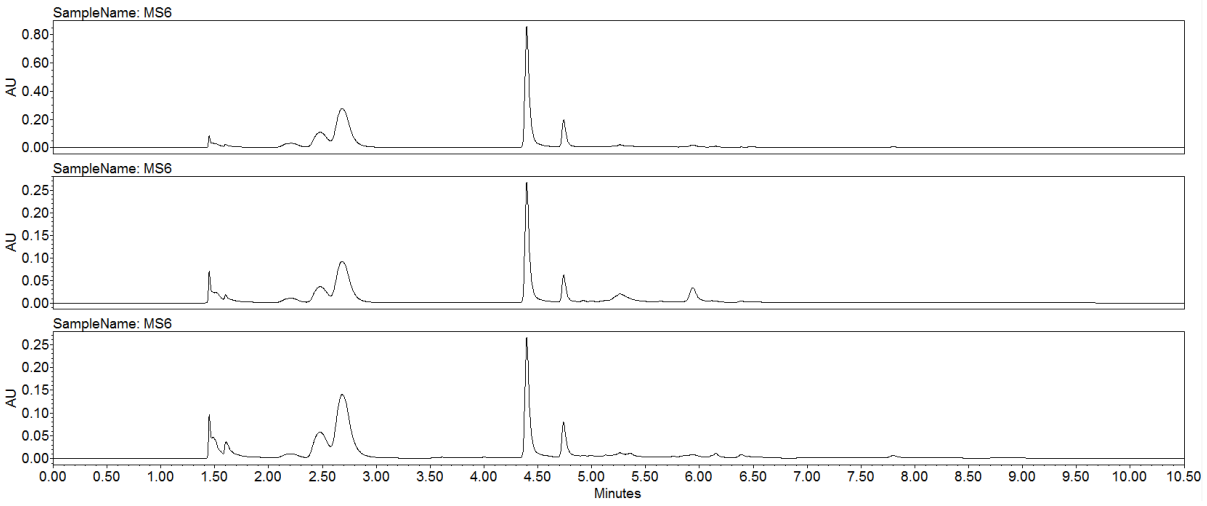
Appendix Figure 2. HPLC chromatograms of standard MS3 (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



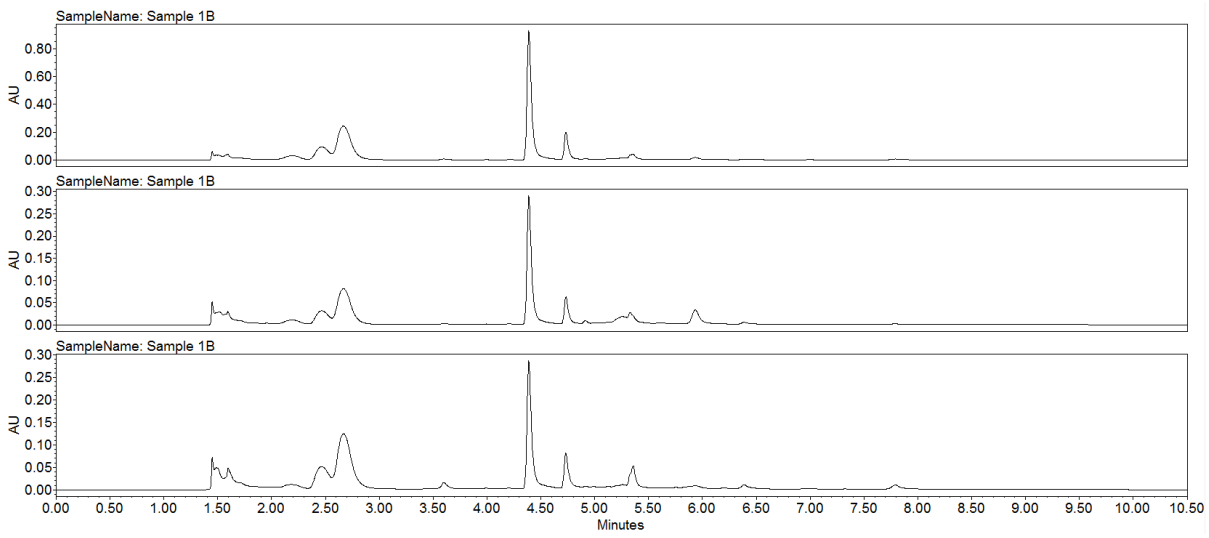
Appendix Figure 3. HPLC chromatograms of standard MS4 (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection at 244 nm (top), 273 nm (middle) and 260 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



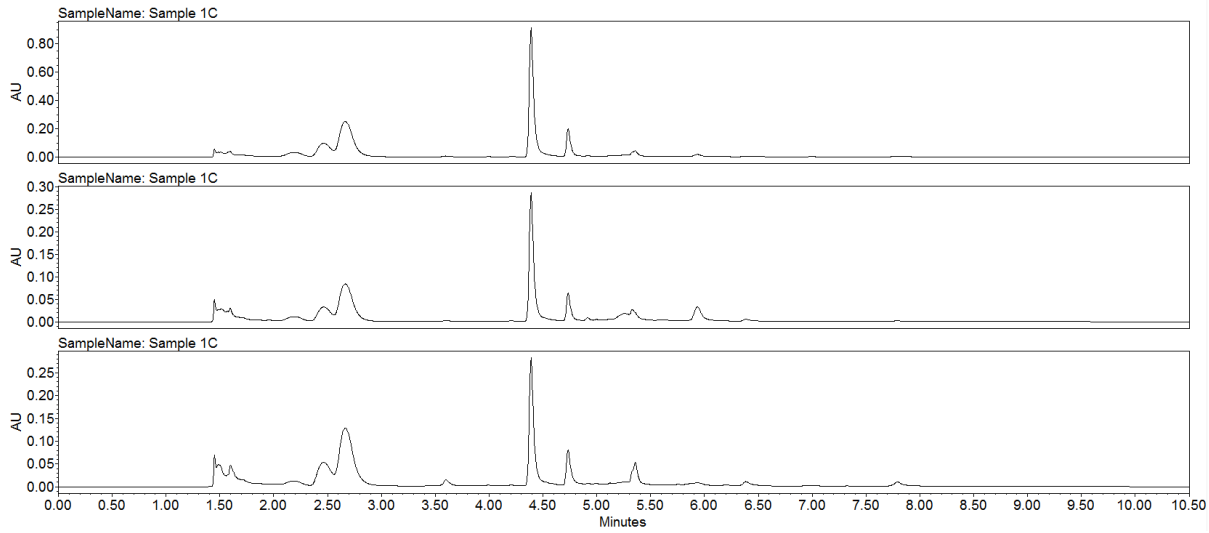
Appendix Figure 4. HPLC chromatograms of standard MS5 (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



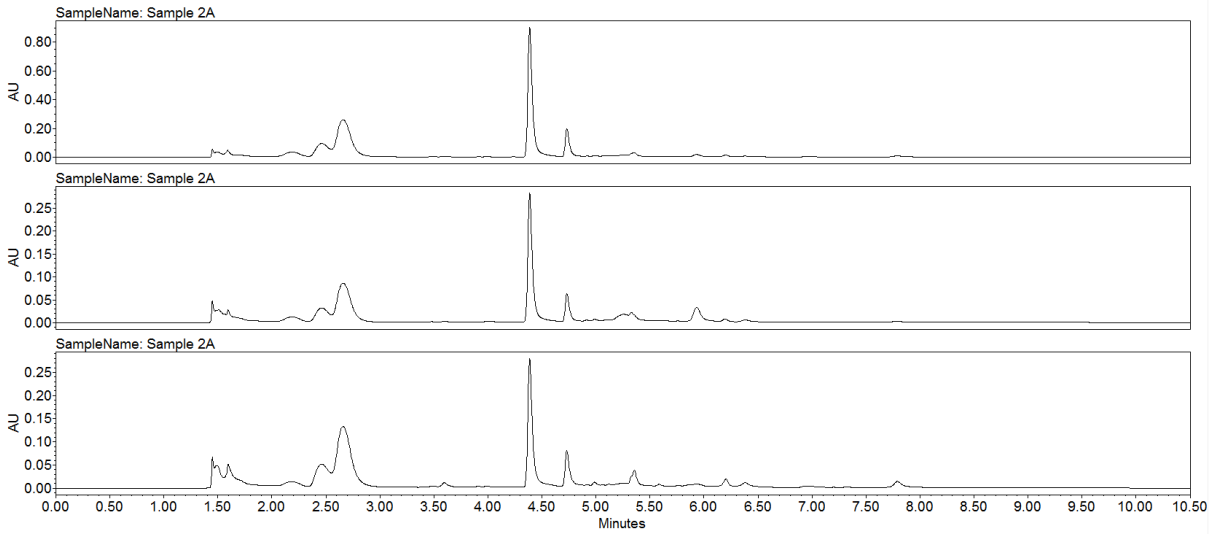
Appendix Figure 5. HPLC chromatograms of standard MS6 (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



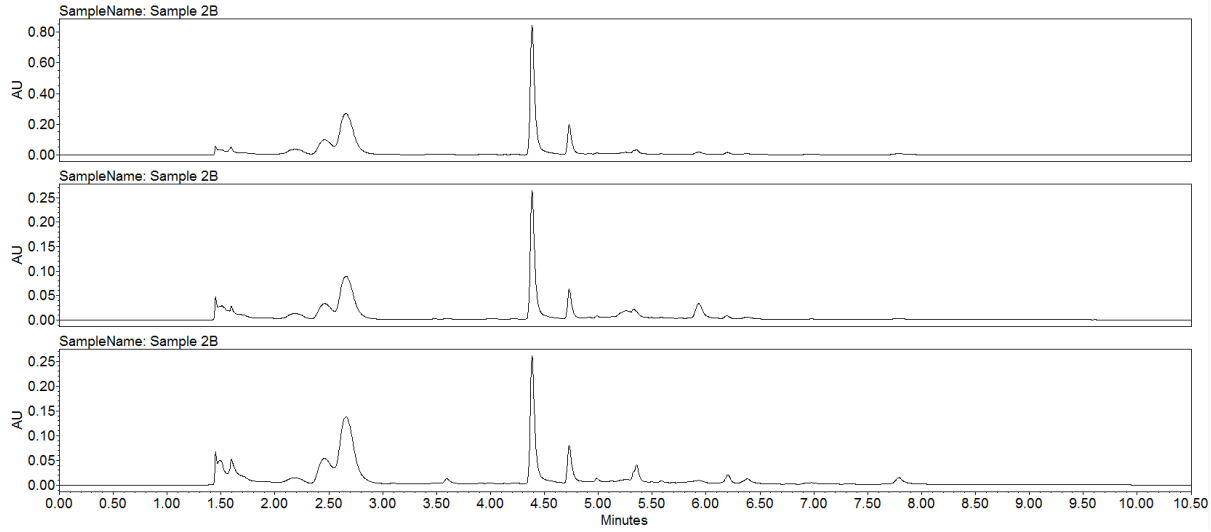
Appendix Figure 6. HPLC Chromatograms of sample 1B (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



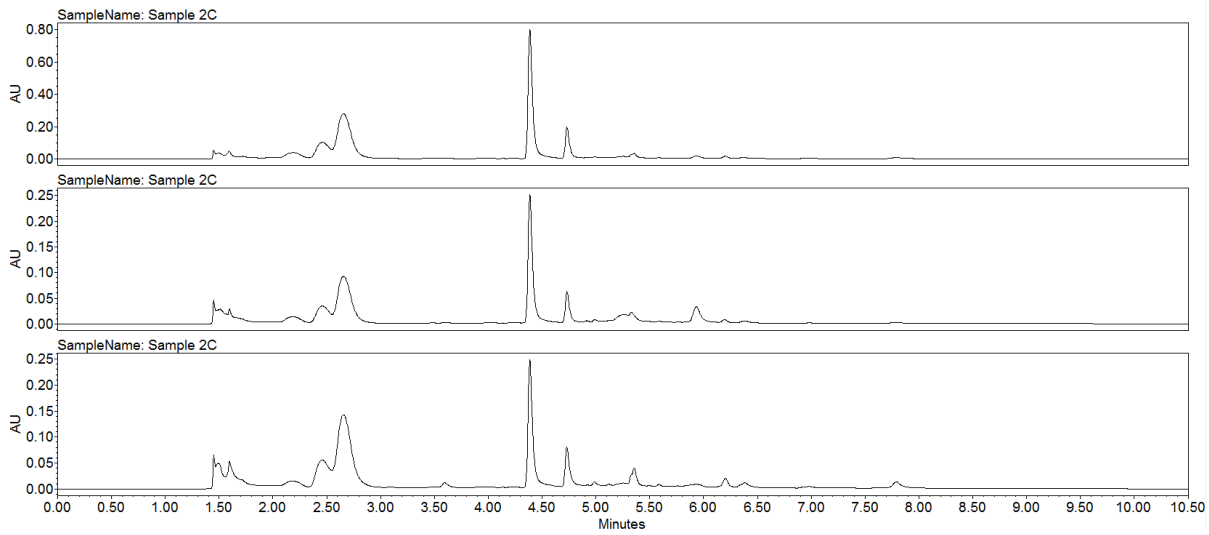
Appendix Figure 7. HPLC Chromatograms of sample 1C (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



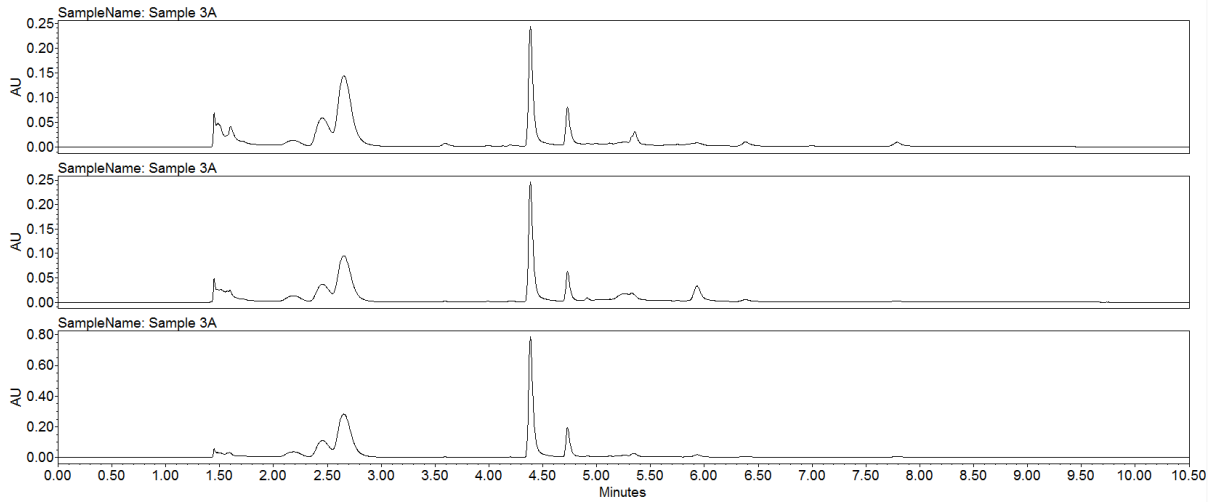
Appendix Figure 8. HPLC Chromatograms of sample 2A (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



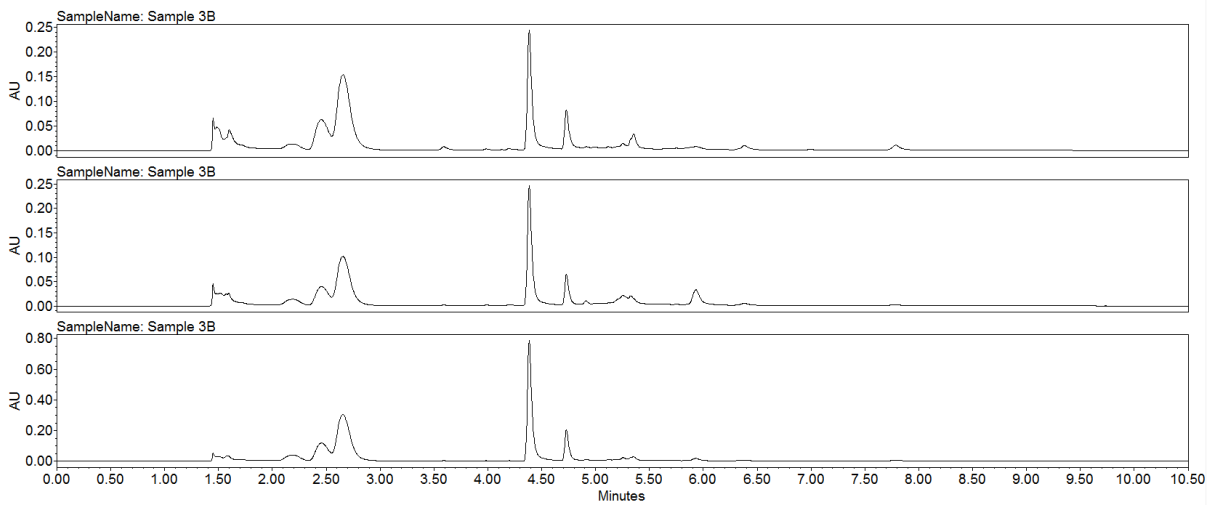
Appendix Figure 9. HPLC Chromatograms of sample 2B (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



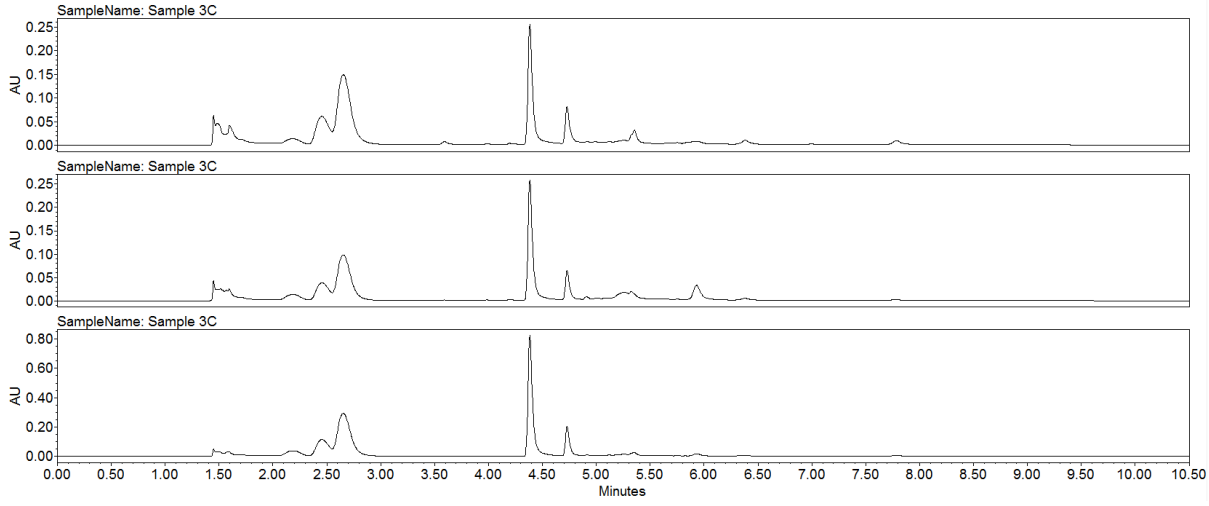
Appendix Figure 10. HPLC Chromatograms of sample 2C (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



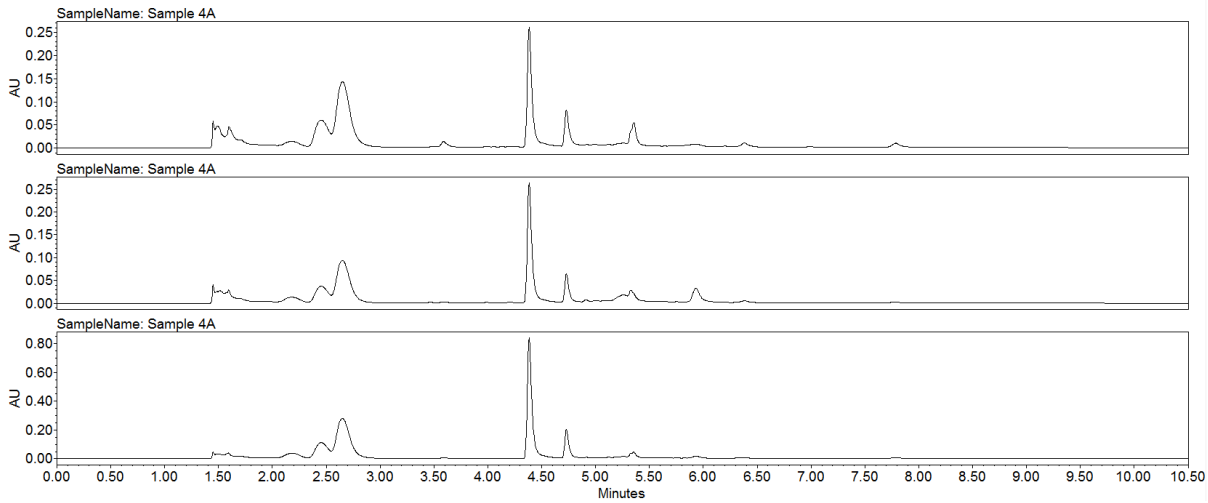
Appendix Figure 11. HPLC Chromatograms of sample 3A (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 244 nm (top), 273 nm (middle) and 260 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



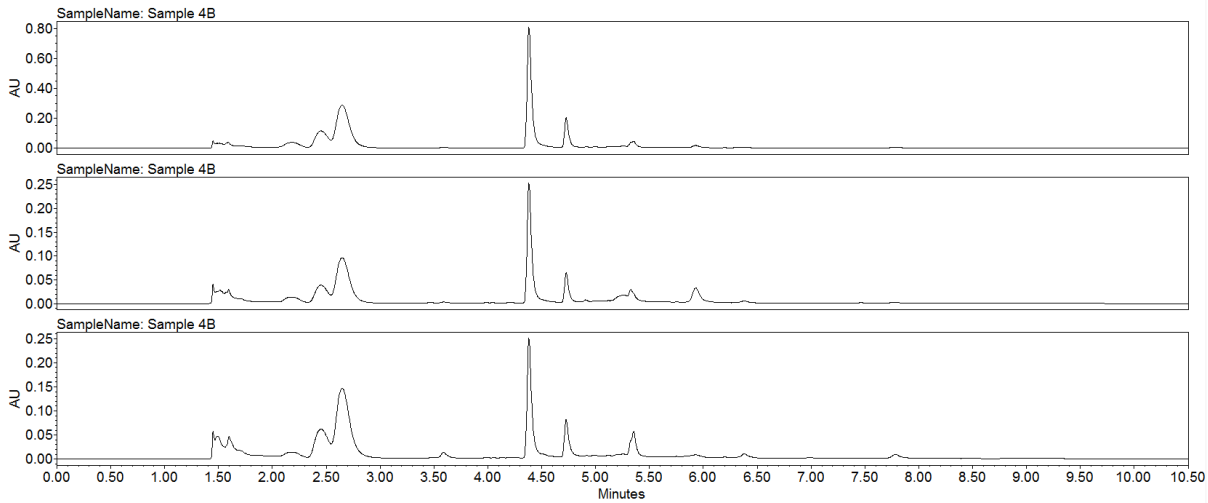
Appendix Figure 12. HPLC Chromatograms of sample 3B (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 244 nm (top), 273 nm (middle) and 260 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



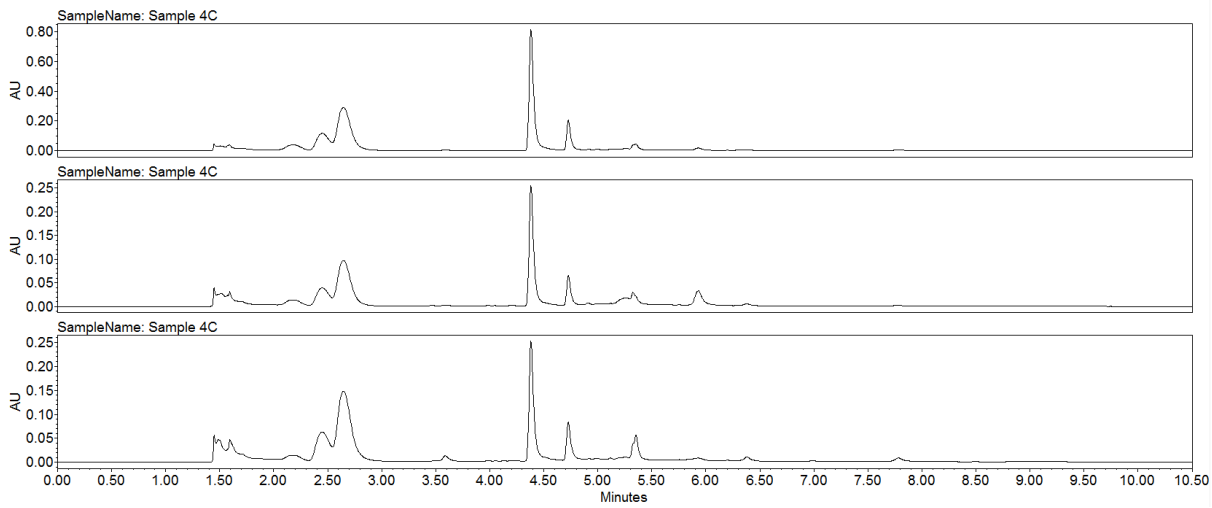
Appendix Figure 13. HPLC Chromatograms of sample 3C (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 244 nm (top), 273 nm (middle) and 260 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



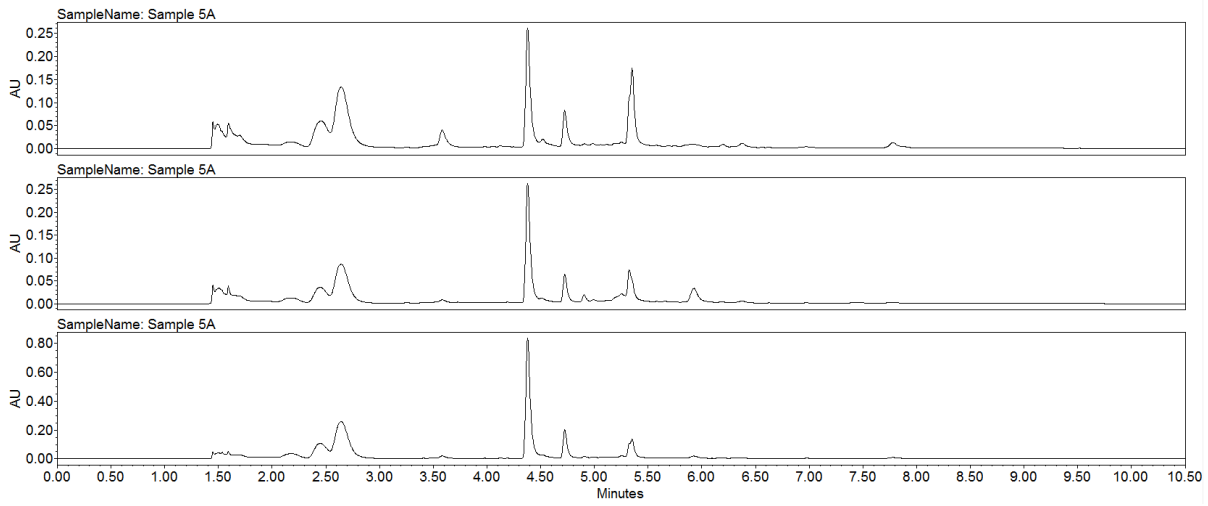
Appendix Figure 14. HPLC Chromatograms of sample 4A (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 244 nm (top), 273 nm (middle) and 260 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



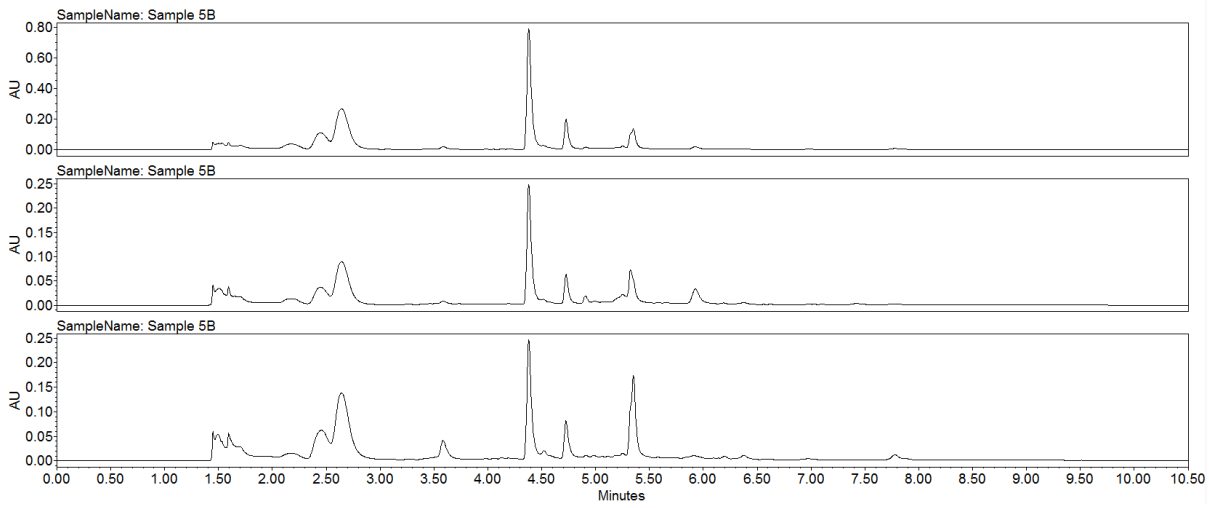
Appendix Figure 15. HPLC Chromatograms of sample 4B (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



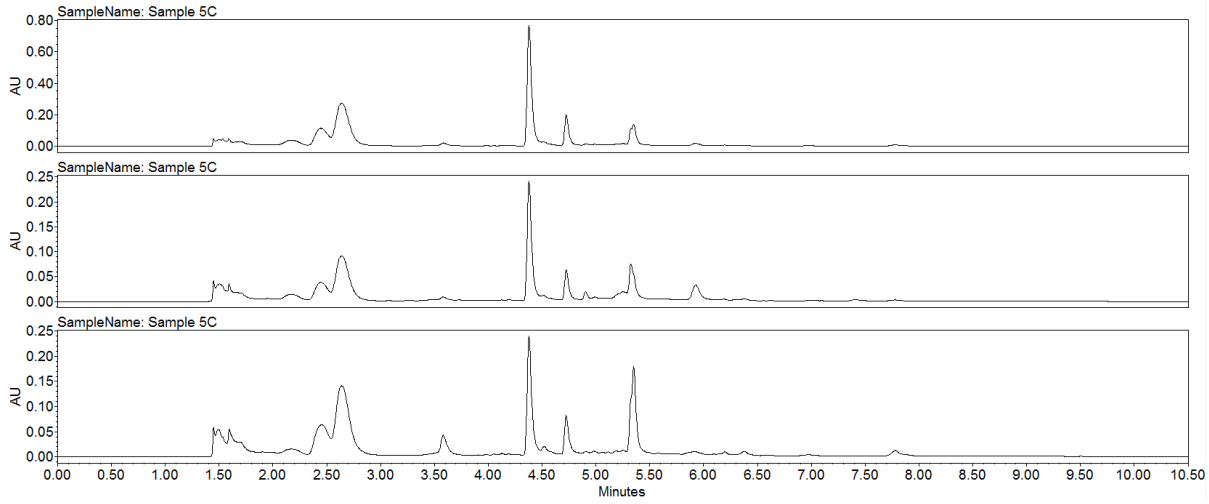
Appendix Figure 16. HPLC Chromatograms of sample 4C (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



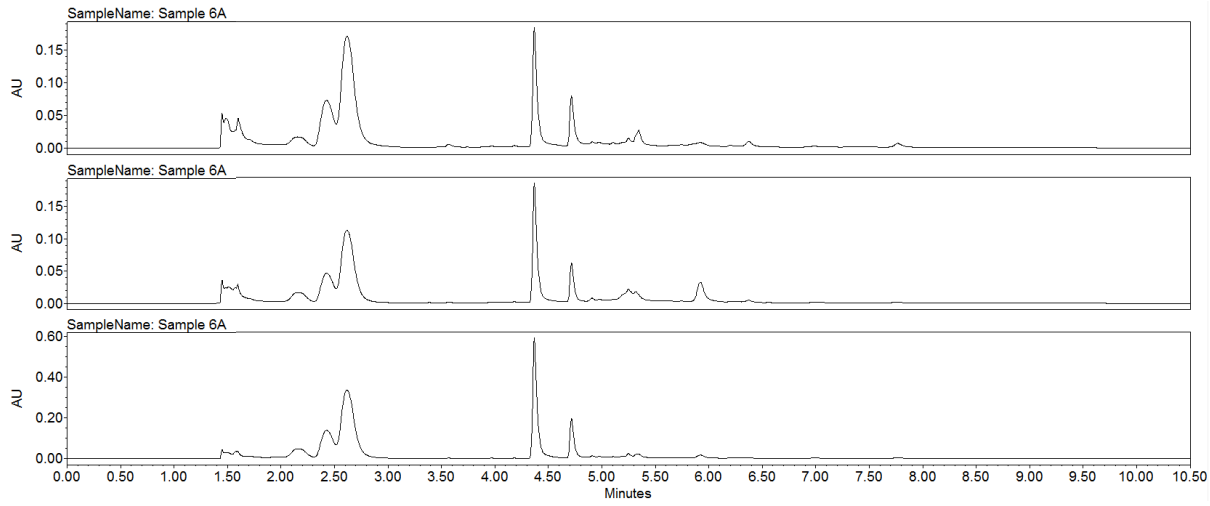
Appendix Figure 17. HPLC Chromatograms of sample 5A (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 244 nm (top), 273 nm (middle) and 260 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



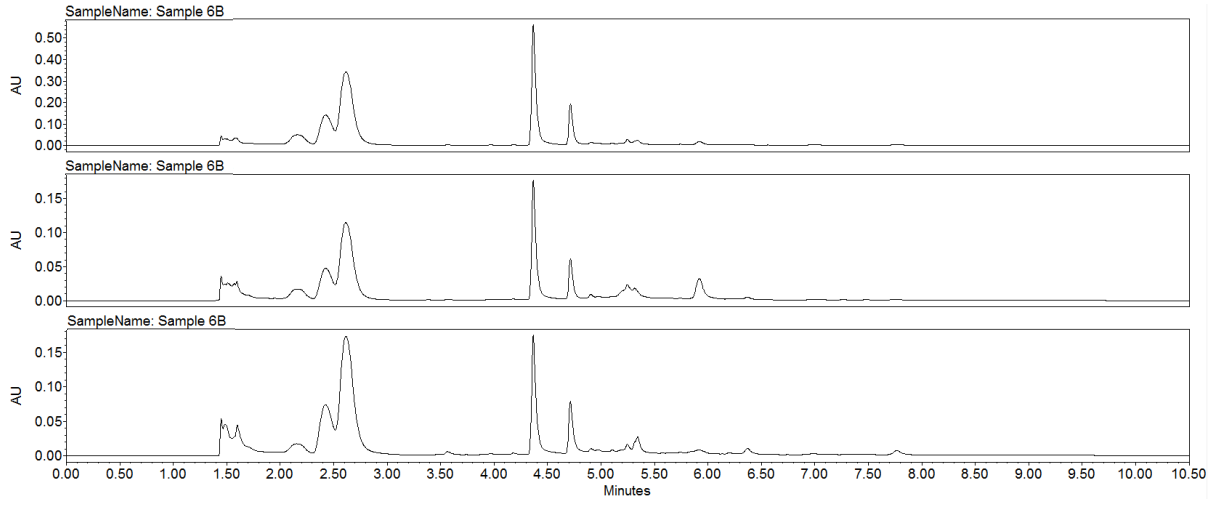
Appendix Figure 18. HPLC Chromatograms of sample 5B (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



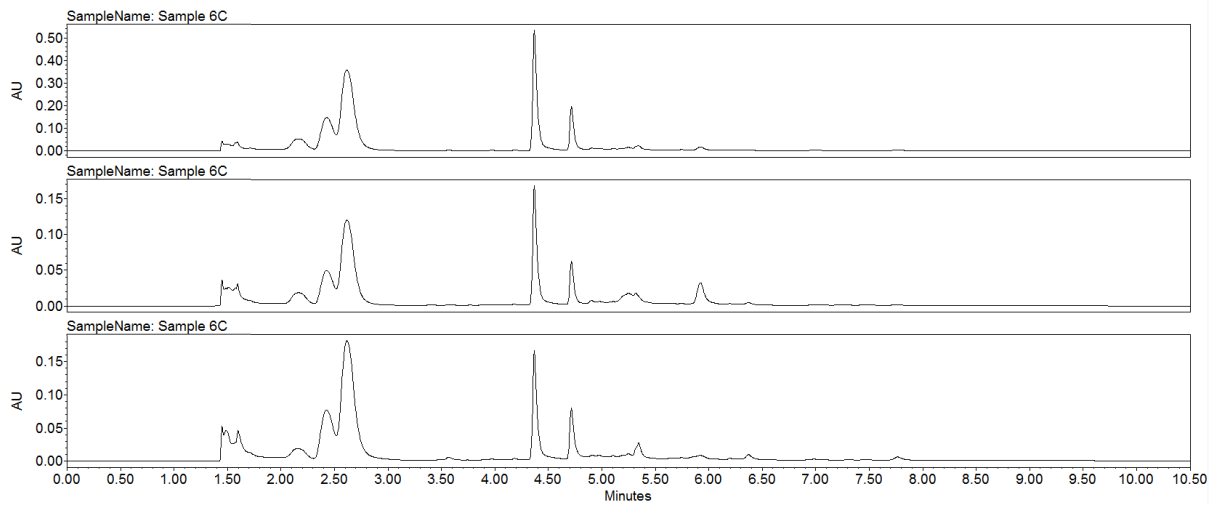
Appendix Figure 19. HPLC Chromatograms of sample 5C (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



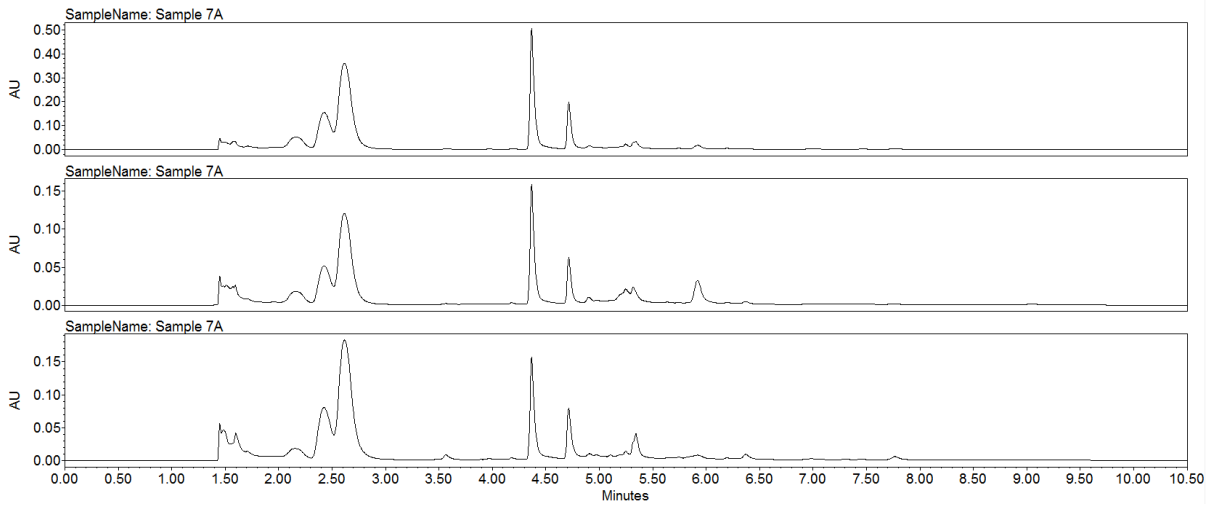
Appendix Figure 20. HPLC Chromatograms of sample 6A (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30°C, with UV Vis detection absorbance at 244 nm (top), 273 nm (middle) and 260 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



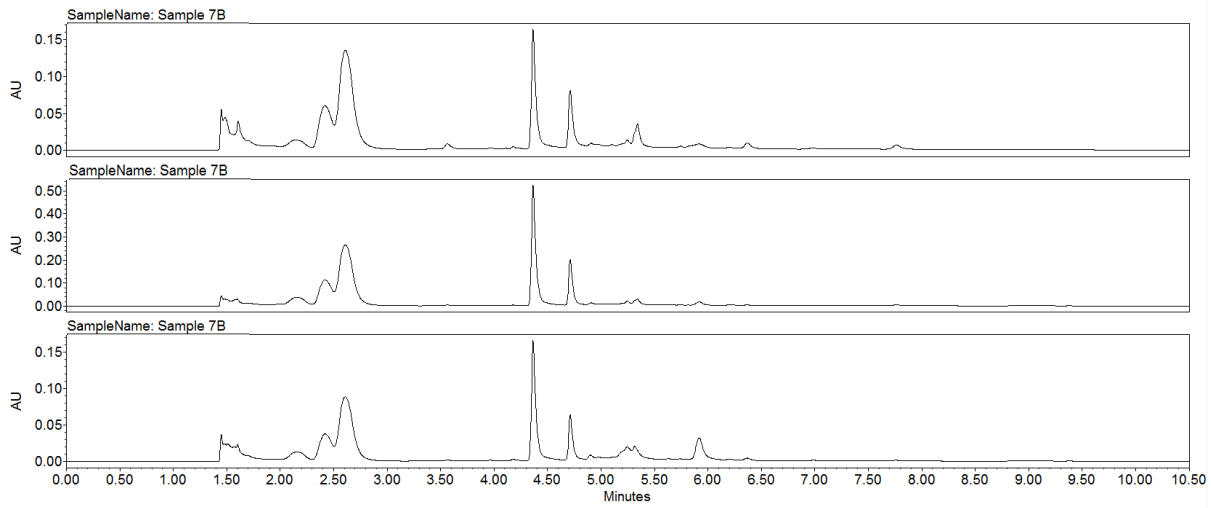
Appendix Figure 21. HPLC Chromatograms of sample 6B (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30°C, with UV Vis detection absorbance at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



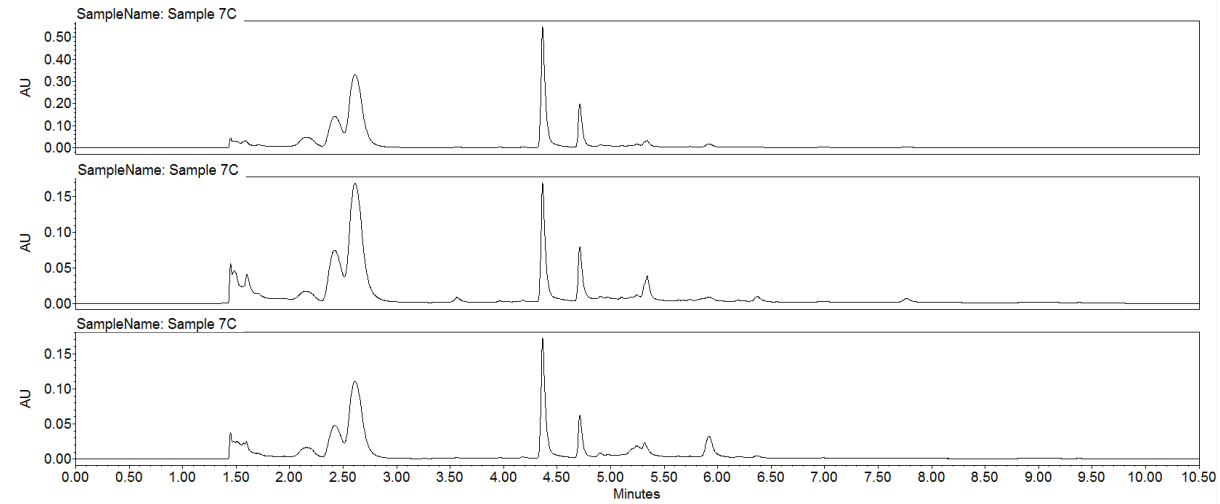
Appendix Figure 22. Analytical chromatograms of Sample 6C (260 nm – top; 273 nm – middle; 244 nm -bottom) HPLC Chromatograms of sample 5C (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



Appendix Figure 23. HPLC Chromatograms of sample 7A (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 244 nm (top), 273 nm (middle) and 260 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



Appendix Figure 24. HPLC Chromatograms of sample 7B (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 260 nm (top), 273 nm (middle) and 244 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.



Appendix Figure 25. HPLC Chromatograms of sample 7C (Phenomenex Kinetex column eluted in 70:30 water:acetonitrile and 100% acetonitrile at 0.8 mL/min at 30ºC, with UV Vis detection absorbance at 260 nm (top), 244 nm (middle) and 273 nm (bottom)). Retention times are shown in Appendix Table 4. Peak areas are shown in Appendix Table 5.

Appendix Table 4. Retention time for standards and samples for 3 in 1

Standard/ Sample	DHA (min)	HA (min)	HMF (min)	MGO 1 (min)	MGO 2 (min)	PFBHA (min)
MS1	4.011	4.749	4.924	7.341	7.810	4.406
MS2	4.007	4.746	4.921	7.338	7.807	4.402
MS3	4.004	4.744	4.919	7.337	7.805	4.400
MS4	4.004	4.743	4.921	7.336	7.804	4.399
MS5	3.999	4.739	4.923	7.334	7.800	4.395
MS6	4.001	4.740	4.921	n.d.	7.799	4.395
1A	n.d.	4.736	4.915	n.d.	7.796	4.391
1B	3.992	4.733	4.911	n.d.	7.791	4.387
1C	3.992	4.734	4.912	n.d.	7.792	4.388
2A	3.987	4.731	4.913	n.d.	7.790	4.385
2B	3.986	4.731	4.912	n.d.	7.788	4.385
2C	3.986	4.731	4.912	n.d.	7.790	4.385
3A	3.987	4.73	4.909	n.d.	7.789	4.384
3B	3.986	4.73	4.909	n.d.	7.787	4.383
3C	3.985	4.729	4.908	n.d.	7.785	4.382
4A	3.985	4.729	4.909	n.d.	7.786	4.382
4B	3.983	4.727	4.907	n.d.	7.784	4.381
4C	3.983	4.727	4.907	n.d.	7.783	4.380
5A	4.050	4.724	4.904	n.d.	7.779	4.378
5B	4.052	4.726	4.905	n.d.	7.779	4.379
5C	4.050	4.725	4.903	n.d.	7.779	4.377
6A	3.968	4.717	4.976	n.d.	7.768	4.368
6B	3.966	4.715	4.974	n.d.	7.766	4.367
6C	3.968	4.717	4.976	n.d.	7.767	4.368
7A	3.967	4.716	4.976	n.d.	7.767	4.367
7B	3.965	4.713	4.975	n.d.	7.763	4.365
7C	3.964	4.714	4.975	n.d.	7.766	4.364
High QC	3.994	4.736	4.913	7.328	7.794	4.391
Low QC	3.996	4.737	4.914	7.328	7.795	4.392

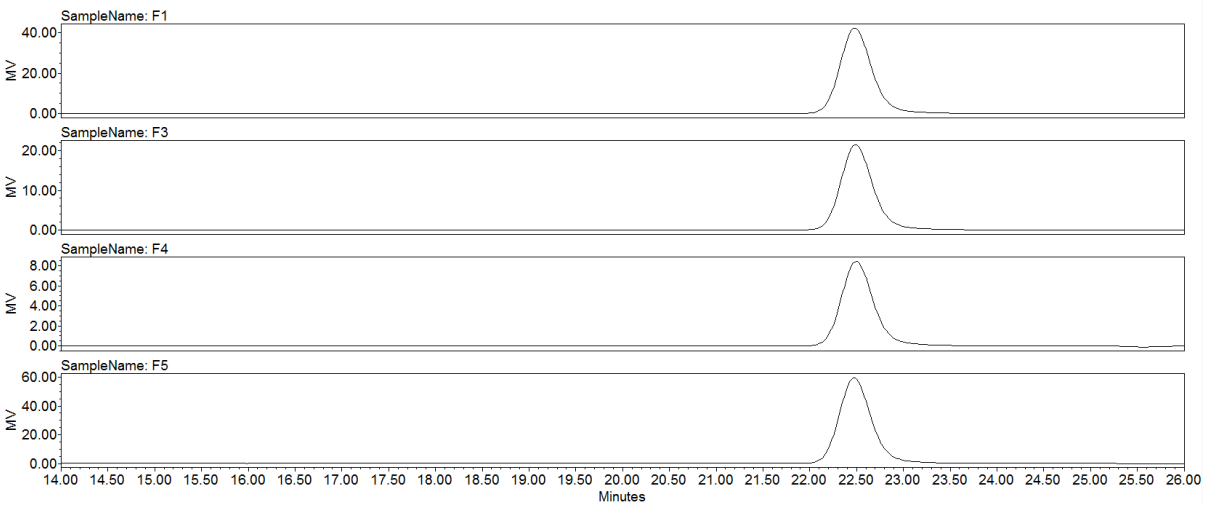
Appendix Table 5. Peak area for standards and triplicate measurements of samples 1-7 for 3 in 1

Standard/ Sample	DHA	HA	HMF	MGO 1	MGO 2	PFBHA
MS1	1275316	528049	105643	436108	10348918	1940133
MS2	840542	527371	86395	324802	7782652	2280343
MS3	647034	532642	59374	221024	5319571	2404402
MS4	323781	536028	23496	105784	2572579	2596726
MS5	51938	531563	11498	5384	116015	2681233
MS6	6932	530114	6408	n.d.	37939	2584723
1A	8237	528555	13354	n.d.	61572	2879342
1B	7178	528706	13356	n.d.	57387	2830450
1C	6757	525412	12996	n.d.	53896	2787911
2A	9896	520970	4482	n.d.	79105	2744018
2B	10243	511371	5182	n.d.	81288	2555412
2C	10023	511452	4758	n.d.	75942	2421899
3A	5496	515048	12753	n.d.	52113	2369973
3B	9878	529310	16503	n.d.	61261	2373139
3C	5977	526913	13028	n.d.	52437	2495496
4A	7937	527213	9490	n.d.	56059	2554394
4B	7362	526725	9275	n.d.	53970	2448894
4C	7112	527176	9202	n.d.	48032	2468486
5A	3277	510724	34634	n.d.	59303	2571873
5B	3275	506063	35317	n.d.	57356	2305688
5C	3285	509669	34605	n.d.	58345	2335669
6A	7454	511978	5683	n.d.	31565	1763501
6B	5742	506752	5719	n.d.	33755	1663802
6C	6626	510702	5490	n.d.	26416	1575020
7A	5817	513332	6281	n.d.	26663	1489079
7B	4992	525354	5505	n.d.	32509	1552349
7C	5755	511637	5735	n.d.	29476	1620335
High QC	401543	528340	36522	232765	4906715	2380465
Low QC	39048	528752	47864	29370	627647	2831720

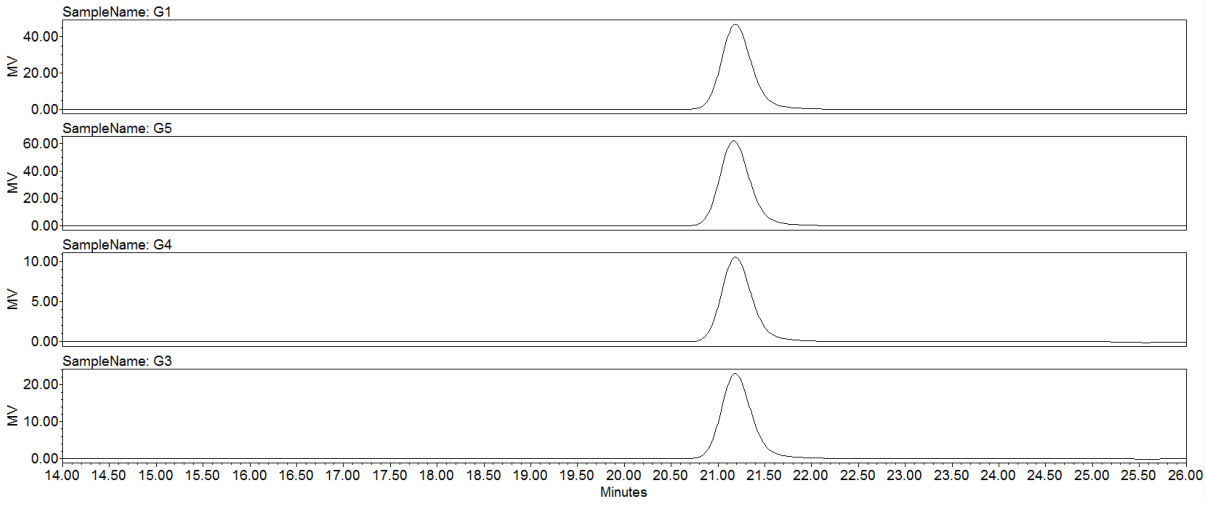
Appendix Table 6. Absorbance at 750 nm for total phenolic analysis of 27 samples of Rēkohu honey

	Absorbance 1	Absorbance 2	Absorbance 3	Absorbance 4	Absorbance 5	Absorbance 6	Average	Standard Deviation
Water Blank	0.05	0.051	0.054	0.057	0.057	0.056	0.054	0.003
0 mg/L GA	0.049	0.051	0.053	0.052	0.055	0.054	0.052	0.002
10 mg/L GA	0.08	0.081	0.09	0.069	0.077	0.091	0.081	0.008
25 mg/L GA	0.16	0.174	0.186	0.172	0.172	0.186	0.175	0.010
50 mg/L GA	0.321	0.346	0.331	0.38	0.316	0.36	0.342	0.025
75 mg/L GA	0.539	0.558	0.556	0.547	0.535	0.569	0.551	0.013
100 mg/L GA	0.658	0.686	0.712	0.716	0.716	0.743	0.705	0.029
125 mg/L GA	0.782	0.787	0.801	0.817	0.863	0.87	0.820	0.038
150 mg/L GA	0.938	0.943	0.886	0.842	0.827	0.854	0.882	0.050
175 mg/L GA	1.157	1.217	1.262	1.22	1.282	1.196	1.222	0.045
200 mg/L GA	1.272	1.34	1.409	1.307	1.37	1.486	1.364	0.076
Sample 1	0.323	0.329	0.324	0.273	0.279	0.274	0.275	0.003
Sample 2	0.31	0.313	0.365	0.26	0.263	0.315	0.279	0.031
Sample 3	0.239	0.205	0.253	0.189	0.155	0.203	0.182	0.025
Sample 4	0.341	0.337	0.337	0.291	0.287	0.287	0.288	0.002
Sample 5	0.569	0.572	0.539	0.519	0.522	0.489	0.510	0.018
Sample 6	0.277	0.277	0.286	0.227	0.227	0.236	0.230	0.005
Sample 7	0.263	0.262	0.259	0.213	0.212	0.209	0.211	0.002
Sample 8	0.264	0.301	0.303	0.214	0.251	0.253	0.239	0.022
Sample 9	0.432	0.37	0.423	0.382	0.32	0.373	0.358	0.034
Sample 10	0.294	0.28	0.269	0.244	0.23	0.219	0.231	0.013

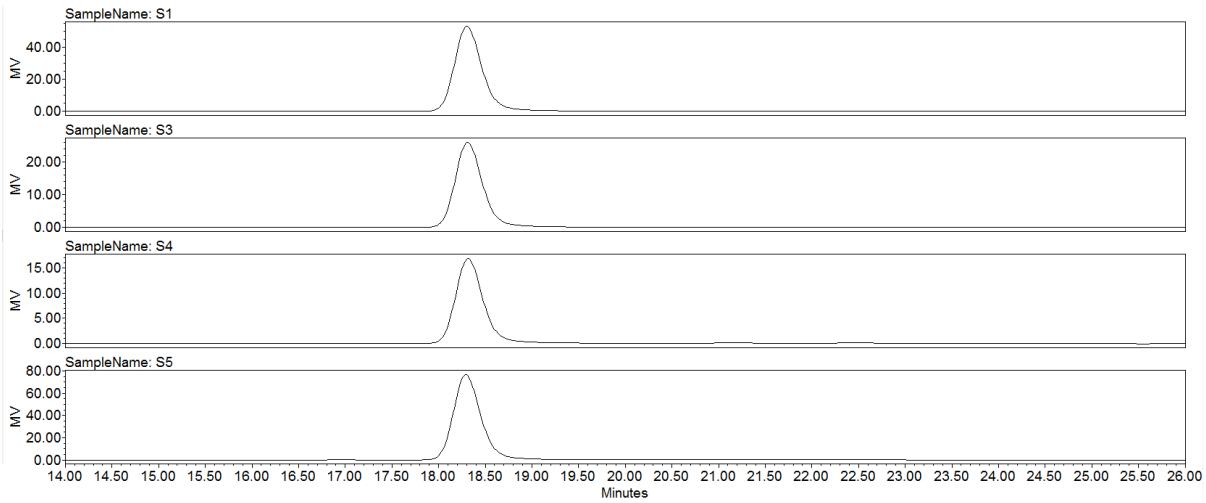
	Absorbance 1	Absorbance 2	Absorbance 3	Absorbance 4	Absorbance 5	Absorbance 6	Average	Standard Deviation
Sample 11	0.386	0.386	0.391	0.336	0.336	0.341	0.338	0.003
Sample 12	0.329	0.358	0.346	0.279	0.308	0.296	0.294	0.015
Sample 13	0.425	0.422	0.358	0.375	0.372	0.308	0.352	0.038
Sample 14	0.28	0.311	0.285	0.23	0.261	0.235	0.242	0.017
Sample 15	0.486	0.425	0.402	0.436	0.375	0.352	0.388	0.043
Sample 16	0.388	0.379	0.368	0.338	0.329	0.318	0.328	0.010
Sample 17	0.341	0.387	0.338	0.291	0.337	0.288	0.305	0.027
Sample 18	0.341	0.34	0.28	0.291	0.29	0.23	0.270	0.035
Sample 19	0.276	0.272	0.263	0.226	0.222	0.213	0.220	0.007
Sample 20	0.429	0.413	0.329	0.379	0.363	0.279	0.340	0.054
Sample 21	0.26	0.26	0.256	0.21	0.21	0.206	0.209	0.002
Sample 22	0.246	0.244	0.237	0.196	0.194	0.187	0.192	0.005
Sample 23	0.274	0.297	0.356	0.224	0.247	0.306	0.259	0.042
Sample 24	0.278	0.264	0.278	0.228	0.214	0.228	0.223	0.008
Sample 25	0.266	0.225	0.26	0.216	0.175	0.21	0.200	0.022
Sample 26	0.214	0.216	0.284	0.164	0.166	0.234	0.188	0.040
Sample 27	0.56	0.56	0.483	0.51	0.51	0.433	0.484	0.044
Manuka 1	0.724	0.841	0.773	0.674	0.791	0.723	0.729	0.059
Manuka 2	0.732	0.796	0.697	0.682	0.746	0.647	0.692	0.050
Manuka 3	0.68	0.697	0.684	0.63	0.647	0.634	0.637	0.009
Manuka 4	0.78	0.733	0.776	0.73	0.683	0.726	0.713	0.026



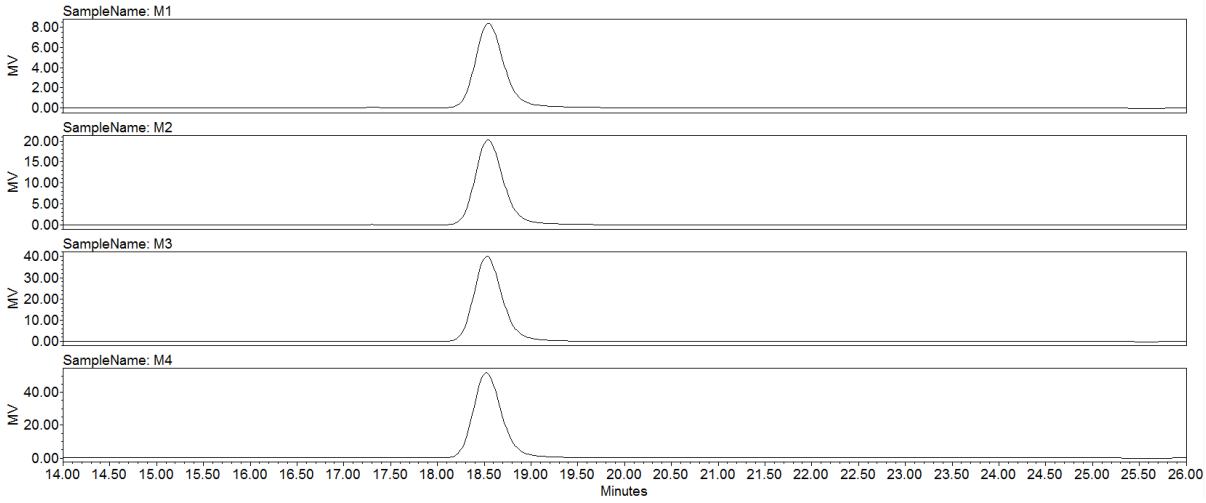
Appendix Figure 26. HPLC chromatograms (zoomed to 14 min – 26 min) of sugar standards for fructose, F1, F3-F5 (F2 is shown in Figure 3.29). (Shodex KS-G, KS-801 and KS-802 columns in series eluted with water at 0.8 mL/min at 80ºC, with refractive index detection. Retention times and peak areas are shown in Appendix Table 7.



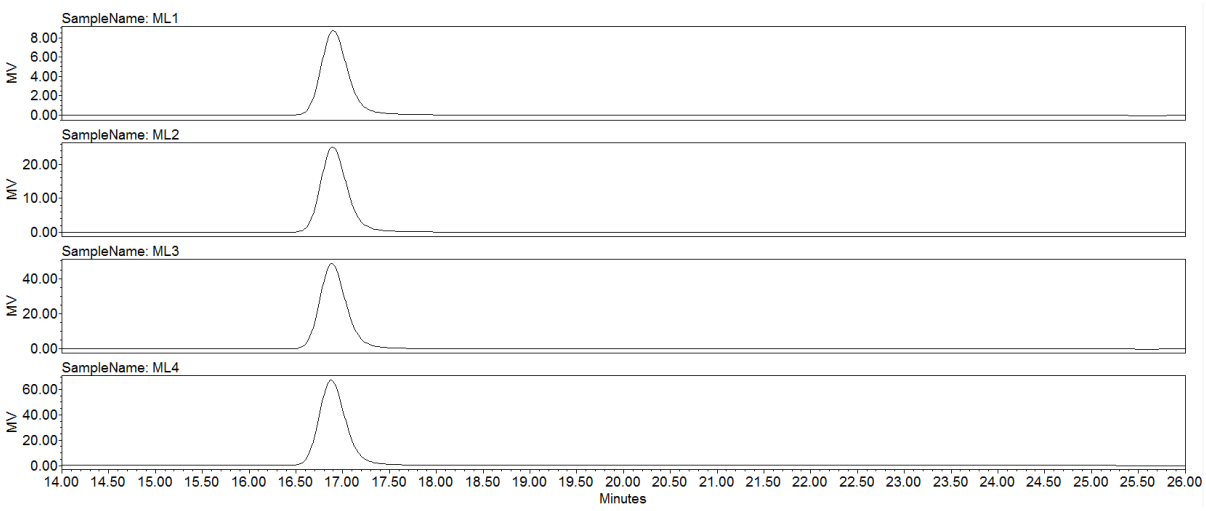
Appendix Figure 27. HPLC chromatograms (zoomed to 14 min – 26 min) of sugar standards for glucose, G1, G3-G5 (G2 is shown in Figure 3.29). (Shodex KS-G, KS-801 and KS-802 columns in series eluted with water at 0.8 mL/min at 80ºC, with refractive index detection. Retention times and peak areas are shown in Appendix Table 7.



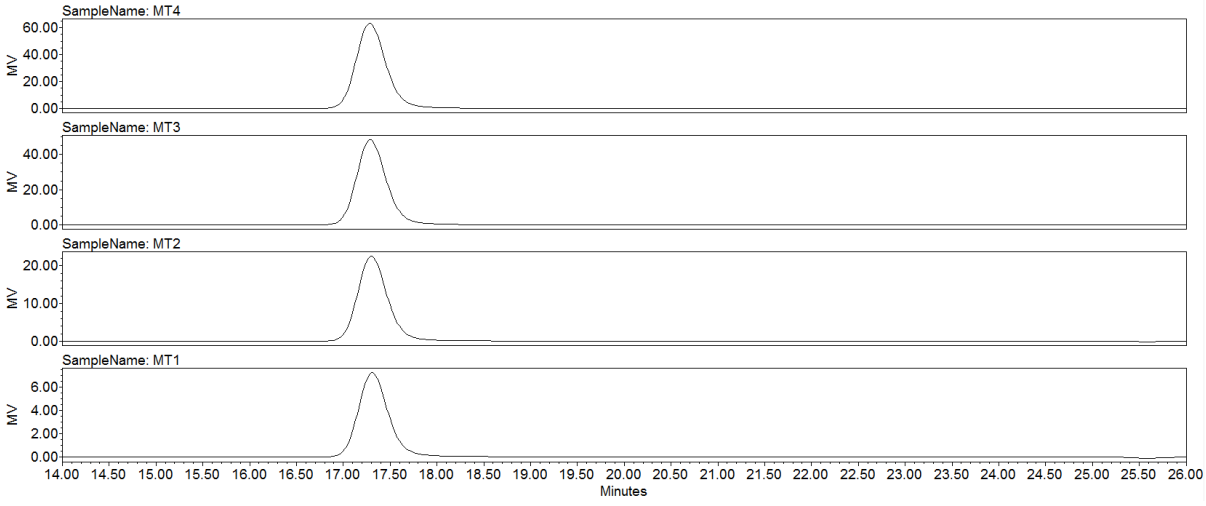
Appendix Figure 28. HPLC chromatograms (zoomed to 14 min – 26 min) of sugar standards for sucrose, S1, S3-S5 (S2 is shown in Figure 3.29). (Shodex KS-G, KS-801 and KS-802 columns in series eluted with water at 0.8 mL/min at 80ºC, with refractive index detection. Retention times and peak areas are shown in Appendix Table 7.



Appendix Figure 29. HPLC chromatograms (zoomed to 14 min – 26 min) of sugar standards for maltose, M1, M3-M5 (F2 is shown in Figure 3.29). (Shodex KS-G, KS-801 and KS-802 columns in series eluted with water at 0.8 mL/min at 80ºC, with refractive index detection. Retention times and peak areas are shown in Appendix Table 7.



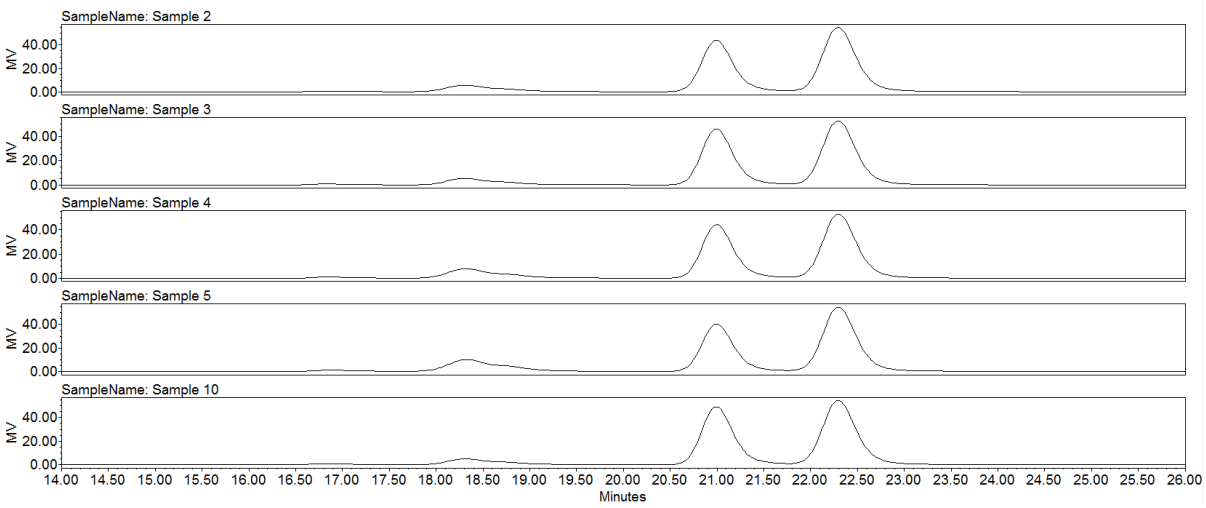
Appendix Figure 30. HPLC chromatograms (zoomed to 14 min – 26 min) of sugar standards for melizitose, ML1, ML3-ML5 (ML2 is shown in Figure 3.29). (Shodex KS-G, KS-801 and KS-802 columns in series eluted with water at 0.8 mL/min at 80ºC, with refractive index detection. Retention times and peak areas are shown in Appendix Table 7.



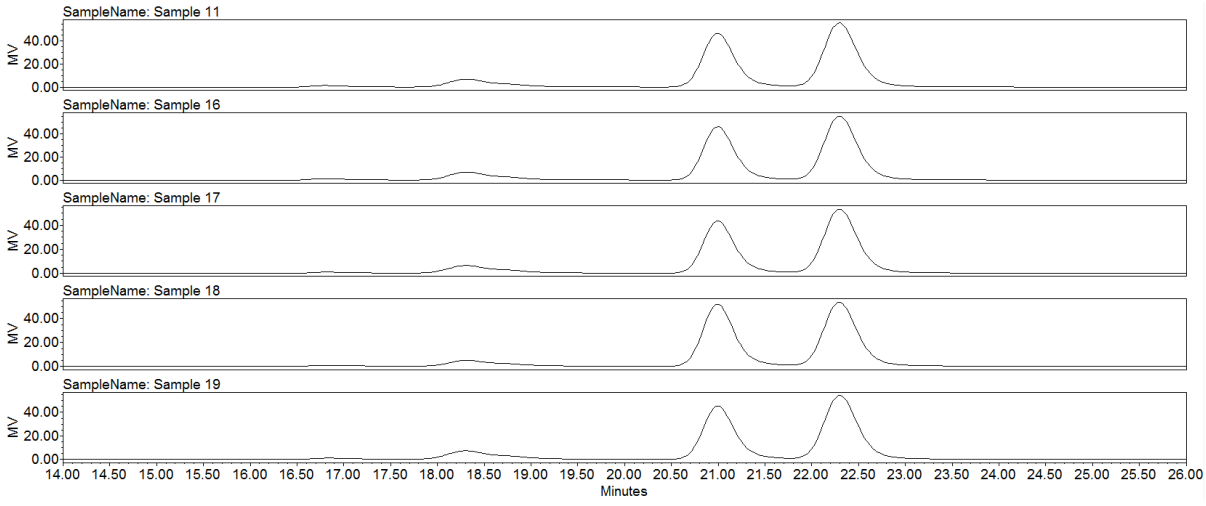
Appendix Figure 31. HPLC chromatograms (zoomed to 14 min – 26 min) of sugar standards for maltotriose, MT1, MT3-MT5 (MT2 is shown in Figure 3.29). (Shodex KS-G, KS-801 and KS-802 columns in series eluted with water at 0.8 mL/min at 80ºC, with refractive index detection. Retention times and peak areas are shown in Appendix Table 7.

Appendix Table 7. Retention time and peak areas for standards used for sugar analysis

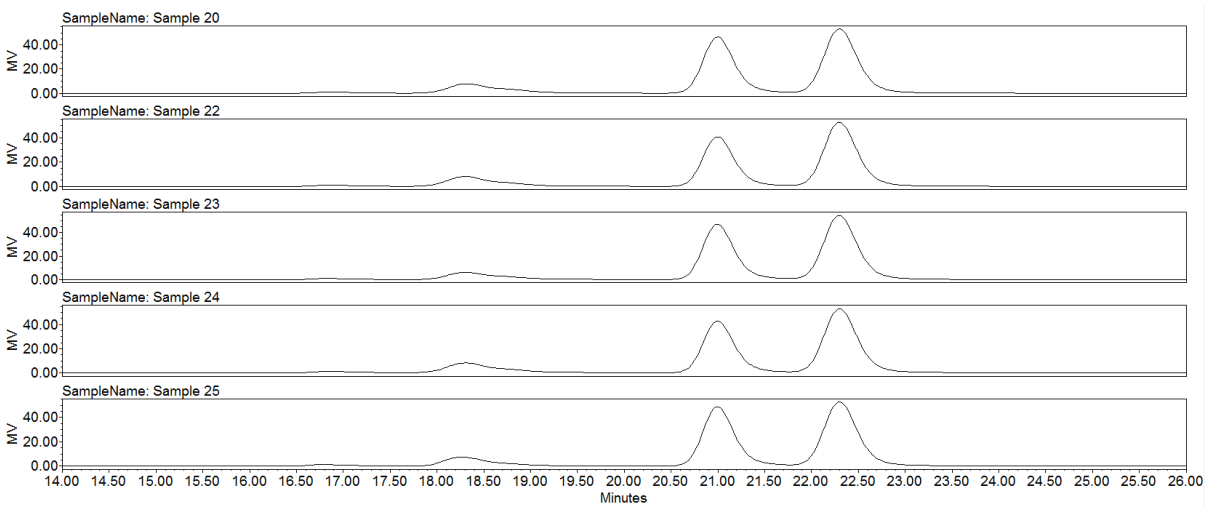
Standard	Retention Time (min)	Peak Area
S1	18.241	1102228
S2	18.227	1861703
S3	18.256	538621
S4	18.267	345984
S5	18.229	1553883
G1	21.099	1083341
G2	21.087	1760594
G3	21.119	531023
G4	21.134	244903
G5	21.09	1435182
F1	22.414	994568
F2	22.406	1816973
F3	22.436	498787
F4	22.457	204553
F5	22.409	1416159
M1	18.504	179445
M2	18.484	416048
M3	18.467	830406
M4	18.457	1114122
M5	18.45	1673652
ML1	16.899	167205
ML2	16.893	489940
ML3	16.883	957674
ML4	16.877	1342981
ML5	16.87	1750875
MT1	17.308	152277
MT2	17.301	487080
MT3	17.288	1057182
MT4	17.283	1399326
MT5	17.278	1804845



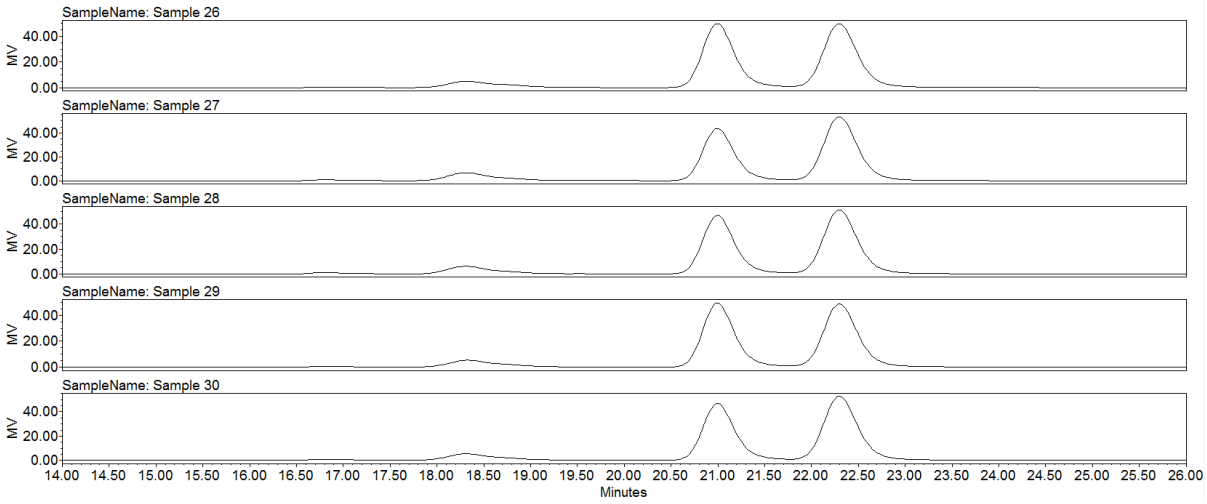
Appendix Figure 32. HPLC chromatograms (zoomed to 14 min – 26 min) of Samples 2-6 (sample 10). Shodex KS-G, KS-801 and KS-802 columns in series eluted in water at 0.8 mL/min at 80ºC, with refractive index detection. Retention times and peak areas are shown in Appendix Table 7.



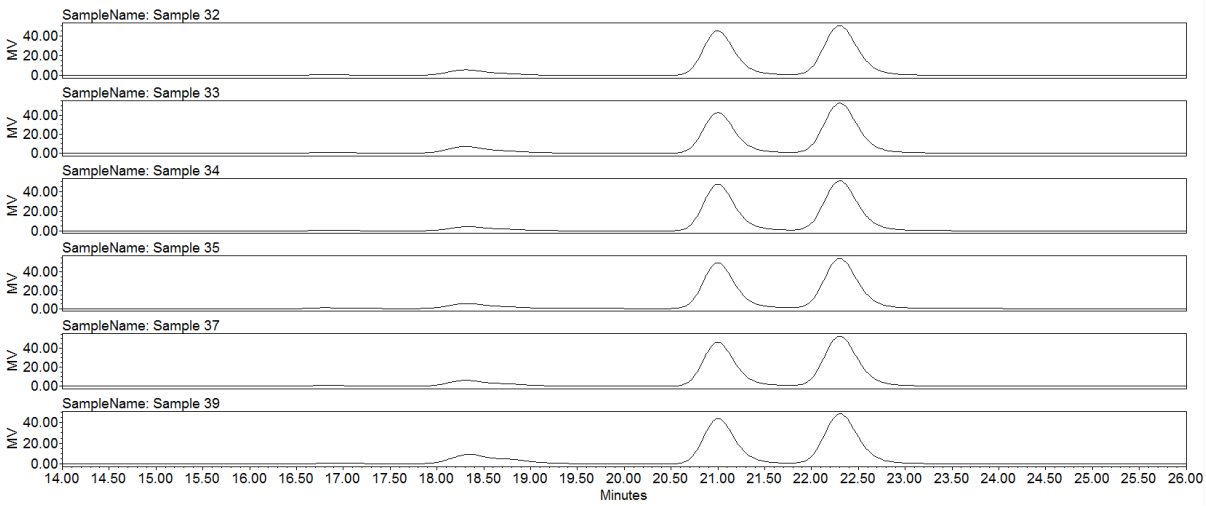
Appendix Figure 33. HPLC chromatograms (zoomed to 14 min – 26 min) of Samples 7-11 (samples 11, 16-19). (Shodex KS-G, KS-801 and KS-802 columns in series eluted in water at 0.8 mL/min at 80ºC, with refractive index detection. Retention times and peak areas are shown in Appendix Table 7.



Appendix Figure 34. HPLC chromatograms (zoomed to 14 min – 26 min) of Samples 12-16 (samples 20, 22-25). Shodex KS-G, KS-801 and KS-802 columns in series eluted in water at 0.8 mL/min at 80ºC, with refractive index detection. Retention times and peak areas are shown in Appendix Table 7.



Appendix Figure 35. HPLC chromatograms (zoomed to 14 min – 26 min) of Samples 17-21 (samples 26-30). Shodex KS-G, KS-801 and KS-802 columns in series eluted in water at 0.8 mL/min at 80ºC, with refractive index detection. Retention times and peak areas are shown in Appendix Table 7.

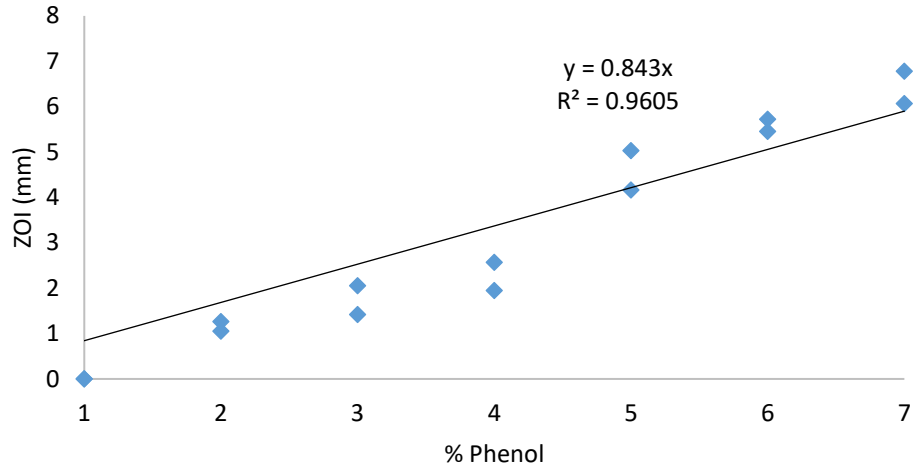


Appendix Figure 36. HPLC chromatograms (zoomed to 14 min – 26 min) of Samples 22-27 (samples 32-37, 39). Shodex KS-G, KS-801 and KS-802 columns in series eluted in water at 0.8 mL/min at 80ºC, with refractive index detection. Retention times and peak areas are shown in Appendix Table 7.

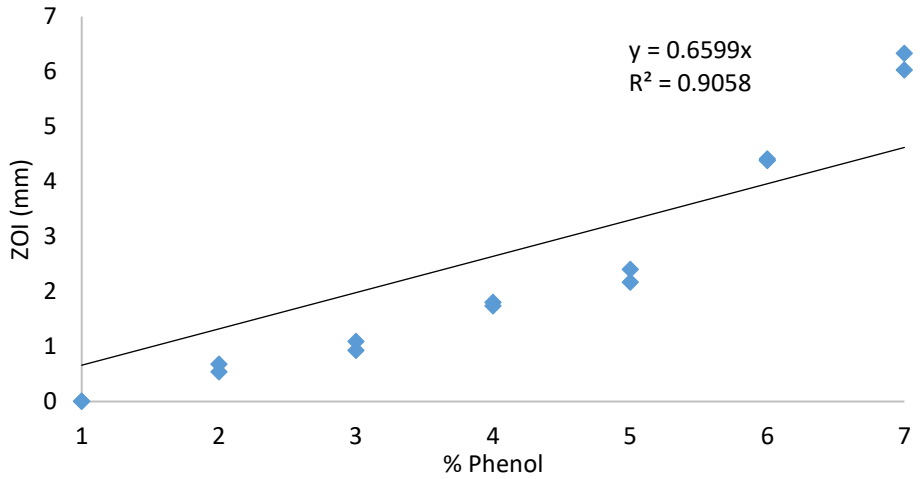
Appendix Table 8. Retention times and peak area for 27 samples of Rēkohu honey

Sample	Fructose		Glucose		Disaccharides		Trisaccharides	
	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area
1	22.296	1354157	20.995	1006915	18.302	231597	16.831	29056
2	22.293	1397534	20.993	1068301	18.308	189401	16.833	16528
3	22.294	1335732	20.993	1115774	18.302	177187	16.822	25890
4	22.298	1347274	20.997	1072183	18.318	264337	16.855	29354
5	22.297	1388527	20.996	978542	18.328	367157	16.888	30196
6	22.295	1387240	20.993	1196922	18.311	158976	16.805	24907
7	22.295	1418898	20.994	1136065	18.309	219925	16.803	17455
8	22.297	1411159	20.995	1123288	18.31	221699	16.803	15554
9	22.297	1359559	20.996	1053888	18.307	217220	16.822	25782
10	22.294	1372739	20.992	1269250	18.311	160605	16.808	24462
11	22.298	1385560	20.996	1099330	18.305	243797	16.838	30362

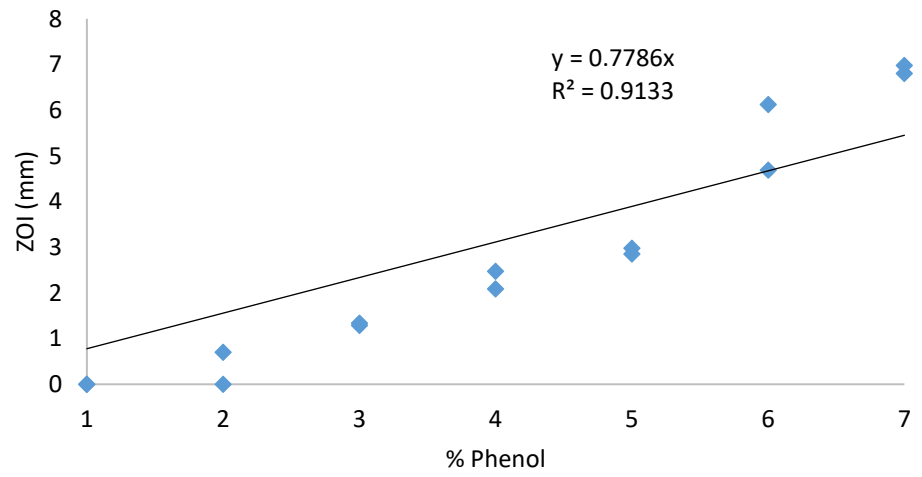
Sample	Fructose		Glucose		Disaccharides		Trisaccharides	
	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area
12	22.299	1349489	20.997	1128642	18.321	273455	16.861	26840
13	22.298	1336539	20.997	986713	18.305	275906	16.866	31727
14	22.297	1382804	20.995	1134801	18.306	208902	16.828	28048
15	22.299	1352018	20.998	1035848	18.306	276259	16.862	31966
16	22.298	1342985	20.996	1180153	18.267	233223	16.786	18700
17	22.295	1272773	20.994	1206388	18.32	168005	n.d.	n.d.
18	22.297	1356604	20.996	1051343	18.312	214149	16.813	25478
19	22.296	1307004	20.994	1135010	18.309	194279	16.787	15704
20	22.297	1243186	20.995	1195270	18.329	172569	16.802	16519
21	22.298	1345805	20.996	1133670	18.315	173271	16.806	25857
22	22.3	1287677	20.998	1089994	18.311	179192	16.802	14295
23	22.301	1335328	21	1032752	18.309	223843	16.823	29707
24	22.302	1291513	21.001	1145245	18.331	135593	16.811	14653
25	22.301	1394415	21	1215309	18.324	175092	16.802	26077
26	22.301	1342399	21	1128027	18.307	205382	16.815	28956
27	22.305	1232339	21.003	1057342	18.347	336642	16.917	26341



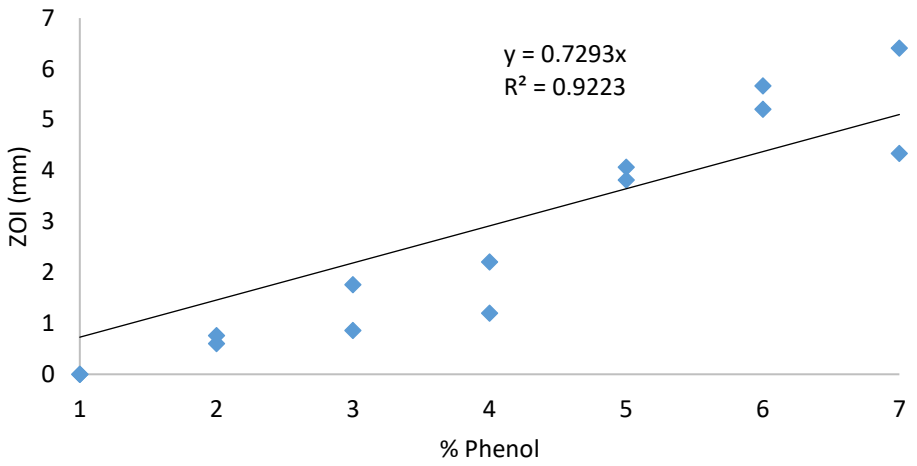
Appendix Figure 37. Standard curve of the antibacterial activity of phenol for plates 3 and 4



Appendix Figure 38. Standard curve of the antibacterial activity of phenol for plates 5 and 6



Appendix Figure 39. Standard curve of the antibacterial activity of phenol for plates 7 and 8



Appendix Figure 40. Standard curve of the antibacterial activity of phenol for plates 1 and 2

Appendix Table 9. T-test analysis for zones of inhibition against *S. Aureus* at 1000 mg/mL, 500 mg/mL, 250 mg/mL and 125 mg/mL for 27 samples of Rēkohu honey and 1 sample of 900+ MGO mānuka honey

Sample	P value	Plate Values Significant at 95% confidence? ($p < 0.05$)	Sample	P value	Plate Values Significant at 95% confidence? ($p < 0.05$)
7 – 1000 mg/mL	0.23	N	20 – 500 mg/mL	0.08	N
8 – 1000 mg/mL	0.03	Y	21 – 1000 mg/mL	0.16	N
9 – 1000 mg/mL	0.16	N	21 – 500 mg/mL	0.37	N
11 – 1000 mg/mL	0.57	N	22 – 1000 mg/mL	0.74	N
11 – 500 mg/mL	0.48	N	22 – 500 mg/mL	0.38	N
12 – 1000 mg/mL	0.95	N	23 – 1000 mg/mL	0.61	N
12 – 500	0.71	N	24 – 1000	0.41	N

Sample	P value	Plate Values Significant at 95% confidence? ($p < 0.05$)	Sample	P value	Plate Values Significant at 95% confidence? ($p < 0.05$)
mg/mL			mg/mL		
13 – 1000 mg/mL	0.79	N	24 – 500 mg/mL	0.60	N
13 – 500 mg/mL	0.86	N	24 – 250 mg/mL	0.26	N
14 – 1000 mg/mL	0.87	N	25 – 1000 mg/mL	0.48	N
15 – 1000 mg/mL	0.89	N	25 – 500 mg/mL	0.30	N
15 – 500 mg/mL	0.46	N	25 – 250 mg/mL	0.23	N
16 – 1000 mg/mL	0.08	N	26 – 1000 mg/mL	0.79	N
16 – 500 mg/mL	0.24	N	Mānuka – 1000 mg/mL	0.48	N
17 – 1000 mg/mL	0.09	N	Mānuka – 500 mg/mL	0.47	N
18 – 1000 mg/mL	0.26	N	Mānuka – 250 mg/mL	0.58	N
19 – 1000 mg/mL	0.13	N	Mānuka – 125 mg/mL	0.32	N
20 – 1000 mg/mL	0.38	N			

Appendix Table 10. Absorbance at 517nm for determination of antioxidant capacity in 27 samples of Rēkohu honey

	Absorbance 1	Absorbance 2	Average
Blank 1	0.351	0.462	0.407
Blank 2	0.044	0.039	0.042
Blank 3	0.042	0.04	0.041
Standard 1 – 0 µg/mL	0.492	0.489	0.491
Standard 2 – 40 µg/mL	0.339	0.358	0.349
Standard 3 – 60 µg/mL	0.318	0.343	0.331
Standard 4 – 80 µg/mL	0.268	0.293	0.281
Sample 1	0.3	0.376	0.338
Sample 2	0.345	0.38	0.363
Sample 3	0.417	0.416	0.417
Sample 4	0.339	0.403	0.371
Sample 5	0.379	0.341	0.360
Sample 6	0.363	0.407	0.385
Sample 7	0.335	0.469	0.402
Sample 8	0.355	0.402	0.379
Sample 9	0.309	0.292	0.301
Sample 10	0.386	0.404	0.395
Sample 11	0.334	0.369	0.352
Sample 12	0.345	0.395	0.370
Sample 13	0.34	0.365	0.353
Sample 14	0.395	0.453	0.424
Sample 15	0.313	0.387	0.350
Sample 16	0.37	0.447	0.409
Sample 17	0.351	0.359	0.355
Sample 18	0.383	0.462	0.423
Sample 19	0.331	0.433	0.382
Sample 20	0.334	0.372	0.353
Sample 21	0.39	0.502	0.446
Sample 22	0.438	0.518	0.478
Sample 23	0.352	0.469	0.411
Sample 24	0.44	0.505	0.473
Sample 25	0.383	0.487	0.435
Sample 26	0.403	0.504	0.454
Sample 27	0.281	0.313	0.297