Characterisation of the aroma profiles of New Zealand monofloral honeys.

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Abstract

New Zealand hosts a variety of monofloral honeys with distinct aromas and flavours. These sensory properties are determined by the volatile organic compounds present in the honey, of which over 600 have been identified. The volatile profile of honey is primarily influenced by botanical origin, geographic origin, storage and processing conditions. Not all compounds in the volatile fraction are represented in the aroma profile of the honey, due to the varying odour thresholds at which compounds can be detected by the consumer. Volatile compounds in New Zealand honeys have previously been studied for authentication purposes, but no New Zealand studies to date have linked the volatile composition with honey aroma. Thus, the aim of this research was to develop and optimise a method for the extraction and analysis of volatile compounds in New Zealand monofloral honeys, to determine the chemical differences responsible for the unique aromas of honeys of different botanical origins.

Various methods were trialled for the extraction of the volatiles, which are present in very low concentrations, from the complex honey matrix. Solvent extraction using dichloromethane was chosen, and samples were analysed by gas chromatography-mass spectrometry in selected ion monitoring mode. This demonstrated excellent sensitivity at the low part-per-billion level and allowed for identification of compounds not seen in total ion chromatograms. The method showed excellent precision and linearity over wide concentration ranges. Eighteen compounds were semi-quantified using this method, with full quantification achieved for four of these: nonanal, cineole, benzyl alcohol and guaiacol. Additional volatiles were screened using total ion chromatogram.

The optimised method was used for the analysis of 44 New Zealand monofloral honey samples spanning 11 floral origins. All 18 compounds were detected in all honey types, but not in all samples. D-limonene, β-ionone and cineole are reported for the first time in any New Zealand honey, and phenylacetaldehyde and nonanal are reported for the first time in kānuka (Kunzea ericoides) honey. Results were comparable with the literature for these honey types, with mānuka honey characterised by o-methoxyacetophenone and kāmahi honey by 4-oxoisophorone. New Zealand clover honey contained low levels of volatiles which has also been reported for international clover honeys. The volatile profiles were also compared with odour thresholds to suggest possible aroma contributors for each honey type. Nonanal was determined to be aroma-active in all honeys analysed, and cineole in all but ling, lotus/blackberry and rātā. Chemometric methods including principal component

analysis, classification and regression trees and linear discriminant analysis were used to identify similarities in samples based on their volatile profiles. Samples were classified by honey type using linear discriminant analysis with 82.9% accuracy, with the most accurate classification achieved for kāmahi and kānuka honeys.

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Abbreviations

%RE: percentage relative error

%RSD: percentage relative standard deviation

AEDA: aroma extract dilution analysis

ANOVA: analysis of variance

BSTFA: *N,O*-Bis(trimethylsilyl)trifluoroacetamide

CART: classification and regression trees

CW: Carbowax

DCM: dichloromethane

DVB: divinyl benzene

FD: flavour dilution factor

FID: flame ionisation detection

GC: gas chromatography

GC-MS: gas chromatography-mass spectrometry

GC-O: gas chromatography-olfactometry

HMF: 5-Hydroxymethyl-2-furfural

HS: headspace

LC-MS: liquid chromatography-mass spectrometry

LDA: linear discriminant analysis

LLE: liquid-liquid extraction

LOD: limit of detection

LOQ: limit of quantification

MPI: Ministry for Primary Industries

MS: mass spectrometry

n.d.: not detected

NIST: National Institute of Standards and Technology

NMR: nuclear magnetic resonance

OAV: odour activity value

OCR: odour contribution rate

PA: poly(acrylate)

PCA: principal component analysis

PDMS: polydimethylsiloxane

PEG: polyethylene glycolRI: retention index

RT: retention time

SDE: simultaneous distillation-extraction

SIM: selected ion monitoring

SMC: system monitoring compound

SNR: signal-to-noise ratio

SPE: solid-phase extraction

SPME: solid-phase micro-extraction

TIC: total ion chromatogram

TMS: trimethylsilyl

USE: ultrasonic extraction

1. Chapter One: Introduction and Literature Review

1.1. Background

Honey is a sweet, syrupy liquid produced by honey bees (*Apis mellifera*) from the nectar of plants. [1,2] It is mainly composed of sugars (primarily fructose and glucose) and water. Other compounds are present in minor concentrations such as lipids, amino acids, flavonoids, proteins, vitamins, minerals and a wide range of volatile compounds. [3,4] It has a sweetness comparable to table sugar and is used as a sweetener in many parts of the world. [1] Honey also has numerous nutritional benefits, including antioxidant and antimicrobial properties, and is used for treatment of cold, flu and gastrointestinal disorders, among others. [4] Honey is widely consumed on its own, added to other products such as toast or tea, and used in cooking and baking.

New Zealand's apiculture industry is a $5 billion industry. The value of New Zealand's honey exports in the 2019-2020 financial year was $425 million, with 88 percent of this revenue attributed to mānuka (*Leptospermum scoparium*) honey. [5] Mānuka is New Zealand's most popular and profitable honey due to its unique non-peroxide antibacterial activity, which is due to methylglyoxal. [6,7] The average price of mānuka honeys was $55.36 per kilogram, while non-mānuka honeys fetched $22.50 per kilogram. [5] However, the country also produces many other monofloral honeys, each with their own distinctive flavours. [8-11] The CODEX Alimentarius 'Standard for Honey' defines monofloral honey as honey that "comes wholly or mainly from that particular source and has the organoleptic, physicochemical and microscopic properties corresponding with that origin." [2] The consumer demand for monofloral honeys has increased in recent years, due to their unique flavour and pharmacological properties.

1.1.1. Authentication of monofloral honeys

The market value of honey has increased due to rising consumer demand for natural food products. Unfortunately, this increased demand has also increased the occurrence of honey adulteration. Monofloral honeys are susceptible to mislabelling due to their increased market value compared to multifloral honeys. [8,10] In 2014, the Ministry for Primary Industries (MPI) reported that 1700 tonnes of mānuka honey was produced in New Zealand, but over 10,000 tonnes of the honey were sold worldwide. [12,13] Although honey is sometimes stored before export, these figures suggest that over 80% of honey labelled as mānuka may have been

labelled fraudulently. In the last twenty-five years, a wide variety of studies have focussed on methods of controlling the authenticity of monofloral honeys to avoid such issues.

Traditionally, melissopalynology (pollen analysis) is used to identify the botanical origin, alongside physicochemical properties such as colour, conductivity, pH and sugar profiles. [14-19] However, melissopalynology relies on considerable experience and training of the operator, and is time intensive. In some cases, such as mānuka and kānuka (*Kunzea ericoides*) , the pollen grains are visually indistinguishable. [20,21] In addition, some honeys such as citrus (*Citrus spp.*) and lavender (*Lavandula angustifolia Mill.*) do not contain pollen due to asexual reproduction of the crop. [22] The concentration of pollen in honey varies between species due to pollen structure, type, and the structure of the flower. For example, most honeys contain between 20,000 and 100,000 pollen grains per 10 g sample, but rewarewa (*Knightia exelsa*) and tāwari (*Ixerba brexioides*) pollen are under-represented in honey (less than 20,000 grains per 10 g sample) whereas mānuka is over-represented (greater than 100,000 grains per 10 g sample). [20] For these reasons, pollen analysis alone cannot be used to identify monofloral honeys with certainty. [23]

Attention has turned to the identification of marker compounds. [10, 24-26] In some cases, a combination of presence of some compounds and absence of others, or a "fingerprint" of a variety of compounds, is used as a marker, while in other cases, the presence of a single unique compound is sufficient to authenticate the botanical origin of the monofloral honey. [8] Marker compounds are typically identified in the volatile fraction of the honey, from which over 600 compounds have been reported. [9,19] These belong to a variety of chemical classes, in particular monoterpenes, norisoprenoids (especially C_{13} norisoprenoids), sesquiterpenoids, and benzene derivatives. [4] Alcohols, esters, fatty acids, aldehydes and ketones are also present. [4] The volatile composition of honey depends on its botanical and geographical origin, as well as other factors such as metabolic activity of the honey bee, storage and technical processing. [8] Several studies have reported the same volatile compounds in monofloral honeys as in nectar from the plants they originated from, indicating their suitability for authentication of honey origin. [11,27,28] For example, to be classified as monofloral mānuka, honey must contain ≥ 400 mg/kg 3-phenyllactic acid, ≥ 1 mg/kg *o*-methoxyacetophenone, ≥ 1 mg/kg 2-methoxybenzoic acid and ≥ 1 mg/kg 4-hydroxyphenyllactic acid. [21] To be classified as the lower-value multifloral mānuka, lower limits have been set for the same four compounds. The volatile profiles of honeys of a wide range of floral origins have been

studied to identify possible marker compounds, and these studies dominate the field of research of honeys volatile fraction.

1.1.2. Honey flavour

Aroma is one of the most important factors influencing honey quality and thus its commercial value. [29] Consumer selection of honey products is often determined by the aroma. [3] Honey aroma has traditionally been assessed qualitatively, using sensory panels. A sensory panel is a group of trained professionals that use a defined method and vocabulary to objectively evaluate all the sensory properties of a food product. [30] For example, in a study of Austrian honeys, the flavour of chestnut (*Castanea sativa*) honey was described as "sweet, bitter, burnt caramel and woody", while fir tree (*Abies* spp.) honey was described as "sweet, slightly bitter, caramel and slightly sour". [9] Other honeys analysed by the same panel were described using much of the same vocabulary, with additional terms such as "honey-flavour", "medicinal", and "slightly fermented" used for some varieties.

While this method of describing honey aroma is irreplaceable, it does have limitations. Sensory panels are time consuming and require highly trained professionals. While defined methods and vocabulary are used in an attempt to make them as objective as possible, the descriptions are influenced by physiological and psychological traits of the panel members and the information provided is qualitative. [3] In order to market monofloral honey flavours to the consumer, and to guarantee that these flavours will be present at detectable levels in the individual batch, quantitative analysis must also be performed. The following section discusses the popular analytical methods involved in this process.

1.2. Characterising the aroma profile of honey

In addition to providing an indicator of botanical origin, the volatile compounds in honey contribute to its aroma. The impact of a compound on the overall aroma of the honey depends on the extent to which its concentration exceeds its odour threshold. [11] The odour threshold is the lowest concentration at which the observer can detect or recognise an odour. [31] For this reason, even compounds present in low concentrations can contribute significantly to the sensory profile of the honey. [11] Quantitative analysis of these volatile compounds provides valuable information on contributors to the sensory aspects of the product. This information can be combined with advanced sensory techniques to determine which compounds contribute to honey aroma and flavour.

1.2.1. Gas chromatography – mass spectrometry

The complexity of the honey matrix makes analysis of the volatile fraction challenging. The volatile compounds responsible for honey aroma must be separated from each other and from the honey matrix, which contains sugars, water, lipids, proteins, vitamins and minerals, among other compounds, prior to analysis. The volatiles are of relatively low molecular weight (below 200 amu) and have a range of functionalities and polarities, although they are typically soluble in non-polar solvents. Total volatile concentration is typically around 50-400 μg kg^{-1}, and individual compound concentrations span several orders of magnitude. [8] This provides an analytical challenge as a highly sensitive instrument is required for their detection. Gas chromatography-mass spectrometry (GC-MS) is the instrument of choice for this work due to its high separation efficiency and sensitivity.

GC has been used for the analysis of honey volatiles since the 1960s. Dörrscheidt and Friedrich (1962) separated 31 compounds from ether extracts of honey by GC with flame ionisation detection (FID). [32] Only four of these were common to the six honey types analysed, indicating the volatile composition may be dependent on the floral source. However, only methyl acetate and methyl butyrate were identified with certainty. Merz (1963) carried out a similar study, separating eight compounds but identifying only 5-hydroxymethyl-2-furfural (HMF). [33] The inclusion of a rudimentary olfactometric detection method (see section 1.2.2 for further explanation) allowed Merz to conclude that the majority of the compounds responsible for aroma were high-boiling volatiles with low vapour pressures. A combination of GC, column, and thin layer chromatography was used by ten Hoopen (1963) to identify formaldehyde, acetaldehyde, acetone, diacetyl and isobutyraldehyde. [34] Among three honey types, these compounds were found in all, but in different amounts, supporting Dörrscheidt and Friedrich's idea that the gas chromatogram may provide a "fingerprint" for characterising floral origin. Eventually, studies were able to identify a greater range of compounds using mass spectrometric detection, when this technique became affordable. This was used as early as 1964 by Cremer and Riedmann, who identified diethyl ether, ethanol, acetaldehyde and acetone using this technique. [35]

When research on aroma compounds in food was in its early stages, the assumption was that all volatile compounds in food contributed to the aroma. [36] GC was the primary method used to analyse volatiles, and if a volatile was identified in the gas chromatogram of a particular sample, it was therefore assumed to have a contribution to the overall sensory properties of the food. Patton and Josephson (1957) were the first to study the relative contributions of

individual compounds to the aroma of food. [37] This was calculated as the ratio of the compounds concentration in the extract to its odour threshold. This value is known as the odour activity value (OAV). Patton and Josephson suggested that compounds present in concentrations above their odour threshold contribute to the aroma of the food. [37] Nearly seventy years later, this relationship still holds in most instances and is used for determining the aroma-active compounds in foods.

To determine the OAV of a compound in a food sample, two values must be determined. The compound's concentration is determined by quantitative GC-MS, while the odour threshold is determined by sensory panel. A range of concentrations of the compound are presented to the panel, and the concentration at which 50% of the panel reports a positive response is identified as the odour threshold. [37] It is not necessary for the odour to be identified or recognised, only detected. The odour threshold values used to calculate OAVs must be determined in a matrix as close to the sample matrix as possible as odour threshold is dependent on the compound's vapour pressure, which is influenced by the compound's interaction with the matrix. [38,39] Odour thresholds in water are typically used for honey samples without issue, as honey is an aqueous solution. Unfortunately, despite well-defined methods for measuring odour thresholds, the literature values can differ significantly. For example, acetic acid's odour threshold varies from 22,000 to 320,000 µg L^{-1} in water. [40] Czerny *et al.* recommended a GC-olfactometry "purity check" of all reference compounds used to calculate odour thresholds, as contaminants with low odour thresholds would affect the accuracy of the calculated odour threshold of the reference compound. [40]

GC-MS is currently one of the most widely used techniques for the analysis of volatile compounds in honey, and most of the research presented in this literature review was performed using this technique, using either OAV calculations or gas chromatography-olfactometry (see section 1.2.2) to determine the contribution of compounds to honey aroma. GC-MS was used in this study, and further information is available in the results section (see Chapter 5).

1.2.2. Gas chromatography – olfactometry

An extensive range of volatile compounds can be characterised by GC, and their contribution to honey aroma determined by calculation of OAVs. However, those compounds that are present in concentrations below the detection limit of the traditional MS or FID detectors may still contribute to the aroma if their odour thresholds are low. Therefore, the GC

chromatogram does not accurately represent the entire aroma profile of the sample. [41,42] The human nose is more sensitive than traditional FID or MS detectors to many aroma-active compounds. Gas chromatography-olfactometry (GC-O), first reported in 1964, uses human assessors to detect and evaluate odour-active volatile compounds eluting from a GC separation. [42,43]

Assessors sniff the eluate using a specially designed port that either takes the place of, or complements, the traditional FID or MS (Figure 1). This allows for the comparison of the detected odours with the eluting compounds to identify the aroma-active compounds in the sample, and the detection of aroma-active compounds that may be present in concentrations too low to be detected on the traditional detectors. [41] As an example, Figure 2 compares a GC-FID chromatogram of hop oil components with the GC-O aromagram obtained by charm analysis. The combination of these two techniques is essential for identifying the key contributors to aroma. However, the intensity of detected aromas cannot be quantitatively measured by sniffing, and is affected by the concentration of the sample. For this reason, several techniques have been developed to determine the relative importance of odourants in an extract.

Figure 1. Odour assessor sniffing the eluting compounds from a GC separation using an olfactometry port. ***Image copyright Sensnenet holding BV, used with permission.*** [44]

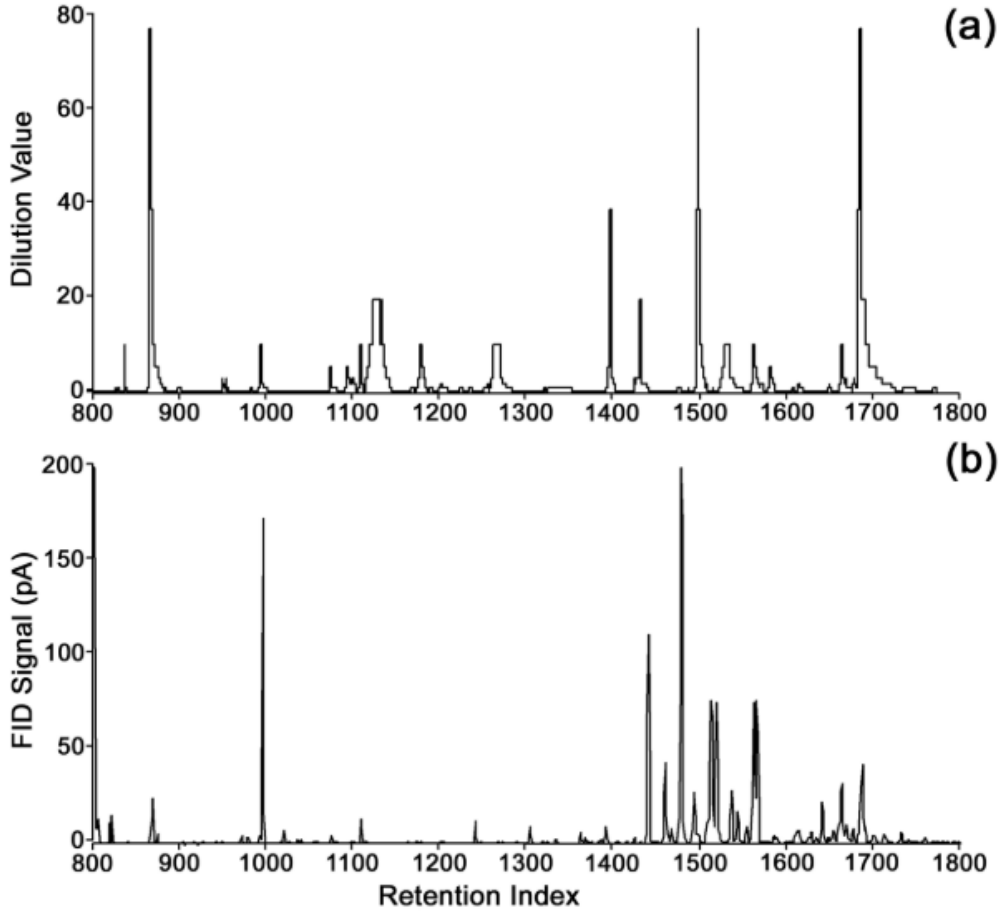

Figure 2. Comparison of (a) the GC-O aromagram generated using charm analysis with (b) the FID chromatogram for a hop essential oil sample. ***Reproduced with permission from Delahunty et al. (2006).*** [42]

Detection frequency experiments operate on the assumption that the proportion of a panel that is able to detect an odour (detection frequency) is related to the actual odour intensity. [42] The panel carry out GC-O on an extract and the number of subjects that detect an odour at a particular retention time (RT) is recorded. Compounds with higher detection frequency are assumed to have a greater contribution to the overall aroma. However, this assumption does not hold when the detection frequency is 100%; for example, compounds with very low odour thresholds that are typically detected by 100% of the population. If the concentration of the odourant increased from this point, the odour intensity would increase but the detection frequency could not. [42]

Numerous dilution experiments have also been developed. In these methodologies, a series of dilutions of a sample are analysed by GC-O and the aroma-active compounds are identified at each level. The first of these is the charm method, developed in 1984. [45] The subject records the RT when they first detect an odour, and when they no longer detect it. Optionally, a

general descriptor of the odour, such as herbaceous or fruity, can be recorded. The experiment is repeated with a series of dilutions until the odour can no longer be detected. The concentration of the sample and the duration and frequency of the response are used to produce a charm chromatogram, where the peak areas represent unitless charm values (Figure 3).[45] The peak area is proportional to the concentration of the compound in the extract.

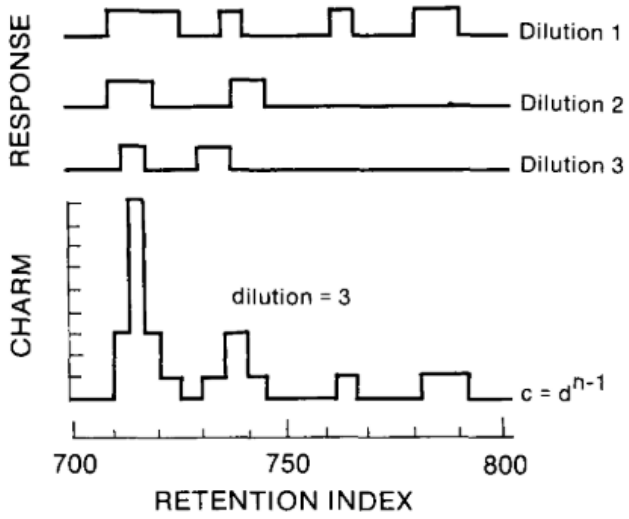

Figure 3. A stylised charm response chromatogram produced from the relationship $c = d^n - 1$, where d is the dilution constant and n is the number of coincident responses at any given index. *Reproduced with permission from Acree et al. (1984).* [45]

The second method used with GC-O is aroma extract dilution analysis (AEDA). The key difference between charm analysis and AEDA is that charm analysis provides chromatographic peaks based on the duration of the aroma response and the frequency of detection, whereas AEDA records only the maximum dilution (flavour dilution factor, FD) at which the aroma is detected; this is equivalent to the height of the charm peak. [42] The results are often presented as a plot of FD against RT, also known as an aromagram.

While AEDA and Charm are useful tools for identifying the key odourants in a sample, they are relative measurements. For example, compounds with an FD value of 32 in one study would not necessarily have the same concentration as compounds from another study with the same FD value. This is because the FD value is relative to the initial concentration of compounds in the honey. Likewise, two compounds with the same FD value in a sample do not necessarily have the same concentration in the sample, or the same odour threshold. This makes it difficult to compare AEDA results between samples and between studies, and to study the effects of the sample matrix or interactions between odourants. OAVs are useful for

this reason, as they provide a quantitative measurement that can be compared between studies.

Although dilution-based GC-O methodologies require extensive analysis time to evaluate multiple dilutions of each sample, these remain the most common methods for characterising aroma profiles in honey. Additional methods of screening for important aroma contributors include the Osme method, olfactometry global analysis and aroma extract concentration analysis, all of which operate on similar principles. The Osme method overcomes a common criticism of the dilution experiments, as perceived odour intensity is recorded. [46] The use of these methods for determining the key aroma compounds in honey have not been reported.

Another experiment involves reconstituting the key aroma compounds, determined by AEDA or other methods, in a synthetic mixture. These mixtures, also known as aroma models, are analysed by a sensory panel to evaluate their similarity to the original sample. In 1987, Buttery *et al.* utilised this approach to identify the major compounds contributing to the aroma of fresh tomatoes. [47] Although over 400 volatile compounds had been isolated from fresh tomatoes at that time, only 16 were found to have an OAV greater than 1. They found that a water solution of these compounds possessed a similar aroma to that of the fresh tomatoes. A similar approach was applied to honey in 2012. [48] A mixture of 20 reference compounds with high FD values in rape (*Brassica napus*) honey was synthesised in a fructose-glucose mixture. A trained sensory panel ranked the similarity of the aroma model to the original sample as 2.6 out of 3. [48] Six of these compounds with OAVs less than 1 were removed and only 7 of 23 panelists noticed the omission, supporting the assumption that compounds with OAV < 1 do not contribute substantially to honey aroma.

It has been suggested that characterising the overall aroma of a mixture of aroma-active compounds may not be as simple as adding the aromas of the aroma-active constituents together. Odours of different quality may mask or suppress each other, whereas similar odours may combine to produce a third distinct odour. [39,42] It is also possible for compounds with OAVs less than 1 to have a synergistic/additive effect, producing an OAV greater than 1 for the combined odour. [42] Additionally, though a high OAV indicates that the compound is a significant contributor to the aroma, it does not predict the perceived intensity of the aroma by the subject. [36]

1.3. Aroma profiles of international monofloral honeys

The volatile fraction of honey has been well studied and a diverse collection of compounds have been identified. However, most of the literature has focussed on identifying those compounds that are unique to honey of a particular botanical origin to control their authenticity. Due to this, the compounds responsible for the aroma and taste of many monofloral honeys have not been identified. All volatile compounds in honey may contribute to the aroma, of which over 600 have been identified, however, it has been suggested that only around 50 are responsible for the unique sensory properties of monofloral honeys. [9,48,49] This review will focus on studies that utilised quantitative sensory analysis techniques, combined with volatile compound analysis, to identify aroma-active compounds in honey (listed in Table 1).

The first such study was published in 1988. [28] Steeg and Montag analysed rape and heather (*Calluna vulgaris*) honeys and compared their results with taste and aroma thresholds to determine which compounds in the volatile fraction of honey were responsible for their distinct flavours. They tentatively identified benzoic acid, phenylacetic acid, phenol, *p*-cresol, guaiacol and eugenol as contributors to honey flavours. [27,28]

Following this, Blank *et al*. conducted a study on linden (*Tilia spp.*), heath (*Erica spp.*) and acacia (*Acacia spp.*) honeys in 1989. [27] The aroma of linden honey has been described as medicinal, woody-balsamic, herbal, and condimental. [9] Following simultaneous distillation-extraction (SDE) at room temperature, the extracts were reported to possess the same odours as the honey samples, indicating the volatile profile was representative of the sample. AEDA revealed 20 compounds with FD factors of 16 or above. The compounds with the highest FD included (*E*)-β-damascenone, *p*-anisaldehyde and phenylacetaldehyde, followed by linden ether, 2-acetyl-1-pyrroline, methional, and linalool. Upon comparison with heath and acacia honeys, it was found that (*E*)-β-damascenone, 2-acetyl-1-pyrroline, methional, phenylacetaldehyde, 1-hexen-3-one, 2-phenylethanol, *p*-cresol, *p*-anisaldehyde, eugenol and vanillin contributed to the odour of all three honeys. *Cis*-rose oxide (floral) and linden ether (floral, mint-like) were only reported in the linden honeys. [27]

Shimoda et al. (1996) analysed the volatile components of haze (*Rhus succedanea*) honey. [50] The volatiles were extracted by adsorptive column chromatography with porous polymer beads as the stationary phase, before GCMS analysis. The volatile concentrations obtained were compared with odour thresholds, but OAVs were not explicitly calculated. The results

suggested that phenylacetaldehyde, linalool, 2-phenylethanol, *p*-anisaldehyde, methyl-*p*-anisaldehydes, trimethoxybenzene, 5-hydroxy-2-methyl-4*H*-pyran-4-one, and lilac aldehydes were the key contributors to the haze honey aroma. Interestingly, the study also used preparative GC to separate the extract into 5 fractions, and qualitative sensory analysis was performed to describe the aroma of each fraction. The authors suggested compounds that likely contributed to the aroma of each fraction. [50] A more useful technique would perhaps be to carry out sensory analysis on the honey sample as a whole, as odour thresholds and volatile concentrations can provide data on which compounds contribute to this.

In 2007, Castro-Vazquez et al. identified the volatiles contributing to the aroma of Spanish citrus honeys. [11] The volatiles were extracted using a micro-SDE process and separated on a polar column. Although terpene compounds such as linalool and its derivatives dominated the volatile fraction in terms of concentration, upon calculation of OAVs two sinensal isomers were found to have the greatest contribution towards the aroma. Sinensal has a fruity, orange-like aroma which, combined with the low odour threshold, explains the orange-like aroma of citrus honeys. (*E*)-β-damascenone, lilac aldehydes, linalool and (*E*)- and (*Z*)-linalool oxides also had high OAVs in the citrus honey. [11]

Table 1. Compounds reported to contribute to honey aroma, with odour thresholds and odour descriptions.

Compound	Odour threshold (μg kg^{-1} in water)	Odour description	Floral honeys in which compound is aroma-active
(*E*)-2-nonenal		cucumber[51]	buckwheat,[51] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb[51]
(*E*)-3-hexenyl hexanoate			cambará[52]
(*E*)-8-hydroxylinalool		fruity	citrus[53]
(*E*)-linalool oxide	6[29]	fresh, sweet, floral,[11] herbal[51]	citrus,[11] buckwheat,[51] lingonberry,[51] sweet clover,[51] willowherb,[51] black mangrove,[39] astralagus[29]
(*E*)-whiskey lactone		chamomile[51]	buckwheat,[51] cloudberry-bog,[51] lingonberry,[51] willowherb[51]
(*E*)-β-damascenone	0.002[54], 0.01[a, 48]	honey-like, fruity, sweet,[27,39] apple[51]	linden,[27] acacia,[27] heath,[27] citrus,[11] buckwheat,[51,55] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb,[51] black mangrove,[39] rape,[48,49] raspberry,[49] heather,[49] alder buckthorn[49]
(*E*,*E*)-2,4-decadienal	0.11[a, 48]	fatty[48]	rape[48]
(*E*,*E*)-2,6-nonadienal		green[51]	buckwheat,[51] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb[51]
(*E*,*Z*)-2,6-nonadienal		green[49]	raspberry,[49] rape,[49] heather,[49] alder buckthorn[49]
(*Z*)-2-penten-1-ol		plastic,[51] solvent[52]	buckwheat,[51] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb,[51] cambará[52]
(*Z*)-3-nonenal		cucumber[51]	sweet clover[51]
(*Z*)-8-hydroxylinalool		fruity	citrus[53]
(*Z*)-linalool oxide	6[29]	fresh, sweet, floral[11,51]	citrus,[11] buckwheat,[51] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb,[51] black mangrove,[39] raspberry,[49] rape,[49] heather,[49] alder buckthorn,[49] astralagus[29,53]
(*Z*)-oak lactone		aniseed[51]	cloudberry-bog,[51] lingonberry,[51] sweet clover[51]
1,3-*p*-methadien-7-al		fatty, spicy[27]	linden[27]
1-heptanol		green[51]	buckwheat,[51] cloudberry-bog,[51] sweet clover,[51] willowherb[51]
1-hexen-3-ol		grass[51]	cloudberry-bog,[51] lingonberry[51]
1-hexen-3-one		metallic, cooked vegetable-like[27]	linden,[27] acacia,[27] heath[27]

Compound	Odour threshold (μg kg^{-1} in water)	Odour description	Floral honeys in which compound is aroma-active
1-nonanol		honey[39]	black mangrove[39]
1-octen-3-ol		mushroom[51]	buckwheat,[51] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb[51]
1-octen-3-one		metal,[51] mushroom[49]	buckwheat,[51] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb,[51] raspberry,[49] rape,[49] heather,[49] alder buckthorn[49]
1-phenyl-1,2-propanedione		sweet, honey-like[52]	cambará[52]
1-propanol		pungent[51]	cloudberry-bog,[51] lingonberry,[51] sweet clover[51]
2,3-butanediol		fruity[51]	cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb[51]
2,3-butanedione	15[56], 2.3[a, 48]	buttery[51]	buckwheat,[51,55] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb,[51] rape[48]
2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF)	31[57]	cowy, sour, balmy[55]	buckwheat[55]
2,6-dimethyl-3,7-octadiene-2,6-diol		fruity, sweet[53]	citrus[53]
2-(2-butoxyethoxy)-ethanol (diethylene glycol butyl ester)			cambará[52]
2-acetyl-1-pyrroline		roasty,[27] popcorn-like[48]	linden,[27] acacia,[27] heath,[27] rape[48]
2-acetylfuran		candy-like[49]	heather[49]
2-ethylhexanol			cambará[52]
2-hexanol		green plant[53]	citrus[53]
2-hydroxybenzaldehyde (salicylic aldehyde)		medicinal[49]	raspberry,[49] rape,[49] heather,[49] alder buckthorn,[49] haze[50]
2-methoxy-4-vinylphenol (4-vinylguaiacol)	2.8[a, 48], 5[29]	spicy[39]	black mangrove,[39] rape,[48] astralagus,[29,53] citrus,[53] linden,[27] acacia,[27] heath[27]
2-methoxy-5-methylphenol		smoky[48]	rape[48]
2-methoxyphenol (guaiacol)	0.34[a, 48]		cashew,[58] rape,[48] astralagus,[53] haze[50]
2-methyl-2-butanol		woody, camphor tree[29]	astralagus[29]

Compound	Odour threshold (μg kg^{-1} in water)	Odour description	Floral honeys in which compound is aroma-active
2-methyl-2-pentanol		cheese[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb[51]
2-methylbutanal	1[59], 3.2[a, 48]	malty[39]	black mangrove, [39] rape[48]
2-methylbutanoic acid	2200[40]	isovaleric acid-like, pungent, solvent[52]	cambará, [52] rape, [48] heather, [49] citrus[53]
2-methylbutanol		solvent[52]	cambará[52]
2-methylpropanoic acid		sweet, solvent, cheese-like, [52] sweaty[48]	cambará, [52] rape, [48] haze[50]
2-phenylethanol	1000[60], 89[a, 48]	honey-like, spicy, [27] floral[51,52]	linden, [27] acacia, [27] heath, [27] buckwheat, [51,55] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb, [51] cambará, [52] rape, [48,49] raspberry, [49] heather, [49] alder buckthorn, [49] haze, [50] astralagus, [53] citrus, [53] cashew[58]
2-phenylethanethiol		rubber[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb[51]
3-(methylthio)propanal (methional)	0.2[61]	cooked potato-like	linden, [27] acacia, [27] heath, [27] buckwheat, [51,55] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb, [51] raspberry, [49] rape, [48,49] heather[49]
3,5-dimethyl-2-ethylpyrazine		coffee-like[49]	raspberry, [49] rape, [49] heather, [49] alder buckthorn[49]
3,9-epoxy-1-*p*-methene		floral, mint-like[27]	linden[27]
3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone)	0.001[62]	caramel, [51] spicy, seasoning-like[55]	buckwheat, [51,55] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb[51]
3-hydroxy-4-phenyl-2-butanone		phenolic, tropical[53]	citrus[53]
3-methylbutanal	0.2[60], 2.1[a, 48]	malty[39,51]	buckwheat, [51,55] cloudberry-bog, [51] lingonberry, [51] willowherb, [51] black mangrove, [39] rape[48]
3-methylbutanoic acid	490[a, 40,48]	isovaleric acid-like, pungent, solvent[52]	cambará, [52] rape, [48] alder buckthorn, [49] haze, [50] buckwheat, [55] cashew, [58] marmeleiro[58]
3-methylpentanoic acid		sweaty[48]	rape[48]

Compound	Odour threshold (μg kg^{-1} in water)	Odour description	Floral honeys in which compound is aroma-active
3-pentanol		fruity[51]	buckwheat,[51] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb[51]
3-phenylpropanoic acid	27[a48]	herbal,[51] flowery, waxy[48]	buckwheat,[51] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb,[51] rape[48]
4-(4-hydroxyphenyl)-2-butanone	5[63]	sweet, candy-like[55]	buckwheat[55]
4-allyl-2-methoxyphenol (eugenol)	1.1[a, 48]	clove-like,[48,49,51] spicy, honey-like[27]	linden,[27] acacia,[27] heath,[27] buckwheat,[51] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb,[51] cashew,[58] raspberry,[49] rape,[48,49] heather,[49] alder buckthorn[49]
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (furaneol)		caramel-like[48]	rape,[48,49] heather,[49] alder buckthorn[49]
4-hydroxy-3-methoxybenzaldehyde (vanillin)	66[a, 48], 25[64]	vanilla-like[48]	linden,[27] acacia,[27] heath,[27] buckwheat,[51,55] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb,[51] marmeleiro,[58] raspberry,[49] rape,[48,49] heather,[49] alder buckthorn[49]
4-hydroxy-4-methyl-2-pentanone (diacetone alcohol)		fatty[53]	astralagus[53]
4-methoxybenzaldehyde (*p*-anisaldehyde)	27[40], 33.1[b65]	mint-like, sweet,[27] aniseed-like[48,51]	linden,[27] acacia,[27] heath,[27] buckwheat,[51,55] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb,[51] rape,[48,49] heather,[49] alder buckthorn,[49] haze[50]
4-methylacetophenone		spicy, almond-like[27]	linden[27]
4-methylphenol (*p*-cresol)	55[66]	horse-like,[48] moldy, fermented[53]	rape,[48] astralagus,[53] linden,[27] acacia,[27] heath,[27] buckwheat,[51,55] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb[51]
9-hexadecenoic acid			cambará[52]
acetic acid	33[29]	vinegar[51]	buckwheat,[51] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb,[51] haze,[50] astralagus,[29,53] citrus[53]
3-hydroxybutanone (acetoin)		fatty[53]	astralagus,[53] citrus[53]
acetophenone		green[49]	raspberry,[49] rape,[49] heather[49]

Compound	Odour threshold (μg kg^{-1} in water)	Odour description	Floral honeys in which compound is aroma-active
benzaldehyde	150[40]	almond, sweet, fruit, [11] honey-like, green, burnt, pungent, bitter[52]	citrus, [11] cambará, [52] black mangrove, [39] rape, [48] haze[50]
benzoic acid		fruity, sweet, slightly bitter[52]	cambará, [52] cashew, [58] marmeleiro, [58] raspberry, [49] rape, [49] heather, [49] alder buckthorn[49]
benzonitrile		solvent, burned plastic, floral, pungent, fruity[52]	cambará[52]
benzothiazole	80[29]	floral[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb, [51] astralagus[29]
benzyl alcohol	620[67]	flowery[53]	citrus, [53] cashew, [58] cambará[52]
butanoic acid	240[61]	cheesy[51]	buckwheat, [51,55] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb, [51] raspberry, [49] rape, [49] heather, [49] alder buckthorn, [49] haze[50]
camphene		camphor[49]	raspberry, [49] rape, [49] heather, [49] alder buckthorn[49]
cis-rose oxide			linden[27]
citral		citrus[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb[51]
coumarin	25[68]	wild flower, herbaceous[55]	buckwheat[55]
D-carvone		thyme[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb[51]
decanal	0.1[39]	sweet-waxy[39]	black mangrove[39]
diethyleneglycol		plastic-like, pungent, alcohol[52]	cambará[52]
dimethyl sulfide	0.3[60]	sulfur, [51] pumpkin[39]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb, [51] black mangrove[39]
dimethyl trisulfide	0.01[69], 0.03[a, 48]	cabbage-like[48]	linden, [27] acacia, [27] rape, [48,49] raspberry, [49] heather, [49] buckwheat[55]
DL-carvone		green[49]	raspberry, [49] rape, [49] heather, [49] alder buckthorn[49]
D-limonene	60[29]	mint, [51] orange-like, fruity[29]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb, [51] astralagus[29]

Compound	Odour threshold (μg kg^{-1} in water)	Odour description	Floral honeys in which compound is aroma-active
lauric acid			cambará[52]
ethyl 2-methylbutanoate	0.3[70]	apple[51]	buckwheat, [51,55] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb[51]
ethyl 2-methylpropanoate	0.1[70]	fruity[55]	buckwheat[55]
ethyl 3-methylbutanoate	0.2[71]	fruity[51]	buckwheat, [51,55] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb[51]
ethyl 3-phenylpropanoate		floral[51]	cloudberry-bog, [51] lingonberry[51]
ethyl acetate			haze[50]
ethyl benzoate		honey[51]	cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb, [51] raspberry, [49] rape, [49] heather[49]
ethyl cinnamate		cinnamon[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb[51]
ethyl dihydrocinnamate		floral[49]	raspberry, [49] rape, [49] heather, [49] alder buckthorn[49]
ethyl hexadecanoate			cambará[52]
ethyl laurate		dill[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb[51]
ethyl pentanoate		fruity[51]	buckwheat, [51] lingonberry[51]
ethyl phenylacetate		honey-like[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb, [51] raspberry, [49] heather[49]
furfural (furaldehyde)	720[29]	almond, sweet, bread, [11] sweet, green, solvent[52]	citrus, [11] rape, [49] heather, [49] astralagus, [29] cambará[52]
furfuryl alcohol		burnt[53]	citrus[53]
furfuryl mercaptan			cashew[58]
geranyl acetone		herbal[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb, [51] raspberry, [49] rape, [49] heather[49]
heptacosane (C_{27})			cambará, [52]
heptanal	3.0[39]	fat, [51] pungent	cloudberry-bog, [51] willowherb, [51] black mangrove, [39] haze[50]
hexacosane (C_{26})			cambará[52]

Compound	Odour threshold (μg kg^{-1} in water)	Odour description	Floral honeys in which compound is aroma-active
hexadecane (C_{16})			cambará[52]
hexanal			haze[50]
hexanoic acid			haze,[50] citrus[53]
hexyl hexanoate		apple[51]	buckwheat,[51] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb,[51] raspberry,[49] rape,[49] heather,[49] alder buckthorn[49]
hexyl octanoate		peppermint[51]	buckwheat,[51] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb[51]
3,7-dimethyl-1,5,7-octatrien-3-ol (hotrienol)	110[29]	fresh, floral, fruity[11,39]	citrus,[11] black mangrove,[39] astralagus[29]
hydrocinnamic acid		floral[49]	raspberry,[49] rape,[49] heather,[49] alder buckthorn[49]
isobutanoic acid		dry[51]	buckwheat,[51] lingonberry,[51] sweet clover,[51] willowherb[51]
3,5,5-trimethylcyclohex-2-en-1-one (isophorone)		herbal,[51] peppermint-like[39]	cloudberry-bog,[51] black mangrove,[39] heather[49]
lilac alcohol (isomer 1)	4-74	fresh, sweet, flowery[11]	citrus[11]
lilac alcohol (isomer 2)	4-74	fresh, sweet, floral[11,51]	citrus,[11] cloudberry-bog,[51] lingonberry,[51] sweet clover[51]
lilac alcohol (isomer 3)	4-74	fresh, sweet, flowery[11]	citrus[11]
lilac alcohol (isomer 4)	4-74	fresh, sweet, flowery[11]	citrus[11]
lilac aldehyde (isomer 1)	0.2-20	fresh, floral[11,51]	citrus,[11] buckwheat,[51] cloudberry-bog,[51] lingonberry,[51] sweet clover,[51] willowherb,[51] black mangrove,[39] raspberry,[49] rape,[49] heather,[49] alder buckthorn[49]
lilac aldehyde (isomer 2)	0.2-20	fresh, flowery[11]	citrus[11]
lilac aldehyde (isomer 3)	0.2-20	fresh, flowery[11]	citrus[11,53]
lilac aldehyde (isomer 4)	0.2-20	fresh, flowery[11]	citrus[11]
linalool	6[72]	floral[27]	linden,[27] acacia,[27] citrus,[11] raspberry,[49] rape,[49] alder buckthorn,[49] haze,[50] astralagus,[29] citrus[53]
linden ether		floral, mint-like[27]	linden[27]
maltol	35000[73]	burnt sugar-like[55]	buckwheat[55]

Compound	Odour threshold ($\mu g\ kg^{-1}$ in water)	Odour description	Floral honeys in which compound is aroma-active
methyl 2-(methylthio)acetate		roasty[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb[51]
methyl 2-methoxybenzoate		honey-like[49]	raspberry, [49] rape, [49] heather[49]
methyl 2-methylbutanoate			haze[50]
methyl 2-methylpropanoate		floral[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry[51]
methyl anthranilate		grape, fruity, [11] caramel[53]	citrus, [11] citrus[53]
methyl butanoate			haze[50]
methyl dodecanoate		dill[51]	buckwheat, [51] lingonberry, [51] sweet clover, [51] willowherb[51]
methyl hexanoate		fruity[51]	buckwheat[51]
methyl salicylate			haze[50]
methyl thiocyanate		roasty, onion[51]	cloudberry-bog, [51] sweet clover[51]
methyl dihydrothiophenone		roasty[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb[51]
methylpropanal		malty[55]	buckwheat[55]
nonacosane (C_{29})			cambará[52]
nonadecane (C_{19})			cambará[52]
nonanal	0.53[b, 74]	fatty-floral[39]	black mangrove, [39] haze, [50] astralagus[53]
nonanol			astralagus[53]
octacosane (C_{28})			cambará[52]
octadecane (C_{29})			cambará[52]
octanal	0.7[39], 0.17[b, 74]	sweet, honey-like[39]	black mangrove[39]
octanoic acid	500[29]		haze, [50] astralagus, [29] citrus[53]
pantoyl lactone (pantolactone)		burnt, caramel[53]	citrus[53]
p-cymene		solvent[51]	cloudberry-bog, [51] sweet clover[51]
pentacosane (C_{25})			cambará[52]
pentacosanol			cambará[52]
pentadecane (C_{15})			cambará[52]
pentadecanoic acid			cambará[52]

Compound	Odour threshold ($\mu g\ kg^{-1}$ in water)	Odour description	Floral honeys in which compound is aroma-active
phenylacetaldehyde	4[61], 2.5[a, 48]		linden, [27] acacia, [27] heath, [27] citrus, [11] buckwheat, [51,55] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb, [51] black mangrove, [39] rape, [48,49] raspberry, [49] heather, [49] alder buckthorn, [49] astralagus, [53] citrus[53]
phenylacetic acid	1000[75], 135[a, 48]	honey-like[48,51]	buckwheat, [51,55] lingonberry, [51] sweet clover, [51] willowherb, [51] rape, [48,49] raspberry, [49] heather, [49] alder buckthorn, [49] cambará[52]
4-*tert*-butylphenol		solvent, pungent, plastic-like, burnt[52]	cambará[52]
sinensal (isomer 1)	0.05	sweet, orange[11]	citrus[11]
sinensal (isomer 2)	0.05	sweet, orange[11]	citrus[11]
tetracosanol			cambará[52]
tetradecane (C_{14})			cambará[52]
trans-4,5-epoxy-(*E*)-2-decenal		metallic[48]	rape[48]
triacontane (C_{30})			cambará[52]
tricosane (C_{23})			cambará[52]
α-methyl citrene		chips, spicy[53]	citrus[53]
α-phellandrene		herbal[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb[51]
α-terpineol	46	green, floral[11]	citrus[11]
β-cryophyllene		woody[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb[51]
γ-butyrolactone		honey[51]	buckwheat, [51] cloudberry-bog, [51] lingonberry, [51] sweet clover, [51] willowherb, [51] raspberry, [49] rape, [49] heather, [49] alder buckthorn[49]
γ-decalactone	1.1[40]	peach-like[48]	cashew, [58] marmeleiro, [58] rape[48]
γ-dodecalactone		herbal[51]	buckwheat, [51] cloudberry-bog, [51] willowherb[51]
γ-octalactone			haze[50]
γ-vinyl-γ-valerolactone		fruity, flowery[53]	citrus[53]
δ-decalactone		coconut-like[49]	raspberry, [49] rape, [49] heather, [49] alder buckthorn[49]
δ-octalactone			cashew[58]

a) odour threshold in an aqueous fructose-glucose solution[48], b) odour threshold in air

The Brazilian cambará (*Gochnatia velutina*) honey was analysed by Moreira and De Maria. [52] Both aqueous and headspace extracts were analysed on polar and nonpolar columns in order to extract volatiles across the full polarity and volatility range. AEDA analysis was performed on the extracts and the distinct odour of cambará honey was attributed to the high FD factors of benzaldehyde, 2,3-methylbutanoic acid benzonitrile and 2-phenylethanol. Although they have relatively high odour thresholds (1000 and 150 μg kg^{-1} in water, respectively), benzaldehyde and 2-phenylethanol are widely reported as contributors to honey aroma. Interestingly, while both compounds were present in the cambará honey at concentrations much lower than their aroma threshold, their FD factors in the headspace extracts were 16 for 2-phenylethanol and 32 for benzaldehyde. This demonstrates that FDs obtained by AEDA cannot be reliably compared with odour thresholds or OAVs.

Buckwheat (*Fagopyrum esculentum*) honey, known for its malty flavour and unpleasant odour, has been the subject of numerous studies. [51,55,76,77] In 2002, Zhou, Wintersteen and Cadwallader published a detailed study of the volatiles of buckwheat honey from the UK and USA. [55] 3-Methylbutanal, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone and (*E*)-β-damascenone were the most potent odourants. It is generally agreed that 3-methylbutanal is the primary contributor to the malty flavour of buckwheat honey. [51,55,76,77] Other contributors included methylpropanal, 2,3-butanedione, phenylacetaldehyde, 3-methylbutyric acid, maltol, vanillin, methional, coumarin, and *p*-cresol. OAVs were also calculated, confirming the results of the AEDA analysis. [55]

An odour detection frequency method, rather than a dilution based method, was used to analyse rape, raspberry (*Rubus idaeus*), heather and alder buckthorn (*Frangula alnus*) honeys extracted by solid-phase microextraction (SPME). [49] The detection frequency method estimated odour intensity based on the proportion of replicates the odour was detected in. The authors defined 33% as the detection frequency required for a compound to contribute to the honey's aroma. Combining this with quantitative GC-MS data by means of statistical analysis allowed tentative conclusions to be drawn, but the arbitrary definition of "odour-active" compounds doesn't allow for comparison with other studies that used more quantitative data. Unique volatiles could be identified for heather (isophorone and 2-methylbutanoic acid) and rape (dimethyl trisulfide) honeys, but the actual contributions of compounds to the honey aroma could not be adequately investigated using this method.

Although the volatile fraction of rape honey has been widely studied, the key aroma compounds were not identified until 2012. [34,48,78] Ruisinger and Schieberle performed AEDA on rape honey extracts obtained by liquid-liquid extraction (LLE) and run on three columns of differing polarities. 28 compounds were identified with FD factors between 4 and 2048. The main contributors to the aroma based on the FD factors were (*E*)-β-damascenone, phenylacetic acid, 4-methoxybenzaldehyde, 3-phenylpropanoic acid and 2-methoxy-4-vinylphenol. Interestingly, of the compounds reported by Steeg and Montag 23 years prior, only benzoic acid and phenol were not identified as aroma contributors. [28]

In addition to AEDA, odour threshold values were calculated for each compound in an aqueous fructose-glucose solution. While odour thresholds calculated in water are still valid as honey is an aqueous solution, matrix-matched odour threshold data is preferable. Indeed, most of the odour threshold values presented in Table 1 were calculated in water. The compounds with the highest OAVs were (*E*)-β-damascenone, 3-phenylpropanoic acid, phenylacetic acid and phenylacetaldehyde. As noted in section 1.2.2, the discrepancy in major compounds identified from each olfactometry method is likely because the FD factors obtained during AEDA are related to the odour threshold of the compound in air, rather than in the fructose-glucose mix used to calculate OAVs. [48] This is a valid point for most of the studies mentioned here, as AEDA requires volatiles to be completely vaporised, and therefore will not be comparable with odour thresholds reported in water.

More recently, Uckun and Selli (2016) analysed the aroma-active compounds in citrus and astralagus (*Astragalus propinquus*, also known as milk vetch or huang zhi) honeys from Turkey. LLE was the chosen extraction method, and the GC analysis was carried out on a wax column. Olfactometry, FID and MS were all connected to GC and AEDA was performed. In this study, compounds with FD factor greater than 8 were considered key contributors to the honey aroma. In both honeys, 2-phenylethanol was the dominant aroma compound, with FD 32 in astralagus honey and 128 in citrus honey. 2-phenylethanol provides a floral odour. Other aroma-active compounds present in both honeys include acetoin, acetic acid, phenylacetaldehyde, and 2-methoxy-4-vinylphenol. Citrus honey had the highest concentration of aroma-active compounds, with 14391.6 μg kg^{-1}, and had 23 compounds with FD > 8. These notably included terpenes such as linalool and (*E*)-8-hydroxylinalool, furfuryl alcohol, methyl anthranilate, γ-vinyl-γ-valerolactone, hexanoic acid and benzyl alcohol. Interestingly, the sinsensal isomers, lilac aldehydes and (*E*)-β-damascenone reported by Castro-Vazquez *et al.* were not identified by Uckun and Selli. This may owe to the

different sample preparation methods used or may be another instance where OAVs cannot be compared with FD factors obtained by AEDA. In contrast, astralagus honey contained 4270.1 μg kg^{-1} of 12 aroma-active compounds. The dominant compounds aside from those already mentioned included guaiacol, nonanal, (*Z*)-linalool oxide and 4-methylphenol. [53]

In another study, astralagus honey samples from three elevation levels were analysed and the total aroma compounds ranged from 14,600 to 29,882 μg kg^{-1}, with 11 aroma-active compounds. [29] This used OAVs rather than AEDA to determine the aroma-active compounds. Interestingly, although the two studies used the same LLE method to extract volatiles, and similar GC-MS methods using the same wax column, there were some key differences in the results achieved. Both studies agreed that (*Z*)-linalool oxide, 2-methoxy-4-vinylphenol and acetic acid contributed to the aroma. [29,53] While 4-hydroxy-4-methyl-2-pentanone and 2-phenylethanol had FD factors of 8 and 32 respectively in Uckun and Selli's study, their calculated OAVs in Turk and Sen's study were less than 1, and thus were not deemed to contribute to the aroma. Acetoin, nonanal, phenyl acetaldehyde, 2-methoxyphenol, 4-methylphenol and nonanol were not detected in the 2021 study. Conversely, while furfural, limonene, linalool, hotrienol, (*E*)-linalool oxide and octanoic acid all had OAVs greater than one, their FD factors were less than 8. 2-methyl-2-butanol and benzothiazole, which both had OAVs greater than 1, were not detected in the 2016 study. [29,53] The differences between these two studies, which used very similar methods aside from the processing of the olfactometric data, shows that AEDA data cannot be reliably compared with OAVs, as AEDA only provides relative data.

In another study that compared AEDA results with OAVs, Pino (2012) investigated the aroma-active compounds in Cuban black mangrove (*Avicennia germinans* L.) honey. [39] HS-SPME was used for the sample extraction and 66 volatiles were identified. Black mangrove honey is locally known as "salty honey" in Cuba and has a sweet, herbal and floral aroma. Based on the results of AEDA the main compounds responsible for these sensory properties are nonanal, decanal, (*E*)-β-damascenone, octanal and phenylacetaldehyde, all with FD factors 512-1024. Other contributing aroma-active compounds with lower FD values included dimethyl sulfide, 2-methylbutanal, lilac aldehyde A, benzaldehyde, (*Z*)- and (*E*)-linalool oxide and 1-nonanol. Quantification of these compounds and calculation of OAVs provided very similar results, the only discrepancy being that benzaldehyde had an OAV less than 1, so it probably did not contribute to the aroma of the black mangrove honey. [39]

In 2018, Tian et al. tried a different approach to establish the relationship between volatile composition and sensory profiles of five monofloral honey types. [3] Samples of astralagus, linden, acacia, loquat (*Eriobotrya japonica*) and zaohua (*Prunus mume*) honey were evaluated by a sensory panel and rated according to five attributes: fresh, sweet, mellow, fruity and fermented. The honey volatiles were extracted using HS-SPME and analysed by GC-MS on a wax column. The correlations between the sensory data and the GC-MS profiles of the five honeys were then analysed by PLSR. [3] While this approach provided some insights into the contributions of different classes of compounds to different aroma descriptors, it is ineffective for understanding the contributions of individual compounds to the aroma profile of the honey.

A 2022 study by Zhu *et al.* compared the volatile profiles of eleagnas (*Elaeagnus angustifolia L.*), jujube (*Ziziphus jujuba Mill.*), apocynum (*Apocynum venetum L.*), lavender and amorpha (*Amorpha fruticosa L.*) honeys. [22] The important contributors to honey aroma were determined from OAVs and odour contribution rates (OCR). OCR is calculated as the ratio of the OAV of a particular compound to the OAVs of all compounds in the sample. Out of 91 volatiles identified in the five monofloral honeys, 27 were deemed to characterise the unique aromas of the honeys. *E*-β-damascenone had the greatest OCR for apocynum, jujube and amorpha honeys, and dominated the aroma profiles, contributing over 90% to the aroma of these honeys. These honeys possess intense fruity aromas, likely owing to this compound. Eleagnus honey, which has a more floral aroma, had linalool, nonanal, decanal, and methyl decanoate as the greatest aroma contributors, while heptanal, hexanol and hexanal contributed to the fresh, green, balsamic and jasmine aroma of lavender honey. [22]

1.4. Volatile profiles of New Zealand monofloral honeys

Monofloral honeys produced in New Zealand have distinctive flavour profiles owing to their botanical origin in native plant species. Tāwari, rewarewa, and pōhutukawa (*Metrosideros exelsa*) honeys are predominantly produced in the North Island. [79] Beech honeydew (*Nothofagus spp.*), and rātā (*Metrosideros umbellata*) honeys are predominantly produced in the South Island. Mānuka and kāmahi (*Weinmannia racemosa*) honeys are produced throughout New Zealand. [79] Other non-native honeys produced in New Zealand include clover (*Trifolium repens*), viper's bugloss (*Echium vulgare*, also known as borage), and thyme.

Extensive studies on the volatile components of New Zealand honeys have been carried out by Tan, Wilkins and Holland between 1988 - 1995. [80-86] In general, honeys from Australia and New Zealand have been found to contain higher concentrations of volatile compounds than those from Europe and America, and are typically more strongly flavoured. [80-85,87,88] For example, Tan *et al.* reported volatiles with concentrations ranging up to 4 mg kg^{-1} in New Zealand honeys, and D'Arcy *et al.* reported concentrations of 0.1 – 51.3 mg kg^{-1} in Australian honeys, whereas the concentrations in American and European honeys are typically in the μg kg^{-1} range. [80,89] The highest concentration reported in American honey by Bouseta *et al.* was 0.6 mg kg^{-1}. [87] Mānuka and kānuka honeys have been found to contain up to 1000 times higher concentrations of organic extractives than other honeys such as New Zealand clover. [80] This leads to the strong flavours of most Australian and New Zealand honeys. As with most overseas studies, New Zealand honeys have had their volatile fractions characterised for the purpose of identifying marker compounds. The main contributors to their aroma profiles have not been identified by sensory techniques. This section discusses the most important volatiles identified in New Zealand native and non-native monofloral honeys, which are also summarised in Table 2. Of these compounds, many have been identified as aroma-active compounds by international studies (section 1.3, Table 1).

Table 2. Volatile compounds previously identified in monofloral honeys produced in New Zealand.

Compound	Honey Floral Type
(*E*)-2,6-dimethyl-2,7-octadiene-1,6-diol	nodding thistle[84]
(*E*)-cinnamaldehyde	pōhutukawa[15,79]
(*E*)-cinnamic acid	clover[80]
(*E*)-methyl 2,6-dimethyl-6-hydroxy-2,7-octanedioate	nodding thistle[84]
(*Z*)-2,6-dimethyl-2,7-octadiene-1,6-diol	nodding thistle[84]
(*Z*)-2,6-dimethyl-6-hydroxy-2,7-octadienal	nodding thistle[84]
1-(2-methoxyphenyl)ethanol	clover, [79] beech honeydew, [15,79] kāmahi, [79] mānuka, [15,79] pōhutukawa, [79] rewarewa, [79] tāwari[79]
1-(3-oxo-1-butenyl)-2,6,6-trimethyl-1,2-epoxycyclohexan-4-ol	thyme[82,86]
1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethylcyclohexane-*trans*-*cis*-1,2,4-triol	thyme[82,86]
1-(4-methoxyphenyl) ethanol	mānuka[90]
1,1'-bicyclopentyl	clover, [79] kāmahi, [79] mānuka, [79] pōhutukawa, [79] rātā, [79] rewarewa, [79] tāwari, [79] viper's bugloss[79]
1,3,5,7-cyclooctatetraene	clover, [79] beech honeydew, [79] kāmahi, [79] mānuka, [79] pōhutukawa, [79] rātā, [79] rewarewa, [79] tāwari, [79] thyme, [79] viper's bugloss[79]
1,3-butanediol	nodding thistle[84]
1,4-dihydroxybenzene (hydroquinone)	thyme, [82,86] viper's bugloss[85,86]
10-oxo-2-*trans*-decenoic acid*	nodding thistle[84]
10-oxodecanoic acid*	nodding thistle[84]
1-hexanol	beech honeydew, [91] clover, [91] kāmahi, [91] mānuka, [91] rātā, [91] rewarewa, [91], tāwari, [91] thyme, [91] viper's bugloss[91]
1-phenylethanol	mānuka[15,79]
2-(hydroxyphenyl)-ethanoic acid*	clover, [86] mānuka, [86] heather[86]
2-(methoxyphenyl)-ethanoic acid	mānuka[86]
2,2,4,4-tetramethylcyclobutane-1,3-dione	clover, [79] beech honeydew, [79] kāmahi, [79] mānuka, [79] pōhutukawa, [79] rātā, [79] rewarewa, [79] tāwari, [79] thyme, [79] viper's bugloss[79]
2,2-dimethylbutanedioic acid	rewarewa[92]
2,5-cyclohexadiene-1,4-dione	thyme, [82,86] viper's bugloss[85,86]

Compound	Honey Floral Type
2,6,6-trimethyl-2-cyclohexene-1,4-dione (4-ketoisophorone)	kānuka[93]
2,6,6-trimethylcyclohexane-1,4-dione	clover, [79] beech honeydew, [79] kāmahi, [79,86] mānuka, [79] pōhutukawa, [79] rātā, [79] rewarewa, [79] tāwari, [79] thyme, [79] viper's bugloss[79]
2,6-dimethyl-1,3,5,7-octatetraene	clover, [79] kāmahi, [79] rātā, [79] rewarewa, [79] tāwari, [79] thyme[79]
2,6-dimethyl-2,7-octadiene-1,6-diol	clover, [79] kāmahi, [79] mānuka, [79] pōhutukawa, [79] rātā, [79] rewarewa, [79] tāwari, [79] thyme[79]
2,6-dimethyl-3,7-octadiene-2,6-diol	clover, [79] beech honeydew, [79] kāmahi, [79] mānuka, [79] pōhutukawa, [79] rātā, [79] rewarewa, [79] tāwari, [79] thyme, [79] viper's bugloss[79]
2,6-dimethyl-6(S)-hydroxy-2-*trans*-2,7-octanedioc acid†	clover, [86] thyme, [86] nodding thistle[86]
2,6-dimethyl-6(*S*)-hydroxy-2-*trans*-2,7-octanedioic acid†	kāmahi[86]
2,6-di-*tert*-4-methylphenol	clover[86]
2,6-di-*tert*-butyl-4-methylphenol	mānuka[83]
2',5'-dimethoxyacetophenone	mānuka[83]
2'-methoxyphenylacetic acid*	mānuka[83]
2′-hydroxyacetophenone	mānuka[93]
2-acetylfuran	mānuka[90]
2-carbomethoxy furan	mānuka[90]
2-decenedioic acid	rewarewa[92]
2-ethyl-2-imidazoline	mānuka[90]
2-ethylhexanoic acid	clover, [79] beech honeydew, [79] pōhutukawa, [79] tāwari[15,79]
2-furancarboxylic acid (2-furoic acid)*	nodding thistle[84,86]
2-hydroxy-2-ethylbutanedioic acid	rewarewa[92]
2-hydroxy-2-isopropylbutanedioic acid	rewarewa[92]
2-hydroxy-2-phenylacetic acid (mandelic acid)	mānuka (TMS ether, TMS ester) [94]
2-hydroxy-2-phenylethanoic acid*	heather[86]
2-hydroxy-2-phenylethanoic acid§	clover, [86] mānuka[86]
2-hydroxy-3-(4'-methoxyphenyl)-propanoic acid§	mānuka[83]
2-hydroxy-3-(4-methoxyphenyl)-propanoic acid*	clover, [80,86] heather, [86] thyme, [82,86] kāmahi, [86] mānuka[83]

Compound	Honey Floral Type
2-hydroxy-3-methylbutanoic acid*	mānuka[86]
2-hydroxy-3-methylpentanoic acid*	clover, [86] mānuka, [83,86] heather[86] kāmahi, [86]
2-hydroxy-3-phenylpropanoic acid*	clover, [80,86] mānuka, [83,86] heather, [81,86] thyme, [82,86] nodding thistle, [84,86] kāmahi[86]
2-hydroxy-3-phenylpropanoic acid§	mānuka[83,86]
2-hydroxy-4-methylpentanoic acid*	clover, [86] mānuka, [83,86] heather[86] kāmahi[86]
2-hydroxybenzoic acid* (salicylic acid)	clover, [86] heather[81,86]
2-hydroxybutanedioic acid (malic acid)	rewarewa, [92] mānuka (TMS ether, di-TMS ester) [94]
2-isopropyl-3-hydroxy-but-2-enoic acid (TMS ether, TMS ester)	mānuka[94]
2-methoxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione	heather, [81,86] thyme, [82,86] kāmahi[86]
2-methoxybenzoic acid*	clover, [80,86] mānuka[83,86] kāmahi[86]
2-methoxybutanedioic acid (methoxysuccinic acid)	rewarewa[92]
2-methoxyphenyl acetate	clover[86]
2-methylbenzofuran	beech honeydew, [79] kāmahi, [79] mānuka[15,79]
2-methylbenzofuran	mānuka[93]
2-methylbutanedioic acid (methylsuccinic acid)	rewarewa[92]
2-methylbutanoic acid	beech honeydew, [91] clover, [91] kāmahi, [91] mānuka, [91] rātā, [91] rewarewa, [91], tāwari, [91] thyme, [91] viper's bugloss[91]
2-methylfuran	beech honeydew, [91] clover, [91] kāmahi, [91] mānuka, [91] rātā, [91] rewarewa, [91], tāwari, [91] thyme, [91] viper's bugloss[91]
2-methyltetrahydrofuran-3-one	mānuka[90]
2-octenedioic acid	rewarewa[92]
2-phenylethanoic acid*	clover, [86] heather, [86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [84,86] kāmahi[86]
2-phenylethanoic acid§	heather[81,86]
2-phenylethanol	clover, [79,86,91] honeydew, [79] kāmahi, [79,91] mānuka, [79,91] pōhutukawa, [79] rātā, [79,91] rewarewa, [79,91] tāwari, [79,91] thyme, [79,82,91] viper's bugloss, [79,91] heather[81,86] nodding thistle, [84,86] beech honeydew, [91] kānuka[93]
3-(3,4-dimethoxyphenyl)-*cis*-prop-2-enoic acid*	clover, [86] mānuka, [83,86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [84,86] kāmahi[86]

Compound	Honey Floral Type
3-(3,4-dimethoxyphenyl)-*trans*-prop-2-enoic acid*	clover, [86] heather, [81,86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [84,86] mānuka[83]
3-(4-hydroxyphenyl)-*cis*-prop-2-enoic acid*	viper's bugloss, [85,86] mānuka[83]
3-(4-hydroxyphenyl)-*trans*-prop-2-enoic acid*	thyme, [82,86] viper's bugloss, [85,86] nodding thistle[84,86]
3-(4-methoxyphenyl)-*trans*-prop-2-enoic acid*	clover, [86] mānuka, [83,86] heather, [86] thyme, [82,86] nodding thistle[84,86]
3,4,5-trimethoxybenzoic acid*	clover, [80,86] mānuka, [83,86] thyme, [82,86] nodding thistle, [84,86] kāmahi[86]
3,4,5-trimethylbenzoic acid*	heather[81,86]
3,4-dimethoxyacetophenone	mānuka[83]
3,4-dimethoxybenzaldehyde	clover, [86] mānuka[83,86]
3,4-dimethoxybenzoic acid*	clover, [80,86] mānuka, [83,86] heather, [81,86] thyme, [82,86] kāmahi[86]
3,5,5-trimethylcyclohex-2-en-1,4-dione	heather[81,86] kāmahi[86]
3,5,5-trimethylcyclohex-2-en-1-one (isophorone)	heather[81,86]
3,5-dimethoxy-4-hydroxybenzoic acid*	mānuka[83]
3,5-dimethoxybenzoic acid*	clover, [80,86] mānuka, [83,86] heather[81,86] nodding thistle, [86] kāmahi[86]
3,7-dimethyl-1,5,7-octatrien-3-ol (hotrienol)	clover, [79] beech honeydew, [79] kāmahi, [79] mānuka, [79] pōhutukawa, [79] rātā, [79] rewarewa, [79] tāwari, [79] thyme, [79] viper's bugloss[79] nodding thistle[84]
3,7-dimethyl-1,5-octadiene-3,7-diol	nodding thistle[84]
3'-aminoacetophenone	thyme[82,86]
3-furancarboxylic acid* (3-furoic acid)	clover, [86] mānuka, [86] heather, [86] thyme, [82,86] nodding thistle, [84,86] kāmahi[86]
3-hexenoic acid*	thyme[82,86]
3-hydroxy-3-(4'-methoxyphenyl)-propanoic acid*	mānuka, [83] nodding thistle[84]
3-hydroxy-3-(methoxyphenyl)-propanoic acid*	clover, [86] mānuka, [86] heather[86] viper's bugloss, [85,86] nodding thistle, [86] kāmahi, [86] thyme[82]
3-hydroxy-3-methylbutanoic acid*	Heather, [86] thyme[82,86]
3-hydroxy-3-methylpentanedioic acid (3-hydroxy-3-methylglutaric acid)	rewarewa[92]
3-hydroxybenzoic acid*	clover, [86] mānuka, [86] heather, [81,86] thyme, [86] kāmahi[86]
3-hydroxybutanoic acid*	mānuka, [86] heather, [86] thyme[82,86]
3-hydroxypropanoic acid*	mānuka, [86] heather[86] viper's bugloss[86]

Compound	Honey Floral Type
3-methoxybenzoic acid*	clover, [86] thyme, [86] viper's bugloss, [86] heather, [81] mānuka[83]
3-methyl-1-butanol (isoamyl alcohol)	beech honeydew, [91] clover, [91] kāmahi, [91] mānuka, [91] rātā, [91] rewarewa, [91], tāwari, [91] thyme, [91] viper's bugloss[91]
3-methyl-2-butenal	pōhutukawa[15,79]
3-methyl-2-oxo-pentanoic acid*	mānuka[83,86]
3-methylpentanoic acid	clover, [15,79] beech honeydew, [79] kāmahi, [79] mānuka, [79] pōhutukawa, [79] rātā, [79] rewarewa, [79] tāwari, [79] thyme, [79] viper's bugloss[79]
3-phenylprop-2-en-1-ol	heather[81,86]
3-phenylprop-2-enoic acid* (cinnamic acid)	heather[81]
3-phenylpropanoic acid* (hydrocinnamic acid)	clover, [86] mānuka, [86] heather, [81,86] thyme, [82,86] nodding thistle[86]
4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one	heather[81,86]
4-(3-oxo-1-butenylidene)-3,5,5-trimethylcyclohex-2-en-1-one	heather, [81,86] thyme[82,86]
4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one	heather[81,86]
4-hydroxy-3,5-dimethoxybenzoic acid* (syringic acid)	clover, [80,86] mānuka, [86] heather, [86] thyme, [82,86] nodding thistle, [84,86] kāmahi[86]
4-hydroxy-3-methoxybenzoic acid* (vanillic acid)	thyme[82,86]
4-hydroxy-3-methyl-*trans*-2-pentenedioic acid	rewarewa[92]
4-hydroxy-4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one	heather[81,86]
4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one	clover, [86] heather, [81,86] thyme[82,86]
4-hydroxy-4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohex-2-en-1-one	heather[81,86]
4-hydroxybenzaldehyde	viper's bugloss[85,86]
4-hydroxybenzoic acid*	thyme, [82] viper's bugloss[85]
4-hydroxybenzyl alcohol	viper's bugloss[85,86]
4-methoxyacetophenone (acetanisole)	mānuka[90]
4-methoxybenzaldehyde	mānuka, [83,90] nodding thistle, [84] viper's bugloss[85]

Compound	Honey Floral Type
4-methoxybenzaldehyde	heather,[81,86] thyme,[82,86] viper's bugloss,[86] nodding thistle[86]
4-methoxybenzoic acid	mānuka[90]
4-methoxybenzoic acid*	thyme,[82] viper's bugloss[85]
4-methoxyphenol	viper's bugloss,[85,86] mānuka[83]
4-methoxyphenylacetone	mānuka[83]
4-methoxypropylbenzene	mānuka[90]
4-methyl-*5H*-furan-2-one	clover,[79] kāmahi,[15,79] mānuka,[79] pōhutukawa,[79] rātā,[79] rewarewa,[79] tāwari[79]
4-oxo-pentanoic acid* (levulinic acid)	heather[86]
5-hydroxymethyl-2-furfural (HMF)	beech honeydew,[91] clover,[86,91] kāmahi,[86,91] mānuka,[86,91] rātā,[91] rewarewa,[91], tāwari,[91] thyme,[82,86,91] viper's bugloss,[85,86,91] heather[86] nodding thistle[84,86]
5-methyloxolan-2-one	clover,[79] beech honeydew,[79] kāmahi,[79] mānuka,[79] pōhutukawa,[79] rātā,[79] rewarewa,[79] tāwari,[79] viper's bugloss[79]
9-hydroxy-2-*trans*-decenoic acid*	nodding thistle[84]
9-β-methyl-2-decalone	mānuka[90]
abscisic acid*	clover,[86] mānuka,[83,86] heather[81,86] viper's bugloss,[85,86] nodding thistle,[84,86] kāmahi[86]
acetaldehyde	beech honeydew,[91] clover,[91] kāmahi,[91] mānuka,[91] rātā,[91] rewarewa,[91], tāwari,[91] thyme,[91] viper's bugloss[91]
acetone	beech honeydew,[91] clover,[91] kāmahi,[91] mānuka,[91] rātā,[91] rewarewa,[91], tāwari,[91] thyme,[91] viper's bugloss[91]
acetophenone	beech honeydew,[79,91] clover,[91] kāmahi,[91] mānuka,[79,83,91] rātā,[91] rewarewa,[91], tāwari,[91] thyme,[91] viper's bugloss[91] pōhutukawa[79]
benzaldehyde	beech honeydew,[79,91] clover,[79,86,91] kāmahi,[79,91] mānuka,[79,86,91] rātā,[79,91] rewarewa,[79,91], tāwari,[79,91] thyme,[91] viper's bugloss,[79,85,86,91] heather[81,86] pōhutukawa[79]
benzoic acid	beech honeydew,[79,91] clover,[79,91] kāmahi,[79,91] mānuka,[79,91] rātā,[79,91] rewarewa,[79,91] tāwari,[79,91] thyme,[79,91] viper's bugloss[79,91] pōhutukawa[79]
benzoic acid*	clover,[80,86] mānuka,[83,86] heather,[81,86] thyme,[82,86] viper's bugloss,[85,86] nodding thistle,[84,86] kāmahi[86]
benzyl alcohol	clover,[79,86,91] beech honeydew,[79,91] kāmahi,[79,86,91] mānuka,[79,86,91] pōhutukawa,[79] rātā,[79,91] rewarewa,[79,91] tāwari,[79,91] thyme,[79,82,86,91] viper's bugloss,[79,91] heather[81,86] nodding thistle[84,86]
butanedioic acid (monoethyl ester)	rewarewa[92]
butanedioic acid (succinic acid)	rewarewa[92]
butanedioic acid†	clover,[86] mānuka,[86] heather,[86] thyme,[82,86] viper's bugloss,[85,86] nodding thistle,[84,86] kāmahi[86]

Compound	Honey Floral Type
cis-3-phenylprop-2-enoic acid*	clover, [86] mānuka, [86] heather, [86] thyme, [82,86] nodding thistle[84,86]
cis-linalool oxide	clover, [79] kāmahi, [79] mānuka, [79,90] pōhutukawa, [79] rātā, [79] rewarewa, [79] tāwari, [79] thyme, [79] viper's bugloss[79]
coumarin	beech honeydew, [91] clover, [91] kāmahi, [91] mānuka, [91] rātā, [91] rewarewa, [91], tāwari, [91] thyme, [91] viper's bugloss[91]
decanedioic acid	rewarewa[92]
decanedioic acid†	clover, [80,86] mānuka, [83,86] heather, [86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [84,86] kāmahi[86]
decanoic acid (C_{10})*	mānuka[83]
diacetone alcohol	nodding thistle, [84] viper's bugloss[85]
dimethyl 2,2-dimethylbutanedioate	kāmahi[86]
dimethyl 2-decenedioate	clover[80]
dimethyl 2-methylsuccinate	mānuka[83]
dimethyl 2-*trans*-octenedioate	nodding thistle[84]
dimethyl sulfide	beech honeydew, [91] clover, [91] kāmahi, [79,91] mānuka, [91] rātā, [15,79,91] rewarewa, [79,91] tāwari, [79,91] thyme, [91] viper's bugloss[91] pōhutukawa[79]
dimethyl sulfoxide	pōhutukawa, [15,79] rātā[15,79]
docosane (C_{22})	clover[80]
docosanoic acid (C_{22})*	clover[80]
dodecanoic acid (C_{12})*	clover, [80,86] mānuka, [83,86] heather, [81,86] thyme, [82,86] nodding thistle, [84,86] kāmahi[86]
ethanol	beech honeydew, [91] clover, [91] kāmahi, [91] mānuka, [91] rātā, [91] rewarewa, [91], tāwari, [91] thyme, [91] viper's bugloss[91]
ethyl acetate	mānuka[90]
furfural	beech honeydew, [91] clover, [91] kāmahi, [91] mānuka, [91] rātā, [91] rewarewa, [91], tāwari, [91] thyme, [91] viper's bugloss[91]
heneicosane (C_{21})	clover, [80,86] mānuka, [83,86] heather, [81,86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [84,86] kāmahi[86]
hentriacontane (C_{31})	clover[80]
heptacosane (C_{27})	clover[80]
heptadecane (C_{17})	mānuka[83]
heptadecanoic acid (C_{17})*	clover, [86] mānuka, [83,86] heather, [81,86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [84,86] kāmahi[86]
heptanedioic acid	rewarewa[92]
hexacosane (C_{26})	clover[80]
hexadec-9-enoic acid*	clover, [86] mānuka, [83,86] heather, [81,86] viper's bugloss, [85,86] nodding thistle[84,86]

Compound	Honey Floral Type
hexadecanoic acid (C_{16})*	clover, [80,86] mānuka, [83,86] heather, [81,86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [84,86] kāmahi[86]
hexadecanoic acid (TMS ester)	mānuka[94]
hexadecanoic acid*	clover[80]
hexanal	beech honeydew, [91] clover, [91] kāmahi, [91] mānuka, [91] rātā, [91] rewarewa, [91], tāwari, [91] thyme, [91] viper's bugloss[91]
hexanedioic acid	rewarewa[92]
hexanedioic acid†	clover, [86] heather, [86] thyme, [82,86] nodding thistle[84,86]
hexanoic acid	thyme[15,79]
hexanoic acid*	heather, [81,86] thyme[82,86]
icosane (C_{20})	mānuka[83]
icosanoic acid (C_{20})*	clover[80]
lilac alcohol (isomer 1)	nodding thistle[84]
lilac alcohol (isomer 2)	nodding thistle[84]
lilac alcohol (isomer 3)	nodding thistle[84]
lilac aldehyde (isomer 1)	nodding thistle[84]
lilac aldehyde (isomer 2)	nodding thistle[84]
lilac aldehyde (isomer 3)	nodding thistle[84]
linalool	beech honeydew, [15,79,91] clover, [79,91] kāmahi, [79,91] mānuka, [79,91] rātā, [91] rewarewa, [91], tāwari, [79,91] thyme, [79,91] viper's bugloss[91] pōhutukawa[79]
linoleic acid*	clover, [80,86] mānuka, [83,86] heather, [81,86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [84,86] kāmahi[86]
methanol	beech honeydew, [91] clover, [91] kāmahi, [91] mānuka, [91] rātā, [91] rewarewa, [91], tāwari, [91] thyme, [91] viper's bugloss[91]
methyl 2-methylsuccinaldehyde	clover, [86] mānuka, [86] heather, [86] thyme, [82,86] kāmahi[86]
methyl *trans*-2-decenaldehydoate	viper's bugloss[85]
myrtenal	clover, [79] mānuka[15,79]
nonacosane (C_{29})	clover[80]
nonadecane (C_{19})	mānuka[83]
nonanal	beech honeydew, [79,91] clover, [91] kāmahi, [91] mānuka, [91] rātā, [91] rewarewa, [91], tāwari, [91] thyme, [91] viper's bugloss[91] pōhutukawa[79]
nonanedioic acid	rewarewa[92]
nonanedioic acid*	nodding thistle[84]

Compound	Honey Floral Type
nonanedioic acid†	clover, [80,86] mānuka, [83,86] heather, [86] thyme, [82,86] nodding thistle, [86] kāmahi[86]
nonanoic acid*	clover, [86] thyme, [82,86] mānuka[83]
octacosane (C_{28})	clover[80]
octacosanoic acid (C_{28})*	clover[80]
octadec-9-enoic acid*	clover, [80,86] mānuka, [83,86] heather, [81,86] viper's bugloss, [85,86] nodding thistle, [84,86] kāmahi, [86] thyme[82]
octadecanoic acid (C_{18})*	clover, [80,86] mānuka, [83,86] heather, [81,86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [84,86] kāmahi[86]
octanedioic acid	rewarewa[92]
octanedioic acid (bis(TMS) ester)	mānuka[94]
octanedioic acid†	clover, [80,86] mānuka, [83,86] heather, [86] thyme, [86] viper's bugloss, [85,86] nodding thistle, [84,86] kāmahi[86]
octanoic acid*	clover, [80,86] heather, [81,86] thyme, [82,86] mānuka[83]
o-methoxyacetophenone	clover, [15,79,80,86] beech honeydew, [79] kāmahi, [79,86] mānuka, [79,83,86,93,94] pōhutukawa, [79] rātā, [79] rewarewa, [79] tāwari, [15,79] thyme, [79,82] viper's bugloss[79]
p-anisaldehyde	kānuka[93]
pantoyl lactone (pantolactone)	clover, [79] beech honeydew, [79] kāmahi, [15,79] mānuka, [79] pōhutukawa, [79] rātā, [79] rewarewa, [79] tāwari, [79] thyme, [79] viper's bugloss[79]
p-benzoquinone	viper's bugloss[15,79]
pentacosane (C_{25})	clover[80]
pentadecanoic acid (C_{15})*	clover, [86] heather, [81,86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [84,86] mānuka[83]
pentanedioic acid	rewarewa[92]
pentanedioic acid†	clover, [86] mānuka, [86] thyme[82,86]
phenol	beech honeydew, [15,79] mānuka, [79] heather[81,86] kāmahi[86]
phenyl 3-(3',4'-dimethoxyphenyl)-*trans*-propenoate	nodding thistle[84]
phenylacetaldehyde	
phenylacetic acid	beech honeydew, [91] clover, [91] kāmahi, [91] mānuka, [91] rātā, [91] rewarewa, [91], tāwari, [91] thyme, [91] viper's bugloss[91]
phenylacetic acid*	clover, [80] mānuka[83]
phenylethanoic acid*	heather[81]
phenylpyruvic acid (TMS ester)	mānuka[94]
picolinic acid*	thyme, [82,86] nodding thistle[84]
propylbenzene	mānuka[83]

Compound	Honey Floral Type
salicylic acid*	mānuka[83]
succinic acid (TMS ester)	mānuka[94]
succinic acid†	clover, [80] mānuka[83]
terpineol	clover, [79] beech honeydew, [79] mānuka, [79] pōhutukawa, [79] rātā, [79] rewarewa, [79] tāwari, [79] viper's bugloss[79]
tetracosane (C_{24})	clover[80]
tetracosanoic acid (C_{24})*	clover[80]
tetradecanoic acid (C_{14})*	clover, [80,86] mānuka, [83,86] heather, [81,86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [84,86] kāmahi[86]
thymol	thyme[15,79]
toluene	mānuka[90]
trans-2-decenedioic acid†	clover, [86] mānuka, [83,86] heather, [86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [84,86] kāmahi[86]
trans-3-phenylprop-2-enoic acid*	clover, [86] mānuka, [86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [84,86] kāmahi[86]
trans-cinnamic acid (TMS ester)	mānuka[94]
trans-*cis*-abscisic acid*	thyme[82,86]
trans-linalool oxide	clover, [79] beech honeydew, [79] kāmahi, [79] mānuka, [79] pōhutukawa, [79] rātā, [79] rewarewa, [79] tāwari, [79] thyme, [79] viper's bugloss[79]
trans-*trans*-abscisic acid*	thyme[82,86]
triacontane (C_{30})	clover[80]
tricosane (C_{23})	clover, [80,86] mānuka, [86] heather, [81,86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [86] kāmahi[86]
trimethylphenol	heather[81,86]
tri-*n*-butylphosphate	mānuka[83]
tritriacontane (C_{33})	clover[80]
undecane (C_{11})	mānuka, [83] nodding thistle, [84] viper's bugloss[85]
xylene	mānuka[83]
α-linolenic acid*	clover, [80,86] mānuka, [86] heather, [81,86] thyme, [82,86] viper's bugloss, [85,86] nodding thistle, [84,86] kāmahi[86]
β-ionone	mānuka[83]

*monomethyl ester †dimethylester

1.4.1. Mānuka honey

Mānuka honey is dark in colour and has a distinctive earthy, mineral flavour. [95] It is produced in regenerating forests and plantations and the harvest famously depends on weather conditions. [96] Wilkins *et al.* [83] found that irrespective of geographic origin or season, mānuka honey can be characterised by a combined concentration of 2-hydroxy-3-phenylpropionic acid and 2-hydroxy-3-(4'-methoxyphenyl) propionic acid > 700 mg kg^{-1}, a combined concentration of syringic acid and 3,4,5-trimethoxybenzoic acid > 35 mg kg^{-1}, and a combined concentration of acetophenone and 2-methoxyacetophenone > 20 mg kg^{-1}. [83] Today, different, non-volatile compounds are used as markers for mānuka, as stated in section 1.1.1; honey must contain ≥ 400 mg/kg 3-phenyllactic acid, ≥ 1 mg/kg *o*-methoxyacetophenone, ≥ 1 mg/kg 2-methoxybenzoic acid and ≥ 1 mg/kg 4-hydroxyphenyllactic acid to be classified as monofloral mānuka. [21] These are analysed by liquid chromatography-mass spectrometry (LC-MS) rather than GC-MS, as they are non-volatile with the exception of *o*-methoxyacetophenone.

Mānuka honey is known for its high levels of volatile compounds. [86] Revell *et al.* found higher concentrations of 2-methylbenzofuran, 1-(2-methoxyphenyl)ethanol, myrtenal and 1-phenylethanol in mānuka than other New Zealand honeys, and proposed them as potential markers. [15,93] However, none of these compounds have been identified as aroma-active compounds in honey. Tan *et al.* noted high concentrations of 2-hydroxy-3-phenylpropionic acid (550 μg g^{-1}), *o*-methoxyacetophenone (10 μg g^{-1}), 2-hydroxybenzoic acid (16 μg g^{-1}) and methyl 4-hydroxy-3,5-dimethoxybenzoate (92 μg g^{-1}). [86]

Mānuka honeys contain a high concentration of aromatic acids. Meloncelli *et al.* (2015) derivatised mānuka extracts with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) to volatilise the semi-volatile organic acids, and detected succinic acid, phenylpyruvic acid, hexadecenoic acid, 2-isopropyl-3-hydroxy-but-2-enoic acid, mandelic acid, malic acid, *trans*-cinnamic acid and octanedioic acid, as their trimethylsilylated derivatives. [94] Syringic acid and 4-methoxyphenyl compounds have also been reported in some studies of mānuka honey. [85] Varying concentrations (32 ± 23 mg kg^{-1}) of syringic acid have been reported depending on the geographic origin, with honey from the East Coast having the highest concentration. [83,97]

1.4.2. Kānuka honey

Kānuka honey is almost identical to mānuka, and is indistinguishable by pollen analysis alone. [96] Beitlich *et al.* (2014) identified 2,6,6-trimethyl-2-cyclohexene-1,4-dione, 2-

phenylethanol and *p*-anisaldehyde in kānuka honey and proposed them as markers compounds for distinguishing kānuka from mānuka, as well as one unidentified compound. [93] However, 2,6,6-trimethyl-2-cyclohexene-1,4-dione and 2-phenylethanol were previously identified in mānuka in 2013. [79] *p*-Anisaldehyde has been identified in international honeys, and contributes to the aroma of linden, buckwheat, haze, acacia and heath honeys, but has not otherwise been identified in New Zealand honeys. [27,50,55]

1.4.3. Rātā and pōhutukawa honeys

Rātā and pōhutukawa honeys, both belonging to the *Metrosideros* genus, are characterised by high concentrations of organosulfur compounds such as dimethyl sulfide and dimethylsulfoxide. [15,79] The odour of dimethyl sulfide is described as "cabbage, sulfur, gasoline" while dimethylsulfoxide is said to smell like garlic. [98,99] These compounds have low odour thresholds so likely contribute to the aroma of the *Metrosideros* honeys. [79] Pōhutukawa honey is known for its butterscotch taste, which is sometimes describes as salty, and has a very light colour. Rātā, while described as a very sweet honey, has the same salty notes. [95,96] 3-methyl-2-butenal and (*E*)-cinnemaldehyde were also identified as possible marker compounds for distinguishing pōhutukawa honey from other New Zealand honeys. [15,79]

1.4.4. Tāwari, rewarewa and kāmahi honeys

Tāwari, rewarewa and kāmahi are known as "bush honeys" as the floral sources for these honeys grow in native New Zealand forests. [79,96] These honeys can be expected to contain similar volatiles due to common nectar sources. Rewarewa has a distinctive reddish colour, with a complex malty flavour. [95,96] Its profile is dominated by aliphatic dicarboxylic acids such as 2-methylbutanedioic acid and 4-hydroxy-3-methyl-*trans*-2-pentenedioic acid, among other organic acids. [92] However, these compounds were methylated in order to be detected by GC-MS, as they are not volatile. It is thus not surprising that they have not been identified as aroma-active in other honeys. Ethanol, methanol and acetone were the major volatiles identified in rewarewa honey by Langford *et al.*(2012). [91] Tāwari honey is described as tasting like rosehip syrup. [95] Tāwari honey displayed higher concentrations of 2-ethylhexanoic acid than other New Zealand honeys. [15] This compound has not been identified as aroma-active in other honeys.

Kāmahi honey has a rich, sweet, buttery taste. Revell *et al.* detected higher concentrations of 4-methyl-5*H*-furan-2-one in these honeys, especially in kāmahi, making it a potential indicator of New Zealand bush honeys. [79] Three novel *nor*-carotenoids were found in kāmahi

honey. [100] The compounds, named kamahines A-C, are diastereoisomeric (Figure 4). It has been suggested that these compounds were not components of the floral origin, but rather formed by oxidative processes in the honey. [100] Other important compounds in kāmahi honey include 2,6,6-trimethylcyclohexane-1,4-dione. [86]

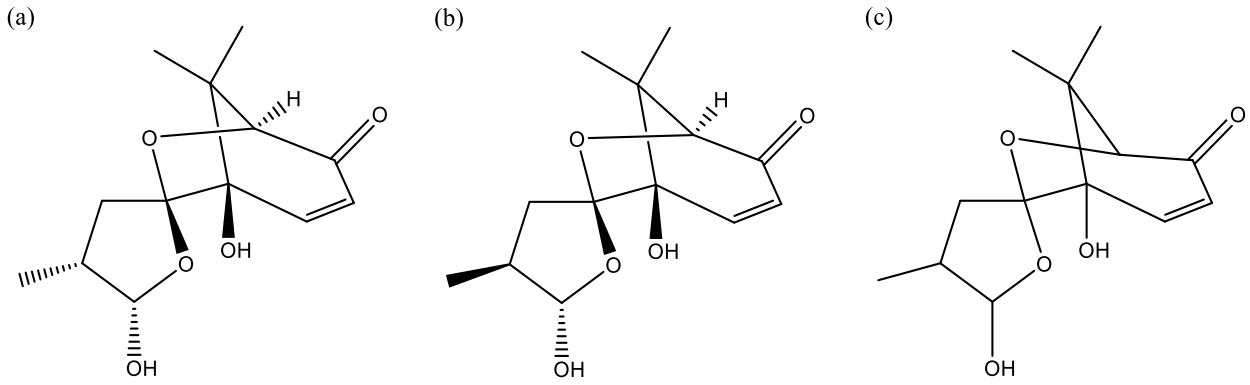

Figure 4. Structure of kamahines. (a) Kamahine A, (b) Kamahine B, (c) Kamahine C.

1.4.5. Honeydew honey

New Zealand beech trees are inhabited by the scale insect (*Ultracoelostoma assimile*), which feeds on the phloem of the tree and secrets a sweet substance called honeydew. Due to the tree's low level of nectar, honey bees instead feed on this honeydew to produce beech honeydew honey. This specialty honey is very dark in colour and has a malty, earthy flavour. Petchell (2009) proposed phenol and 1-(2-methoxyphenyl)ethanol for discrimination of honeydew honeys from New Zealand nectar honeys, although these compounds have been found in mānuka, heather and kāmahi honeys. [15,79]

As well as botanical origin, geographical location also influences the volatile profile of the honey. For example, Meloncelli *et al.* (2015) reported that syringic acid was present in mānuka honey originating from New Zealand, but not in that originating from Tasmania. [94] Non-native honeys such as clover, thyme and heather produced in New Zealand display different volatile profiles to internationally produced honeys of the same botanical origin.

1.4.6. Clover honey

Clover honey has a mild, sweet, fudge-like taste. [95,96] Clover honey typically contains low concentrations of volatiles, with extractable organic substances (excluding beeswax hydrocarbons and fatty acids) typically below 50 μg g^{-1}. [86] Tan *et al.* identified a wide range of hydrocarbons and straight-chain monobasic and dibasic acids from the methylated extracts of clover honeys. [80,86] The dominant aroma compounds were 2-hydroxy-3-phenylpropionic acid (phenyllactic acid, 2.5 – 66.9 μg g^{-1}), phenylacetic acid, (*E*)-cinnamic acid, and benzoic

acid.[80] However, the concentration of 2-hydroxy-3-phenylpropionic acid was noticed to dramatically increase in samples containing mānuka pollen.[86] More recent research by Grainger (2015) did not identify it in clover honey; rather, it was only identified in mānuka and kānuka honeys.[6] These pieces of information suggest that the presence 2-hydroxy-3-phenylpropionic acid in clover honey is due to contributions from mānuka nectar rather than clover. Additionally, other floral types will always contribute to honeys classed as monofloral, as it cannot be guaranteed that the bees will not feed on multiple sources. Petchell (2009) found clover honey to be characterised by the presence of 3-methylpentanoic acid and *o*-methoxyacetophenone.[15] Though the aroma-active compounds in clover honey have not been reported to date, a review by Machado (2020) shows that the dominant volatiles differ significantly between clover honeys produced in different countries.[8] This makes an analysis of the aroma profile of New Zealand clover honey worthwhile, as international studies are unlikely to represent the profile of New Zealand clover accurately.

1.4.7. Thyme honey

Thyme honey is produced in the Central Otago region of the South Island, and has a strong herbal taste. It has a rich volatile profile and phenylacetaldehyde and benzyl nitrile have been proposed as marker compounds in an international study.[101] In contrast, thymol and hexanoic acid have been used to identify thyme from other New Zealand honeys.[15] The different geographical origin of these honeys may contribute to the different volatile fractions, but the difference in sample extraction methods is also likely to contribute, as the New Zealand study used SPME whereas a purge and trap method was used to isolate volatiles from the Grecian honey.[15,101] Other important compounds in New Zealand thyme honey are 1-(2-oxo-*trans*-1-butenyl)-2,6,6-trimethylcyclohexane-*trans,cis*-1,2,4-triol, 3-hexenoic acid, 3'-aminoacetophenone, 1-(3-oxo-1-butenyl)-2,6,6-trimethylcyclohexan-4-ol, and 1-(3-oxo-*trans*-1-butenyl)-2,6,6-trimethylcyclohexane-*trans-cis*-1,2,4-triol.[82,86,102]

1.4.8. Viper's bugloss honey

Proposed marker compounds for viper's bugloss honey are *p*-benzoquinone[15] and hydroquinone.[85] Although average concentrations of volatile compounds are low compared to the other native honey types, other important compounds were identified including 2,5-cyclohexadiene-1,4-dione, 4-methoxyphenol, hydroxybenzyl alcohol, hydroxybenzaldehyde, and 1,4-dihydroxybenzene, which has been found in high concentrations compared to other New Zealand honeys (16 – 28 μg g^{-1}).[85,86] The appearance of *para*-disubstituted aromatic compounds can be attributed to the floral origin, as these compounds are common in plants

and are thought to be secondary metabolites. [85] This honey has a delicate, floral flavour and is often used as a sweetener due to its high fructose content. [103]

1.4.9. Nodding thistle honey

Several linalool derivatives have been identified in New Zealand nodding thistle (*Carduus nutans*) honey. [84] Linalool and its derivatives, including *cis*- and *trans*-linalool oxides, hotrienol, (*E*)- and (*Z*)-hydroxylinalool, and the lilac aldehydes and alcohols are the most abundant monoterpenes found in honey. [104] These compounds can be produced during honey storage in addition to their origins in the plant matter, making them unsuitable as marker compounds. [105] The dominant linalool derivatives identified in New Zealand nodding thistle honey were *(E)*-2,6-dimethyl-3,7-octadiene-2,6-diol, *(E)*-2,6-dimethyl-6-hydroxy-2,7-octadienoic acid and (Z)-2,6-dimethyl-6-hydroxy-2,7-dienal. Lilac alcohol and aldehyde isomers were also found and proposed as markers, although they are also found in citrus and gardenia honeys internationally. [24,83,89,106] This demonstrates the risk of using these compounds as markers. Lilac aldehydes have been identified in Italian thistle honey, while linalool oxide, which is an isomer of lilac aldehyde, was identified in American thistle honey. [88]

1.4.10. Ling/heather honey

Analysis of New Zealand ling/heather honey revealed C_{13} norisoprenoids, which are degraded carotenoids, as the dominant volatile compounds. [81,86] Carotenoids degrade by enzymatic or non-enzymatic pathways to produce norisopenoid compounds with a 3,5,5-trimethylcyclohex-2-ene backbone. These norisoprenoids, such as damascenones, damascones and ionones (Figure 5), have low aroma thresholds and contribute to the aroma of many food products, including wine and honey. [107] (*E*)-β-damascenone and β-ionone are the major aroma contributors of flowers, and are consequently found in many honeys, particularly those from Australia and New Zealand. [80-82,89,100,108]

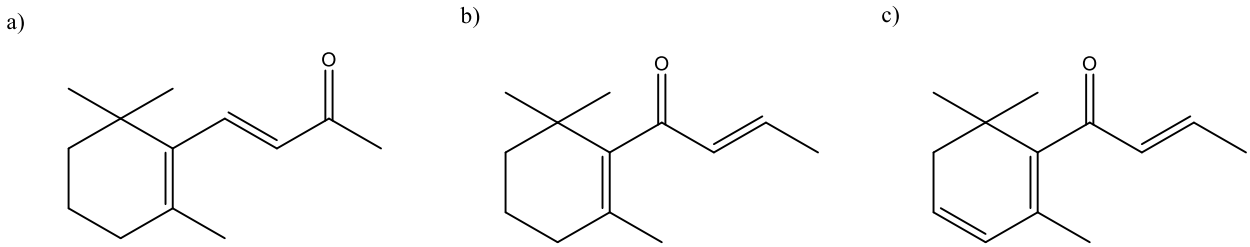

Figure 5. Structures of selected aroma-active norisoprenoids. a) β-ionone, b) β-damascone, c) β-damascenone.

The dominant norisoprenoids in New Zealand heather honey were 4-(3-oxo-1-butenylidene)-3,5,5-trimethylcyclohex-2-en-1-one, 4-hydroxy-4-(3-hydroxy-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one, and 4-hydroxy-4-(3-oxo-1-butenyl)-3,5,5-trimethylcyclohex-2-en-1-one. These compounds have not been identified as aroma-active in heather honey produced internationally, though isophorone (3,5,5-trimethylcyclohex-2-en-1-one), which is similar in structure, was identified as an aroma contributor in Estonian heather honey by Seisonen *et al.* (2015). Other major norisoprenoid components not present in the New Zealand heather include dehydrovomifoliol, 3-oxo-α-ionol and (*E*)-β-damascenone. [109] In 2021, the most abundant volatiles in international heather honey were reported to be hotrienol, phenylacetaldehyde and *cis*-linalool. [110] New Zealand heather honey has been described to have a "mild but pronounced flavour" and is reddish in colour, to which the degraded carotenoids are thought to contribute significantly. [81,111] Other prominent compounds in the New Zealand heather honey included benzoic acid (82 μg g^{-1}), 2-phenylethanoic acid (188 μg g^{-1}) and 2-hydroxy-3-phenylpropionic acid, [86] of which benzoic acid and 2-phenylethanoic acid have previously been identified as aroma contributors to heather honey. This data suggests there may be a difference in the volatile profile of heather honey depending on geographical origin.

Langford *et al.* (2012) used selected ion flow tube mass spectrometry rather than GC-MS to quantify 22 volatile compounds in nine monofloral New Zealand honeys. [91] The compounds included methylbutanoic acid, benzoic acid, phenylacetic acid, 2-methylfuran, furfural, HMF, methanol, ethanol, 3-methyl-1-butanol, 1-hexanol, phenylmethanol, 2-phenylethanol, acetaldehyde, hexanal, nonanal, benzaldehyde, phenylacetaldehyde, acetone, acetophenone, dimethyl sulfide, linalool and coumarin. Many of these compounds had not been identified before in New Zealand honeys, and statistical analysis on the quantitative results allowed discrimination between the honeys. [91]

1.5. Aim of this research

Mānuka honey is New Zealand's most famous and valuable monofloral honey, with the average price of these honeys more than double that of non-mānuka honeys. [5] However, it is clear that other New Zealand native monofloral honeys have distinct, appealing flavours. These flavours are well described by sensory analysis, but the compounds responsible for the aromas have not been well studied. An analytical method to quantify the concentrations of compounds responsible for the flavour of a honey would contribute to a better understanding of the reasons for flavour differences between honeys. [112] This would open up the potential for marketing of honeys with unique flavour combinations, or honeys with stronger or milder flavours, allowing New Zealand's other honeys to share in mānuka's success. [113]

The aims of this research were thus:

1. Develop, optimise and validate a method for the analysis of volatile compounds in honey.
2. Apply the method for the analysis of volatile compounds in New Zealand native monofloral honeys, and link this with sensory information to identify the key aroma contributors in each honey.
3. Examine the differences between the volatile profiles of New Zealand monofloral honeys of different botanical origins.

1.6. Thesis outline

Chapter Two outlines the experimental procedures used in this study, including the extraction and analysis of volatile compounds from honey, as well as pollen analysis and statistical analysis.

A wide variety of methods have been used for the extraction of volatile compounds from honey. **Chapter Three** includes a short review of these methods, followed by the development and optimisation of a method. Three different sample preparation techniques were investigated, including static headspace, headspace solid-phase micro-extraction, and liquid-liquid extraction. The chosen method was optimised with a series of experiments, and the instrumental method was also optimised.

Chapter Four outlines the procedures that were undertaken to validate the sample preparation and GC methods and the results of these.

After validation, a set of New Zealand native monofloral honeys were analysed using the chosen method and their volatile profiles are reported in **Chapter Five**. The data was compared with available sensory information to identify the key aroma contributors in each honey, followed by chemometric analysis to separate and classify the honeys by their floral origin.

Final conclusions and recommendations for future work are discussed in **Chapter Six**.

2. Chapter Two: Methods

This chapter outlines the experimental processes used for method validation (Chapter 4) and for the analysis of honey samples (Chapter 5).

2.1. Materials

2.1.1. Honey samples

A summary of the sample details is given in Table 3. While all samples were labelled as monofloral, visual melissopalynological analysis was carried out on recently obtained samples that were sourced directly from beekeepers to confirm their origin. As commercial suppliers typically conduct this analysis prior to sale, the commercially purchased honeys were not sent for visual pollen analysis. Honey samples were subsampled upon arrival, and stored at 4 °C in the absence of light in a walk in chiller. Prior to extraction, samples were thawed at room temperature.

Table 3. Details of honey samples analysed.

Sample Code	Region	Year of Collection	Floral Origin
NZ004	Manawatu	2018	Kāmahi
NZ011	Waikato	2018	Kāmahi
NZ012	Coromandel	2018	Mānuka
NZ013	Coromandel	2018	Rewarewa
NZ016	unknown	2015	Rewarewa
NZ017	unknown	2015	Mānuka
NZ019	Gisborne	2019	Tāwari
NZ035	Waikato	2019	Ling (heather)
NZ036	Waikato	2019	Lotus/blackberry
NZ037	Waikato	2019	Mānuka
NZ105	Bay of Plenty	2015	Pōhutukawa
NZ182	Bay of Plenty	2012	Clover
NZ202	Bay of Plenty	2015	Clover
NZ216	Auckland	2017	Rātā
NZ231	Bay of Plenty	2013	Rewarewa
NZ232	Bay of Plenty	2013	Rewarewa
NZ233	Bay of Plenty	2015	Rewarewa
NZ246	Bay of Plenty	2013	Pōhutukawa
NZ247	Bay of Plenty	2014	Pōhutukawa
NZ248	Bay of Plenty	2015	Pōhutukawa
NZ251	Gisborne	unknown	Honeydew
NZ270	unknown	2019	Pōhutukawa
NZ271	unknown	2021	Rewarewa
NZ272	unknown	2021	Kāmahi
NZ273	unknown	2020	Rātā
NZ274	unknown	2022	Rewarewa

Sample Code	Region	Year of Collection	Floral Origin
NZ275	unknown	2019	Kāmahi
NZ276	unknown	unknown	Pōhutukawa
NZ277	unknown	unknown	Kānuka
NZ280	Matawai	unknown	Tāwari
NZ281	Buller	2018	Beech Honeydew
NZ282	Marlborough	2021	Kāmahi
NZ283	Marlborough	2019	Kānuka
NZ284	unknown	2017	Honeydew
NZ287	Matawai	2022	Tāwari*
NZ298	unknown	2020	Mānuka
NZ299	unknown	2020	Mānuka
NZ323	Gisborne	2021	Tāwari
NZ329	New Plymouth	2021	Clover
NZ334	Far North	2020	Kānuka*
NZ342	unknown	2021	Kāmahi†
NZ360	North Canterbury	2021	Honeydew
NZ361	North Canterbury	2021	Honeydew
NZ364	Bay of Plenty	2020	Tāwari*
NZ372	unknown	unknown	Honeydew
NZ375	unknown	unknown	Clover
NZ376	unknown	2022	Kānuka
NZ386	unknown	unknown	Clover
NZ387	unknown	unknown	Clover

* Botanical origin confirmed by visual pollen analysis. †Though this honey was labelled as monofloral kāmahi, visual pollen analysis indicated it was classed as multifloral.

2.1.2. Standards

(*E*)-β-damascenone (analytical standard), *p*-anisaldehyde (98%), linalool oxide (≥97.0% GC grade, mixture of isomers), 2-methoxyacetophenone (99%), 3-methylvaleric acid (97% GC grade), 2-phenylethanol (99% GC grade), nonanal (95%), 2,6,6-trimethyl-2-cyclohexene-1,4-dione (4-oxoisophorone) (98%), syringaldehyde (98%), D-(-)-pantolactone (99%), *n*-dodecane (99%) and octanoic acid (98%) were obtained from Sigma-Aldrich. β-ionone (97%) and (*R*)-(+)-limonene (97%) were from Aldrich Chemical Company. Vanillic acid was from Sigma Chemical Company. Linalool (97%) and 1-hexanol (99%) were from Alfa-Aesar. Benzaldehyde (98%) was obtained from Acros. Phenylacetaldehyde (50% in benzyl alcohol) was from Fluka AG, Buchs SG. Cineole, succinic acid (99%), benzyl alcohol (98%), eugenol and guaiacol were from BDH Chemicals Ltd. Benzoic acid was from Univar. Purity and supplier information was unavailable for α-pinene and β-pinene. Purity was assumed to be 98% for all compounds where purity information was unavailable, and this value was carried through for all calculations.

2.1.3. Solvents and consumables

The solvents used in this study were dichloromethane (DCM), diethyl ether and ethyl acetate. DCM (≥99.8%, GC grade) and diethyl ether ((≥99.8%, GC grade) were obtained from Merck. Ethyl acetate (analytical reagent grade) was from Ajax Laboratory Chemicals. Acetone was used as a wash solvent for the GC injection needle, and was obtained from Merck.

Milli-Q water (18.0 mΩ) was used for pollen DNA analysis, and was obtained using a Milli-Q purification system from Merck. For all other applications, distilled water was used.

Sodium chloride (NaCl) used for static headspace and SPME experiments was from Sigma-Aldrich.

Glass microfibre filter paper sourced from Whatman (GF/C grade, 55 mm diameter, 1.2 μm pore size) and from Advantec (GA-55 grade, 55 mm diameter, 0.6 μm pore size) were used for filtering of extracts.

2.2. Preparation of standard solutions

10,000 mg kg^{-1} solutions of each of the external standards were prepared by adding 133 mg of the pure compound (actual weight adjusted depending on purity) and making up to 20 mL with DCM.

External standards were grouped into two categories based on their ranges of linearity on the GC-MS selected ion monitoring (SIM) method. Group A included α-pinene, β-pinene, ᴅ-limonene, cineole, linalool oxide, linalool, 4-oxoisophorone, (*E*)-β-damascenone and β-ionone. Stock solutions of 10 and 1 mg kg^{-1} containing all the Group A compounds were made by serial dilution of the 10,000 mg kg^{-1} solutions. Group B included benzaldehyde, benzyl alcohol, phenylacetaldehyde, 2-phenylethanol, *o*-methoxyacetophenone, guaiacol, nonanal and eugenol. A stock solution of 10 mg kg^{-1} was made by dilution of the 10,000 mg kg^{-1} solutions of the Group B compounds.

These three stock solutions were then used to make the daily calibration standards according to Table 4. The calibration range for the Group A compounds was 0.01 – 1 mg kg^{-1}, and for the Group B compounds was 1.5 – 3.5 mg kg^{-1}. The solutions were made so that all compounds were present in the same solution, but with the Group A compounds at lower concentrations than the Group B compounds. This allowed the analysis of a both calibration ranges simultaneously, reducing the time required for analysis of the calibration curve.

Table 4. Dilution scheme used to make weekly calibration standards from stock solutions.

Vial	Volume of 10 mg kg^{-1} A stock (µL)	Volume of 1 mg kg^{-1} A stock (µL)	Volume of 10 mg kg^{-1} B stock (µL)	Volume of DCM (µL)	Final concentration of A compounds (mg kg^{-1})	Final concentration of B compounds (mg kg^{-1})
1	-	10	50	940	**0.01**	**0.5**
2	-	25	75	900	**0.025**	**0.75**
3	-	50	100	850	**0.05**	**1**
4	-	100	150	750	**0.1**	**1.5**
5	-	200	200	600	**0.2**	**2**
6	-	350	250	400	**0.35**	**2.5**
7	50	-	300	650	**0.5**	**3**
8	100	-	350	550	**1**	**3.5**

Initially, the idea of matrix-matching the calibration solutions was considered, by spiking an artificial honey matrix with the external standards. The Food and Drugs Administration guideline on analytical method validation states that "The sponsor should prepare the calibration standards in the same biological matrix as the samples in the intended study." [114] However, as the matrix components of the live samples were removed during sample extraction, the calibration standards were matrix-matched to the analysed extracts, which were DCM solutions.

2.3. Sample Extraction

Honey samples were stirred thoroughly and weighed (2.000 g ± 0.050 g) into 15 mL plastic extraction tubes. Water (2 mL) was added and the samples were homogenised using an orbital shaker (10 min). DCM (4 mL) was added and the samples were shaken (30 min). Samples were centrifuged (30 min, 3900 rpm, 4 °C). The upper, aqueous layer was transferred to a new tube and the extraction was repeated two more times by adding DCM to the aqueous fraction, resulting in three 4 mL extracts. After the third extraction, the aqueous layer was discarded and the organic extracts were frozen overnight (−18 °C). Extracts were combined and filtered using glass microfibre filter paper into clean tubes. A 1 mL aliquot of the filtered extract was transferred into a 1.5 mL amber glass GC vial for analysis.

2.4. GC-MS

Analysis of the honey extracts was performed using a Hewlett-Packard 6890 Series GC system, coupled to a Hewlett Packard 5973 mass selective detector. The system was equipped with a PAL autosampler. 2 µL of the extracts were injected in pulsed splitless mode (injection

pulse pressure 25 psi until 1 min, then purge flow to split vent at 54 mL/min) at an injector temperature of 250 °C. The chromatographic separation was performed on an HP-5 MS column with length of 30 m, 0.25 mm inner diameter and 0.5 μm film thickness. The carrier gas was helium, with a flow rate of 1 mL/min. The oven temperature was held at 40 °C for three minutes, before increasing at 2 °C/min to 154 °C. The temperature was then increased at 20 °C/min to the final temperature of 250 °C and held for 5 minutes. The transfer line and ion source temperatures were 280°C and 230 °C, respectively. The mass selective detector was operated in positive ion mode; the ionisation voltage was 70 eV.

2.4.1. Quantification of selected compounds

The MS was operated in selected ion monitoring (SIM) mode for the quantification of selected compounds (Table 5). Solvent delay was 3.5 minutes, and the detector was turned off after 60 minutes.

Data processing was achieved using MassHunter QuantAnalysis MS software. Peaks were integrated using the Agile 2 integrator, though manual integration was also used when required. Compounds were identified based on the ratio of qualifier ion intensity to the quantifier ion and comparison of RT with that of the pure compound. Quantification was achieved using calibration curves of external standards, which were produced for each batch using the calibration standards described in section 2.2.

Table 5. SIM method parameters.

Time segment start time (min)	Target compound	Quantitative ion (*m/z*)	Qualifier ions (*m/z*)
3.5	α-pinene	92.8	118.8 133.7
12	benzaldehyde	105.8	104.8 76.8
13.3	β-pinene	92.8	90.8 135.8
16	D-limonene	67.9	120.8 106.8
	cineole	153.8	138.8 124.8
17.3	benzyl alcohol	107.8	78.8 76.8
17.85	phenylacetaldehyde	90.8	119.8 91.8

Time segment start time (min)	Target compound	Quantitative ion (*m/z*)	Qualifier ions (*m/z*)
19.6	linalool oxide (isomer 1)	93.8	92.8 58.9
20.5	linalool oxide (isomer 2)	93.8	92.8 58.9
	guaiacol	123.8	108.8 80.8
21.5	linalool	92.8	120.8 70.9
22.15	nonanal	56.9	97.9 69.9
22.5	2-phenylethanol	90.8	121.8 91.8
24	oxoisophorone	151.8	95.8 67.9
34	*o*-methoxyacetophenone	149.7	134.8 130.7
37	eugenol	163.8	148.8 130.8
40	(*E*)-β-damascenone	120.8	189.8 68.8
45	β-ionone	176.8	191.8 42.9

2.4.2. Total ion chromatogram screening

The MS was operated in total ion chromatogram (TIC) mode for the identification of other volatile compounds. The scan range was *m/z* 30-300. Data was processed using MassHunter Qualitative Workflows and Qualitative Navigator software, and tentative identification was achieved by comparison with the National Institute of Standards and Technology (NIST) mass spectral database.

2.5. General methods

2.5.1. Statistical analysis

Basic data processing was carried out in Microsoft Excel, including calculation of recoveries, means and standard deviations. Other statistical tests including one-way analysis of variance (ANOVA), *t*-tests, principal component analysis (PCA), classification and regression trees (CART), linear discriminant analysis (LDA) and display of data using individual value distribution plots were carried out using Minitab (LLC, 2021).

2.5.2. Cleaning of equipment

All glassware was washed with hot soapy water and rinsed with tap water before being soaked overnight in concentrated nitric acid. The acid was rinsed off with tap water (10 min). Glassware was rinsed with tap water and distilled water, and oven-dried.

Plasticware, including 15 mL tubes used for extraction, was washed with hot soapy water and rinsed with distilled water before being oven-dried.

3. Chapter Three: Method Development and Optimisation

A wide variety of methods have been employed for the extraction and analysis of volatile compounds from honey. Section 3.1 provides an overview of these methods and how popular methods have evolved over time. Static headspace (HS), headspace-solid phase micro-extraction (HS-SPME), and liquid-liquid extraction (LLE) were investigated as possible extraction methods. The experiments performed to select and optimise an extraction method are detailed here in sections 3.2 to 3.4, with GC-MS method development discussed in section 3.5.

3.1. Review of sample preparation methods

As previously discussed, the volatiles responsible for honey aroma must be extracted prior to analysis, as they are present in low concentrations in a highly complex matrix. The use of heat must be avoided where possible during the extraction. For most applications, heat increases the efficiency of the extraction, however in honey it can lead to the formation of artefacts, for example, from the degradation of sugars by non-enzymatic browning reactions or condensation. [115] This rules out hydrodistillation, a traditional extraction method. [116]

3.1.1. Liquid-liquid extraction

Liquid-liquid extraction (LLE), also referred to as solvent extraction, is one of the most commonly used methods of extracting volatiles from honey. [117] It allows the extraction of compounds with a wide range of volatilities in a single step. [118] In most cases, the honey sample is first diluted in water and an internal standard is added before liquid-liquid extraction with an organic solvent. [80]

Traditionally, large sample and solvent volumes are required due to the low concentration of volatiles in the honey matrix, which is a disadvantage from a green chemistry perspective. The method used by Castro-Vazquez *et al.* (2003) required a 50 g honey sample, extracted in 60 mL of DCM. [117] In a more extreme example, Blank *et al.* (1989) extracted 200 g honey in 250 mL DCM. [27] In recent years, studies have focussed on miniaturising the technique to reduce the volume of sample and solvent required. For example, a method developed by Kus and Jerkovic (2018) employing dehydration homogenous liquid-liquid extraction required only 5 g of honey. [119] Meloncelli *et al.* (2015) extracted 2 g honey in 2 mL ethyl acetate, although the extracts were derivatised prior to GC-MS analysis, which allowed detection of

semi-volatile compounds by GC-MS. Meloncelli *et al.* used N,O-bis(TMS)trifluoroacetamide to convert hydroxyl groups and carboxylic acids to trimethylsilyl (TMS) ethers and esters, respectively. [94] Tan *et al.* methylated New Zealand honey extracts with etheral diazomethane. [80] Derivatisation is useful for studies aiming to identify marker compounds or simply characterise the volatile fraction of the honey. In the case of this research, however, which aims to characterise only those compounds that are already sufficiently volatile to be detected in the honey aroma, derivatisation is unnecessary.

In a variation of LLE, the diluted sample and organic solvent are subjected to high-energy ultrasonic waves, which facilitate mixing through acoustic cavitation. [120] This process is known as ultrasonic solvent extraction (USE) and has shown increasing popularity in recent years. Jerkovic *et al.* found that USE extracts were more representative of the volatile fraction of the honey compared to extracts obtained by hydrodistillation, and did not contain thermally derived artefacts. [121] They noted that it was useful for extracting semi-volatile compounds, and thus could be used to complement HS-SPME. Their method, which required three 30 min ultrasonic extractions, was used to characterise the volatile profiles of black locust (*Robinia pseudoacacia L.*), sweet chestnut, mint (*Mentha spp.*), apple (*Malus domestica Borkh.*) and red clover (*Trifolium pratense L.*) honeys in the authors' further studies. [121-124] Alissandrakis *et al.* were able to identify 110 compounds from USE extracts of various honeys, compared to just 24 from HS-SPME extracts. Sharin *et al.* only managed to identify 19 compounds from USE extract of Malaysian stingless bee honey, though loss of compounds may have occurred during the sample evaporation, which was carried out at 35 °C using a rotary evaporator. [125]

3.1.2. Simultaneous distillation extraction

In 1964, Likens and Nickerson developed a specialised apparatus (Figure 6) for the extraction of volatile hop oil components from beer using pentane. [126] The process combined steam distillation, in which volatiles are carried from an aqueous sample to a condensing chamber before being collected, with continuous LLE, and is called simultaneous distillation-extraction (SDE). Two flasks are fitted to the apparatus; one containing the sample (dissolved in water) and the other containing the immiscible solvent. The two flasks are brought to their respective boiling points using water baths and the vapours are combined in the condensing chamber, allowing extraction of the volatile components into the solvent. The aqueous phase is returned to the large distilling flask through arm B for continued extraction. The solvent is returned through arm A and concentrated in the small tube. [51,76,77,109,116,126,127]

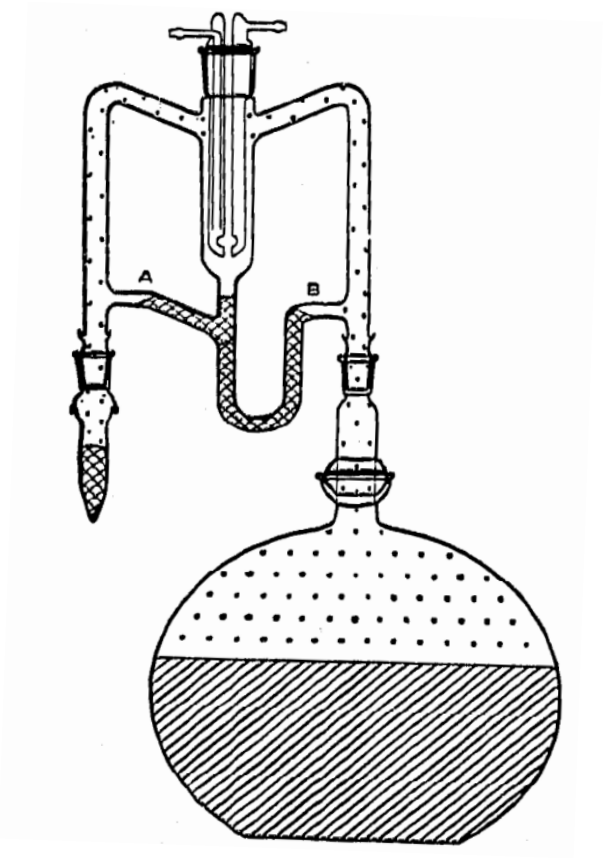

Figure 6. Likens-Nickerson apparatus for steam distillation extraction. ***Reproduced with permission from Nickerson and Likens (1966).*** [126]

This extraction method was particularly useful for extracting volatiles present in low concentrations, making it ideal for honey. However, it required a prohibitively large sample size (9 L of beer was used in the original method) and required extracts to be concentrated using a Vigreux (fractionating) column or nitrogen flow. [11,27] These concentration steps often led to loss of volatiles as they are carried off with the evaporated solvent. In 1981, Godefroot *et al.,* used a miniaturised apparatus to extract volatiles from plant material using DCM. [128] They were able to extract the volatiles from 1 g of plant material into only 1 mL of solvent, removing the requirement for solvent evaporation. This method, and the miniaturised apparatus known as the Godefroot-Sandra-Verzele apparatus, were very popular for the extraction of volatiles from honey and inspired many variations.

Due to the raised temperature required for distillation, the formation of artefacts has been observed. [87,90,115] The method was revised again in 1992 to operate at reduced pressure, which allowed the isolation of volatiles at room temperature. [129] The authors demonstrated that heat-generated artefacts were not produced during the distillation, such as furfural (a Maillard

reaction product). Still, for the extraction of volatiles from honey, this method required an 80 g sample size and concentration of the extract under a stream of nitrogen gas.

Another variation of the method involves a preliminary LLE step to remove sugars from the honey prior to heating. This was first proposed by Bicchi *et al.* (1983), who reported that the extract obtained by preliminary acetone extraction followed by SDE possessed a stronger honey-like aroma than extracts obtained by SDE, LLE and Soxhlet extraction. [115] In 1995, Bouseta *et al.* refined the method, using DCM for the extraction and SDE, and operating under an inert atmosphere to prevent oxidation of monoterpenes. [130] Although the method is lengthy, it allowed accurate quantitation and excellent recovery for over 70 compounds. [130]

SDE was the dominant extraction method for honey volatiles until the late 1990s when solid-phase extraction (SPE) began to rise in popularity. The technique used by Overton and Manura was over 100 times more sensitive than SDE for honeys from a variety of sources. [88]

3.1.3. Solid-phase extraction

In 1996, Shimoda *et al.* compared the method of Bicchi *et al.* with a column extraction. [50] A honey sample (100 g) was diluted in water (500 mL) and passed through a column packed with porous polymer beads. Water-soluble compounds were washed away with deionised water before the volatiles were eluted with diethyl ether and collected. This process is known as adsorptive column chromatography. Shimoda *et al.* reported that their extract possessed a stronger honey-like aroma than that obtained by the SDE method of Bicchi *et al.* [50] Of course, this is not a quantitative measure of the extract's representativity of the sample, but it does provide an indication that the compounds contributing to the honey aroma have been extracted effectively. They were able to identify 130 compounds using this method, with many in the medium to high-boiling region of the chromatogram.

Little variation is seen in the column chromatography methods used for extraction of honey volatiles. The column is almost exclusively packed with porous polymer beads, and the aqueous phase is washed away with water before elution of the analytes with an inorganic solvent. The choice of solvent is the main variation between studies. While Shimoda *et al.* used diethyl ether, Moreira and De Maria used acetone. [52]

SPE follows the same principles as column extraction and has been used for the extraction of volatiles from honey. Commercially available cartridges are selected and preconditioned with solvent before the diluted sample is loaded on. Analyte compounds adsorb to the cartridge sorbent, while interfering compounds are washed off. The analytes are then eluted with

solvent and the extract is concentrated prior to analysis. Although there are a variety of cartridges available for SPE depending on the properties of the analyte and matrix, polymer-based sorbents are most useful for the extraction of honey volatiles. These are suitable for nonpolar compounds that may have polar functionalities, such as aromatics. [131] The typical nonpolar solvents that have been described for other methods are suitable for SPE. Commonly used cartridges and solvents have been summarised in Table 6.

The mode of extraction is very similar to LLE, except that a solid phase is used to extract the volatiles rather than a liquid. Indeed, volatile profiles obtained by SPE and LLE are very similar, especially when the same solvent is used. [117,132] Each method has its advantages. SPE has the advantage of extracting volatiles from honey without the use of heat, and Naef *et al.* preferred SPE to LLE due to presence of waxy emulsions in the DCM extract obtained by LLE, which also caused issues in this study (see section 3.4.1). However, Castro-Vazquez *et al.* found that esters were poorly extracted by SPE, and standard deviations were greater than for LLE. [117] Uckun and Selli also found that LLE extracts were found to be more intense and closer in aroma to the original sample than the SPE extracts. [53] Single-use SPE cartridges are expensive and produce a lot of waste, which is not preferable from a green chemistry perspective. [119] Due to these disadvantages, SPE has not been widely employed for the extraction of volatiles from honey, and is rarely reported in the literature in recent times.

Table 6. Methods used for SPE of honey volatiles.

Cartridge name	Stationary phase	Solvent	References
OASIS (Waters)	Divinyl benzene-N-vinyl pyrrolidone	DCM	53,117
OASIS-HLB (Waters)	Divinyl benzene-N-vinyl pyrrolidone	Diethyl ether	133
LiChrolut EN (Merck)	Styrene-divinylbenzene	DCM	132,134
Bond Elut	Polypropylene-divinylbenzene	DCM	135,136

A common disadvantage with the methods described thus far is that the honey extract must be concentrated prior to analysis. This is commonly performed by solvent evaporation under a gentle stream of nitrogen, fractional distillation in a Vigreux column or similar, or in a rotary evaporator operating at reduced pressure. There is potential for significant loss of volatiles when the solvent is evaporated, resulting in an extract that is not representative of the original honey sample.

3.1.4. Headspace analysis

Headspace sampling is a simple technique that allows the extraction of the most volatile compounds into the vapour phase (headspace) above the sample. [137] Honey is dissolved in water and equilibrated at a given temperature in a gas-tight vial before the headspace is sampled and injected directly onto the GC column. It is advantageous compared to the aforementioned methods as it eliminates the need for complicated sample extraction and clean up. Static headspace sampling has not been widely employed for the analysis of volatile compounds in honey due to its low sensitivity, and low reported recoveries of semi-volatile compounds. [137,138]

Headspace sampling relies on the formation of an equilibrium between the sample matrix (honey dissolved in water) and the headspace. This equilibrium is affected by the partition coefficient, *K*. [139] *K* (Equation 1) describes the ratio between the concentration of a compound in the aqueous sample phase, C_S, and the concentration in the gas phase, C_G.

$$K = \frac{C_S}{C_G}$$

Equation 1. Calculation of partition coefficient in headspace equilibrium systems.

The value of *K* for a particular compound in a given system depends on the compound's affinity for each phase, which is affected by the sample matrix, the compound's volatility and the temperature.

Dynamic headspace purge-and-trap techniques have been used with much greater sensitivity. [87] This technique involves passing a carrier gas through the honey solution, adsorbing the volatiles onto a sorbent and desorbing them onto the GC column. [137] The selection of operating conditions is crucial for accurate profiling of honey volatiles with dynamic headspace extraction. Typically, nitrogen or helium gas is used for purging the volatiles from the honey solution, and it must be sufficiently heated. Bouseta et al. (1995) used a temperature of 75 °C while Radovic *et al*. (2001) used only 45 °C. [87,140] The latter method was later proved inadequate due to carry-over effect, although this is likely due to the choice of adsorption column. Bouseta *et al.* used Orbo-32, an activated coconut charcoal tube, whereas Radovic et al. used a Tenax TA trap. [87,140,141] Tenax TA is a porous polymer adsorption tube that is recommended for low concentration, high-boiling volatiles. [141] Other studies have used Carbopack B and Porapak Q traps, with no observed carry-over effect. [23,52] Carbopack B is a graphitised carbon black, recommended for use for C_6-C_{12} volatiles. [23,142]

Porapak Q is a porous copolymer of divinylbenzene and is suitable for the separation of organic analytes in water. [143] Dynamic headspace extraction allows for greater reproducibility than the other techniques discussed as it can be fully automated, and the elimination of any preliminary sample extraction makes the process faster and simpler. It removes the need for toxic organic solvents as the honey can simply be dissolved in water, and high sensitivity has been reported for highly volatile compounds. However, the method still needs to be optimised for semi-volatile compounds such as some terpenes. [137] As terpenes are a key class of aroma-active compounds in honey, it is important to have a sample extraction method that is sensitive to these compounds.

3.1.5. Solid-phase micro-extraction

In 1990, a new technique was developed by Arthur and Pawliszyn that improved upon the principle of SPE. [144] Solid-phase micro-extraction (SPME) is a solventless technique that involves the direct extraction of volatiles from an aqueous sample (or its headspace) onto a fused silica fibre with a selective coating. The fibre is exposed to the sample for a determined length of time before the analytes are thermally desorbed into the injection port of the GC-MS.

There are two modes of action for the extraction. Polydimethylsiloxane (PDMS) and poly(acrylate) (PA) are liquid coatings that extract by absorption. [145] Analyte molecules initially attach to the surface of the fibre coating, and diffuse into the coating (Figure 7). Alternatively, some fibres extract by adsorption, such as PDMS/DVB (divinylbenzene), Carbowax/DVB and Carboxen. These fibres have mixed solid porous coatings, the surface of which analyte molecules adsorb to. They are best suited to volatiles with low concentrations, as the calibration curve is non-linear at higher concentrations. [145] This makes these fibres ideal for extraction of volatiles from honey.

Numerous SPME fibres have been evaluated for the extraction of volatile compounds from honey. Divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm) has typically been favoured as the best performing fibre for extraction of honey volatiles in comparative studies. [4,76] This semi-polar fibre is recommended by the manufacturer for volatile and non-volatile flavour compounds, and shows a high extraction capacity for monoterpenes, C_{13} norisoprenoids, aliphatic and aromatic hydrocarbons, sesquiterpenoids, and ethyl esters. [4,146] Compared with PDMS, Carbowax/divinylbenzene (CW/DVB), CAR/PDMS and PA fibres, it was most suitable for the extraction of honey volatiles, based

on reproducibility, extraction time efficiency, and number of identifiable compounds extracted. [4,76] This fibre is used widely in the literature, [9,10,76] with some noting that it allowed extraction and enrichment of a broad range of compounds of varying polarities, and resulted in highly reproducible chromatograms. [9]

As adsorption is a competitive process, a molecule with a higher affinity for the fibre coating can replace molecules with lower affinity. [145] This can pose a problem when extracting compounds from complex matrices such as honey, as non-target compounds from the matrix may be preferentially adsorbed and saturate the fibre. This problem can be avoided by exposing the fibre to the sample headspace. [147] When the fibre is exposed to the headspace, two equilibria are established: between the sample and headspace, and between the headspace and the SPME fibre. Headspace analysis (HS-SPME) is more suited to the analysis of highly volatile compounds, and requires a shorter equilibration time than direct SPME extraction. [4,145,148] It also results in a cleaner chromatogram as there is no interference from the solvent and matrix. This improves detection limits, however, the method must be carefully optimised to achieve the desired sensitivity.

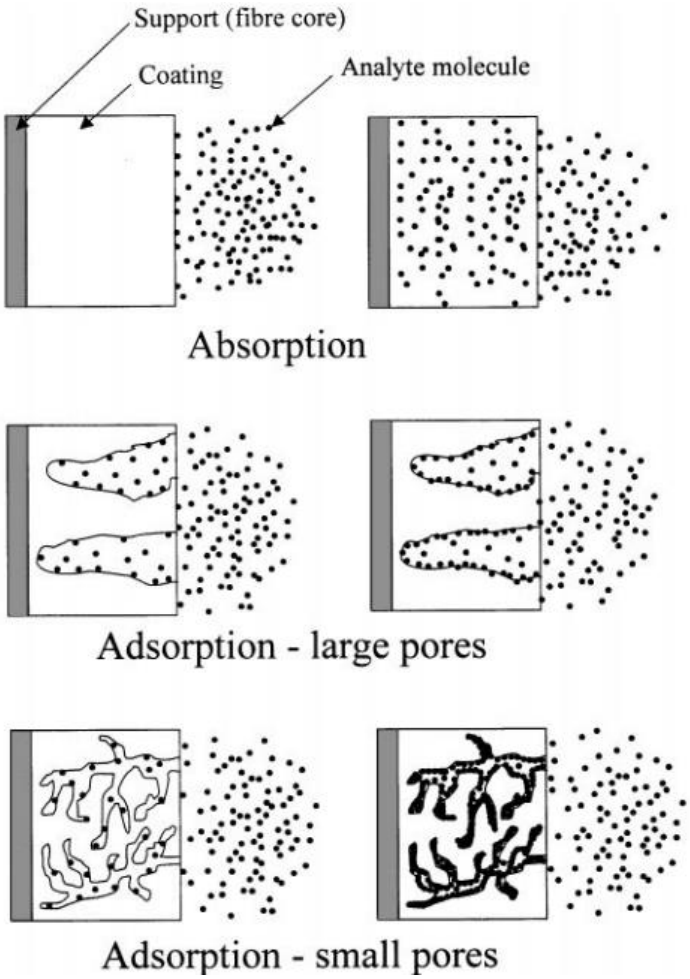

Figure 7. Comparison of absorption and adsorption extraction mechanisms (cross-sections of the coated fibres). Diagrams on the left illustrate the initial stages of the processes. Diagrams on the right illustrate the steady state condition. ***Reproduced with permission from Górecki et al (1999).*** [145]

SPME has many advantages over other extraction methods. Little to no sample preparation is required, and the use of toxic, high purity solvents can be avoided. [49,144] It can be used to analyse a high number of compounds in a single sample, which is advantageous due to the complex nature of the volatile fraction of honey. Its low cost, time efficiency, precision and low detection limit also make SPME an attractive extraction method. [147] SPME allows the extraction of volatiles without the need for added heat, avoiding the formation of artefacts. [117] HS-SPME also eliminates problems encountered in other headspace sampling methods, such as non-selectivity for gas-phase compounds and adsorption of analytes onto the sampling syringe. [147,148] For these reasons, SPME has been the method of choice for the overwhelming majority of studies of honey volatiles in the last 20 years (Table 7).

Table 7. Popular SPME fibres used for the extraction of volatiles from honey.

SPME fibre coating	Composition	Polarity	Extraction mechanism	References
PDMS	Polydimethylsiloxane	Low	Absorption	26,149
PA	Poly(acrylate)	High	Absorption	109,149,150
CW/DVB	Polydivinylbenzene in Carbowax (polyethylene glycol)	High	Adsorption	
CAR/PDMS	Carbon molecular sieve and polydimethylsiloxane	Medium	Adsorption	15,79,109,127,151
DVB/CAR/PDMS	Divinylbenzene/Carboxen/ polydimethylsiloxane	Medium	Adsorption	3,4,9,10,49,51,55,76,77,105,141,152-157
PDMS/DVB	Polydimethylsiloxane with divinylbenzene			110,122,124

SPME is not without disadvantages. The optimisation of the extraction method is crucial to achieve the desired results, and this can be time-consuming and expensive. Factors that must be considered are the extraction time and temperature, preparation of the sample (with regards to dilution and any additives such as salt or sugar) and desorption time and temperature.

While a number of studies[51,76,77,109,116,127] simply diluted the honey sample in water, others used concentrated solutions of sodium chloride to decrease the solubility of the volatiles, forcing them into the sample headspace in a process called "salting out". [4,158] The addition of sodium chloride has been shown to increase sensitivity. Dekebo *et al.* found that the addition of salt resulted in a difference in the volatile profile of the honey extract. [153] Dilution of the honey is necessary to decrease the density of the sample matrix, aiding in evaporation of volatiles. [4]

The fibre must be exposed to the sample for a period of time sufficient for the analyte to reach equilibrium between the sample and the fibre. [159] Often the sample is heated during this time, however for honey volatiles this poses a risk of thermal degradation of analytes or side reactions occurring.

3.2. Methods

This section details the initial methods used for static headspace, HS-SPME and liquid-liquid extraction throughout the method development process. As each experiment was completed, the results were used to optimise method parameters for the subsequent experiments. As

such, it is important to note that the methods changed slightly with each experiment. Samples were analysed in triplicate for all experiments.

3.2.1. Static Headspace

For the first experiment regarding sample dilution, clover honey was used. Clover honey typically contains low concentrations of volatiles, so is useful to investigate matrix effects while effectively providing a "blank canvas" to calculate spike recoveries from. For subsequent experiments, an artificial honey mixture was used containing fructose (83.2 g), glucose (74.6 g), sucrose (5.8 g) and water (36.4 mL). Spikes (10 mg kg^{-1}) of linalool, dodecane, guaiacol, 2-phenylethanol, hexanol, syringaldehyde and benzaldehyde were added to the honey and left to interact with the matrix (30 min) prior to sample dilution.

A preliminary method was used, adapted from de Lima *et al.* and Rowland *et al.* [160,161] As optimised parameters were determined in each experiment, the method was amended for subsequent experiments to include these. Honey (2.000 g ± 0.050 g) was mixed with water (2 mL) and sodium chloride (0.800 g ± 0.050 g) in a 20 mL headspace vial and sealed with a screw-top magnetic cap with septum. The sample was equilibrated (60 min, 60 °C) prior to sampling. The injection volume was 1 mL.

3.2.2. HS-SPME

HS-SPME was tested on clover honey. Honey (2.000 g ± 0.050 g) was added to a 20 mL headspace vial, along with water (2 mL) and sodium chloride (0.800 g ± 0.050 g). The agitator on the instrument autosampler was set to 250 rpm, (alternating 5 s on and 2 s off) to mix the samples. A DVB/Carboxen/PDMS SPME fibre was used, with the following method from Agilent Technologies. [162] The SPME fibre was preconditioned (60 min, 270 °C) prior to sampling. The fibre was exposed to the sample headspace (30 min, 80 °C) before thermal desorption in the injection port of the GC (2 min, 250 °C).

3.2.3. Liquid-liquid extraction

Mānuka honey was selected for the LLE experiments as it typically contains higher levels of volatile compounds than clover honey and is likely more representative of the levels of volatiles expected in New Zealand monofloral honeys. [80] The sample used was NZ018 (Table 3). For initial trials of a solvent extraction method, the following method was used, which was adapted from the methods of Castro-Vázquez *et al.*, Graddon *et al.*, and Uckun and Selli. [53,117,163]

Honey (5.000 g ± 0.050 g) was diluted in distilled water (5 mL) in a 50 mL falcon tube. *N*-dodecane (100 μL, 100 mg kg^{-1} in DCM) was added as a system monitoring compound (SMC). The mixture was homogenised using an orbital shaker (10 min). DCM (20 mL) was added and the mixture was shaken (10 min) before being centrifuged (4 °C, 3000 rpm, 5 min). The aqueous layer was pipetted off the top into a separate tube and the extraction process was repeated two more times by adding DCM to the aqueous layer, resulting in three 20 mL extracts. These were combined and evaporated to dryness under a gentle stream of nitrogen gas, before being reconstituted to 1 mL in DCM. 1-Hexanol (100 μL, 100 mg kg^{-1}) was added as internal standard. Extracts were stored at 4 °C and vortexed prior to analysis by GC-MS.

The effect of the filtration and evaporation steps were tested in turn on 20 mL aliquots of standards (0.5 mg kg^{-1} solutions of 19 external standards, reconstituted to a final concentration of 10 mg kg^{-1} in 1 mL). These compounds were 1-hexanol, benzaldehyde, (*E*)-β-damascenone, *p*-anisaldehyde, phenylacetaldehyde, benzoic acid, benzyl alcohol, dodecane, eugenol, linalool, nonanal, 3-methylvaleric acid, guaiacol, 2-phenylethanol, β-ionone, and linalool oxide.

For each experiment, samples were analysed in triplicate along with spikes (spike level 10 mg kg^{-1} for solvent choice experiment and 2 mg kg^{-1} for all others) of 1-hexanol, benzaldehyde, 3-methylvaleric acid, benzyl alcohol, phenylacetaldehyde, linalool oxide, guaiacol, linalool, nonanal, 2-phenylethanol, 4-oxoisophorone, benzoic acid, octanoic acid, dodecane, anisaldehyde, eugenol, (*E*)-β-damascenone, β-ionone and syringaldehyde. Recoveries were calculated using calibration curves for each compound.

3.2.4. GC-MS

For static headspace, HS-SPME and initial LLE experiments, TIC mode was used. The oven programme was as follows: an initial temperature of 40 °C was held (3 min), before increasing to 180 °C at 2 °C min^{-1}. Finally, the temperature was increased to 250 °C at 10 °C min^{-1}.

After the SIM method was developed (see Section 3.5.4) allowing for the omission of the solvent evaporation step, this was used for the LLE experiments on solvent volume and centrifugation time. The solvent choice experiment for LLE was repeated using SIM to confirm the choice in solvent, as the results of the first experiment may have been affected by

analyte loss due to evaporation. The oven programme detailed in section 2.4 was used for these experiments.

3.3. Results of preliminary trials – static headspace and HS-SPME

Despite its simplicity, static headspace (HS) sampling has not been widely employed for the analysis of volatile compounds in honey due to its low sensitivity. The concentration of volatiles in the HS depends mainly on ionic strength, temperature, pressure and the amount of time required for equilibrium to be reached. [164] Experiments were carried out to optimise these parameters in an attempt to improve sensitivity.

3.3.1. Sample preparation

Sample preparation for headspace analysis of honey is very simple, involving three components: honey, water, and salt. Water is needed to release volatiles into solution, and the addition of sodium chloride improves the migration of polar compounds into the vapour phase by decreasing their solubility in the aqueous phase. [164] This process is known as salting out. Rowland *et al.* used the ratio 2:5:5 (w/w/w) for salt, honey and water, whereas de Lima *et al.* simply specified the use of a "saturated NaCl solution". [160,161] Thus, four variations of the sample preparation were trialled (Table 8) with varying amounts of water and sodium chloride added to honey.

Table 8. Ratios of honey, water and salt trialled for headspace extraction.

Sample name	Honey (g)	Water (mL)	Sodium chloride (g)
Honey	2	-	-
Honey + water	2	2	-
Honey + salt	2	-	0.8
Honey + water + salt	2	2	0.8

The effects of these ratios of honey, water and salt can be seen in Figure 8. Peak areas varied significantly for the different compounds, indicating that the extraction efficiencies differed based on the affinity of each compounds for the sample matrix and vapour phases. The largely non-polar 1-hexanol and benzaldehyde displayed the greatest peak area, which makes sense given that they are largely non-polar and thus possess little affinity for the aqueous phase. 2-Phenylethanol and guaiacol were only detected in one of the honey and salt samples. These compounds form hydrogen bonds with water, explaining their greater affinity for the sample matrix.

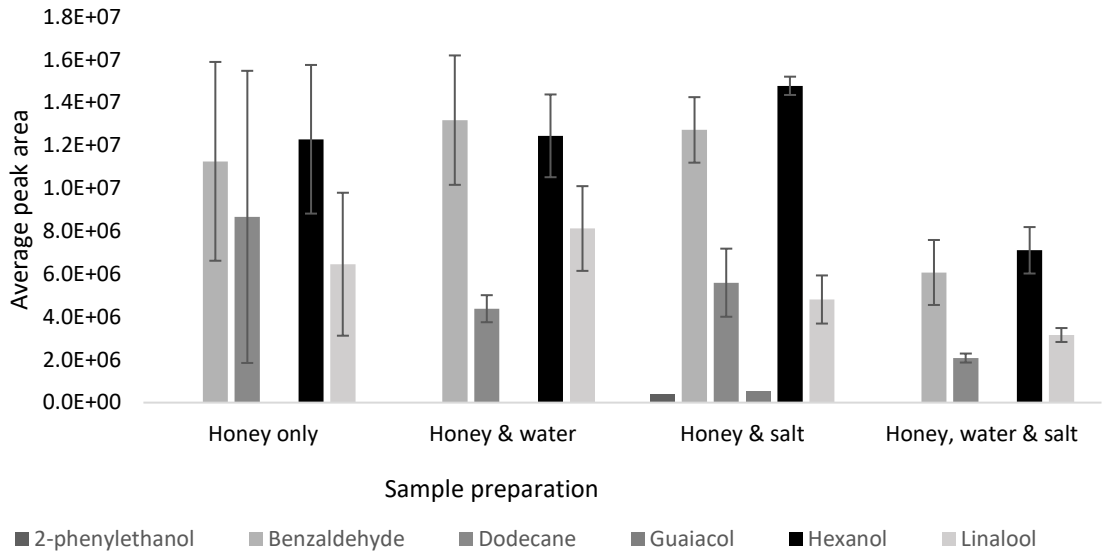

Figure 8. Average peak areas of aroma compounds in artificial honey prepared using different sample preparation methods (n = 3). Error bars represent one standard deviation in result.

Peak areas varied slightly between sample preparation methods, demonstrating that the ratios of honey, water and salt affected the partitioning of volatiles between the sample matrix and headspace. Precision was very low for the honey with no salt or water added, as demonstrated by the high standard deviations for all compounds. This may be due to the sample homogenisation, which was more difficult to achieve for the thick honey mixture than the aqueous solution. This variation decreased when water was added but was most noticeable when salt was added. The peak areas also typically increased when water and salt were each added (honey + water and honey + salt), demonstrating the effect of these additions at manipulating the partition coefficient. Interestingly, the addition of both salt and water decreased the peak areas of all compounds, although the standard deviation was also decreased. Although both salt and water are usually required to "salt out" analytes into the sample headspace, these results are consistent with previous research by Petchell, who found that sensitivity was maximised when no water was added to honey. [15] Water content in honey is typically 15-21%, so this was likely sufficient for achieving equilibrium and additional water simply diluted the solution, increasing the partitioning coefficient. [19]

Ultimately, the honey + salt preparation was chosen due to increased peak areas for most compounds compared to honey only, and lower standard deviations than the honey only and honey + water methods.

3.3.2. Equilibration temperature

Partitioning of volatiles between the aqueous and vapour phases is a function of temperature, as the vapour pressure of the liquid increases with increasing temperature. [160] This affects both the compounds of interest and the matrix, meaning that as temperature is increased, the concentration of volatiles in the headspace can be expected to increase, but the concentration of undesired matrix components can also increase. [139] Equilibration temperatures for static HS methods are typically 50-60 °C, [55,160,161] whereas dynamic HS methods used 45-70 °C. [87,140] Equilibration temperatures between 40-80 °C (in increments of 10 °C) were tested because this encompasses the typical range seen in the literature.

The results of this experiment are shown in Figure 9 and Figure 10. Guaiacol, linalool and syringaldehyde displayed predictable results, with peak area increasing with temperature. No degradation of the compounds were noticed with increasing temperature, although as previously stated, excessively high temperatures should be avoided to minimise any degradation reactions that could occur between volatile compounds and sugars in the honey matrix. As the peak area did not level off at a particular temperature, it can be predicted that this would keep increasing if higher temperatures were used, and the compounds must be exceptionally stable in this matrix even with added heat. Because the peak area did not reach a maximum, the compounds were not completely extracted at the trialled temperatures. The more readily volatilised compounds benzaldehyde, dodecane and 1-hexanol showed a marked decrease in peak area above 60 °C. This demonstrated that these compounds were unstable above 60 °C and higher temperatures should be avoided. Additionally, the variability of the results was concerning, with extremely large standard deviations. It is possible that these compounds reached equilibrium very quickly, due to their low affinity for the sample matrix and thus the variation was caused by poor sample homogenisation or increased pressure in the sample headspace due to the increased temperature. 60 °C was chosen as the equilibration temperature as it produced the highest peak areas for all compounds and is a moderate temperature. de Lima *et al* also chose 60 °C after method optimisation. [160]

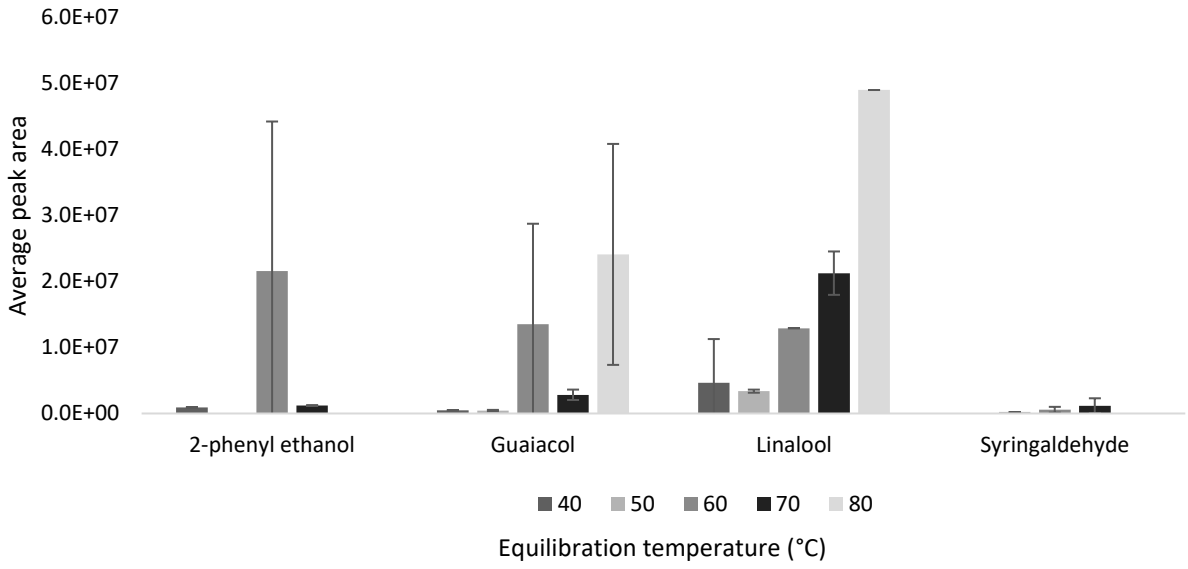

Figure 9. Average peak area of 2-phenylethanol, guaiacol, linalool and syringaldehyde at different equilibration temperatures in artificial honey (n = 3). Error bars represent one standard deviation in result.

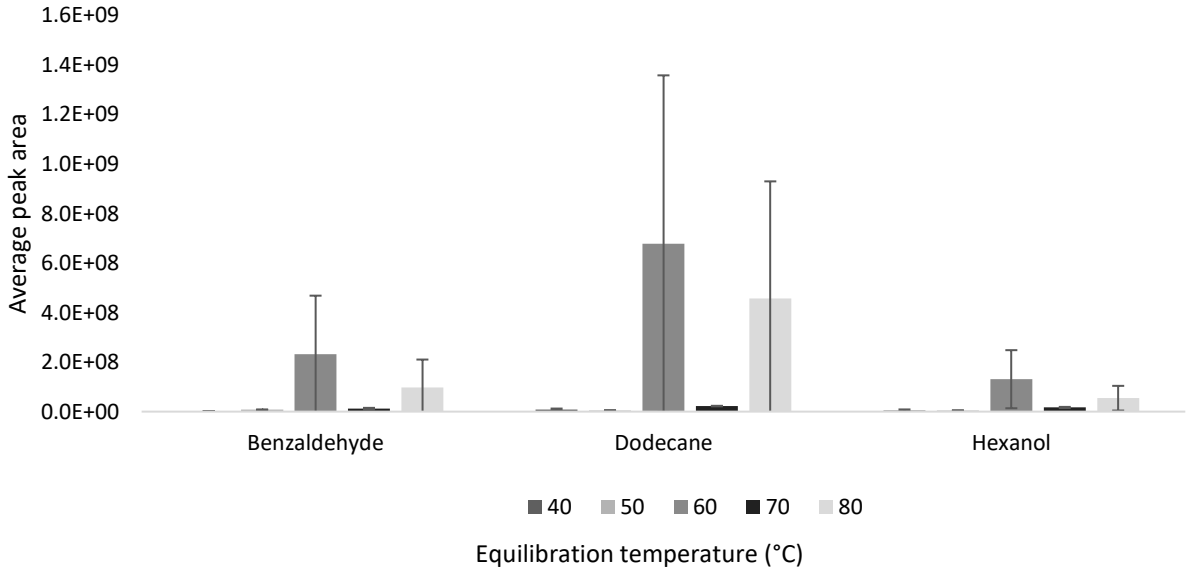

Figure 10. Average peak area of benzaldehyde, dodecane and hexanol at different equilibration temperatures in artificial honey (n = 3). Error bars represent one standard deviation in result.

3.3.3. Equilibration time

The length of time required for establishment of an equilibrium between the aqueous and vapour phases depends on the chemical properties of the analyte, and since a variety of volatiles were to be analysed this is difficult to estimate. Equilibration times between 10 and 45 minutes have been previously reported in the literature. [55,160,161] Equilibration time is also

affected by the temperature and whether sample agitation is employed. [139] Thus, it was important to carry out an experiment to determine the optimal equilibration time for this system. Equilibration times from 10-80 minutes were tested using an equilibration temperature of 50 °C (this experiment was carried out simultaneously with the previous experiment concerning determination of equilibration temperature).

Figure 11 shows that peak area of all compounds increased steadily with increasing equilibration time up to 50 minutes, where it levelled off or decreased in some cases (significant decreases were observed for dodecane, guaiacol and hexanol). This indicates that the time required for equilibrium to be reached in the system is 50 minutes. Thus, 50 minutes was chosen as the optimal equilibration time.

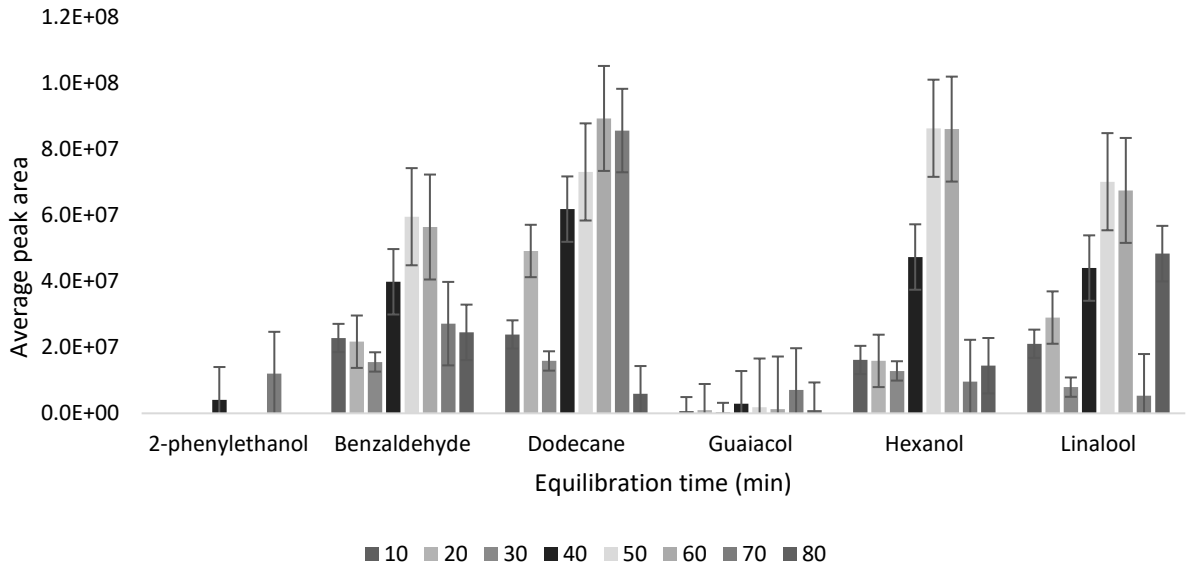

Figure 11. Average peak area of aroma compounds at different equilibration times in artificial honey (n = 3). Error bars represent one standard deviation in result.

The decreased peak areas seen for some compounds after extended periods of time (≥70 min) may have occurred due to increased pressure in the sample vial. After such a long time, undesired compounds may have also reached equilibrium, increasing pressure in the vapour phase. Indeed, some of these vials were noticed to have ruptured septa.

3.3.4. Agitation

Shaking, or agitation, of the sample vial can decrease the equilibration time as compounds are more exposed to the phase interface. [139] Agitation was used by Zhou *et al.* in the GC-O analysis of headspace extracts (500 rpm, 5 s on, 2 s off). [55] The effect of agitation (250 rpm, alternating 5 s on and 2 s off) was investigated. As shown in Figure 12, the peak area of most

compounds appeared highest when no agitation took place. This may be due to the viscous nature of the honey matrix; agitation may have had more of an effect on disturbing the vapour phase than mixing the sample matrix. However, the standard deviations of all measurements were very high. A two sample t-test revealed that for all compounds with sufficient data (benzaldehyde, dodecane, hexanol and linalool) there was no significant difference between the peak areas of samples that were agitated and samples that were not ($p > 0.05$). Therefore, agitation was not used.

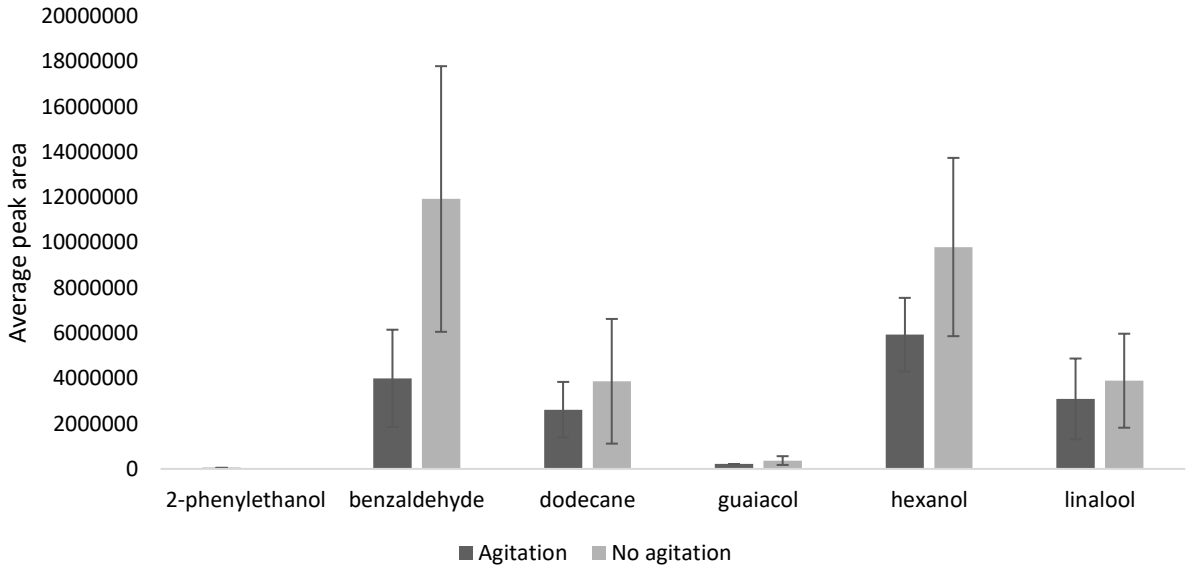

Figure 12. Average peak area of aroma compounds in artificial honey with and without sample agitation (n = 3). Error bars represent one standard deviation in result.

3.3.5. Evaluation

Even after optimisation of the method, static headspace sampling lacks the necessary sensitivity for analysing volatiles in honey by GC-MS. All experiments were performed on matrix-matched solutions of aroma compounds at 10 mg kg^{-1}. Lower concentrations were not able to be detected at all (Figure 13), and even at 10 mg kg^{-1} there were problems detecting 2-phenylethanol, syringaldehyde and guaiacol. Aroma-active compounds in honey are typically present at much lower concentrations than this, so static headspace would not be useful for extracting these compounds.

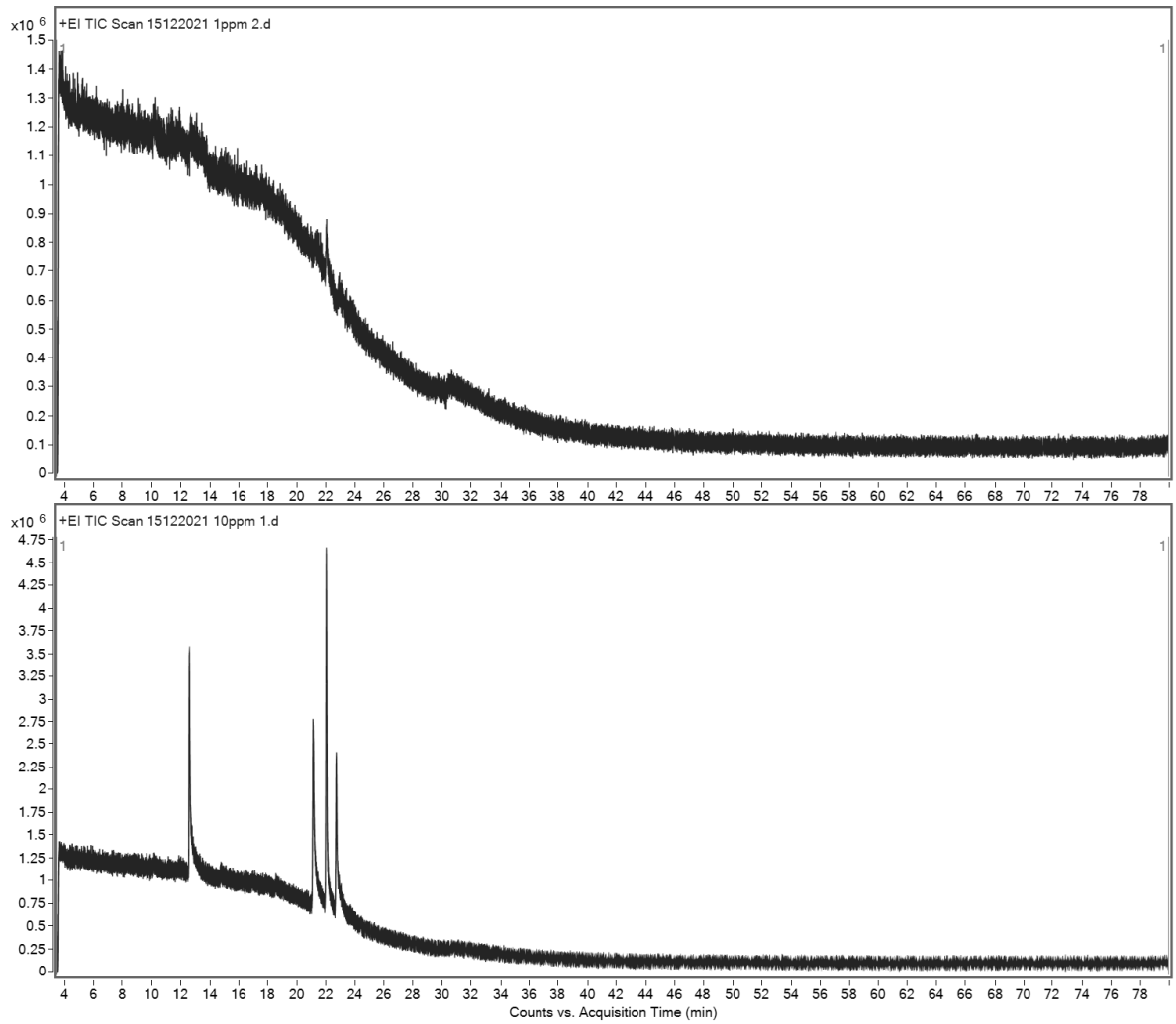

Figure 13. Chromatograms of artificial honey containing aroma compounds spiked at 1 mg kg^{-1} (top) and 10 mg kg^{-1} (bottom). No peaks are visible at 1 mg kg^{-1}, while at 10 mg kg^{-1} benzaldehyde (12.60 min), guaiacol (21.13 min), linalool (22.02 min) and 2-phenylethanol (22.69 min) are visible.

3.3.6. HS-SPME

Due to the popularity of HS-SPME for the extraction of aroma-active volatile compounds from honey in recent years, the technique was trialled for use in this study. A DVB/Carboxen/PDMS fibre was selected as this was the most popular fibre used in the literature (see section 3.1.5). The method used had been successfully used for extraction of volatiles from honey by Chen *et al.*; although the method performance was not evaluated, up to 52 compounds were identified from honey. [162] However, in this trial, clover and artificial honey samples extracted via this method did not display any peaks on the chromatogram.

HS-SPME also proved insufficiently sensitive for this purpose. As this initial trial displayed even worse results than that of the static HS method with no detected compounds at all, it was decided that running experiments to optimise the SPME method would have required too

great a time investment, for likely minimal improvements. It is worth noting that the static headspace and HS-SPME experiments were performed with the MS detector in TIC mode, which has lower sensitivity than SIM mode. However, switching to SIM mode was unlikely to make a significant difference as there were no noticeable interferences, so the chromatogram would not have been "cleaner" in SIM mode.

3.4. Optimisation of a liquid-liquid extraction method

Due to the low sensitivity of both headspace vapour injection methods, a liquid injection was investigated. Liquid injection affords improved sensitivity, but it also makes necessary a potentially lengthy and complex process of sample extraction and clean-up to remove unwanted compounds prior to injection. A series of experiments were carried out to optimise an extraction method, with the purpose of improving sensitivity and analyte recovery, as well as minimising the sample and solvent volumes and the extraction time. This section details the results of the experiments, which covered solvent choice, solvent volume, sample filtration and concentration.

3.4.1. Initial extraction method

For initial trials, the method described in section 3.2.3 was followed. However, the extraction was made challenging by the formation of a waxy emulsion between the aqueous and organic layers (Figure 14), which was difficult to completely remove using a pipette. A similar layer was also noticed by Graddon *et al* and Naef *et al.* [133,163]

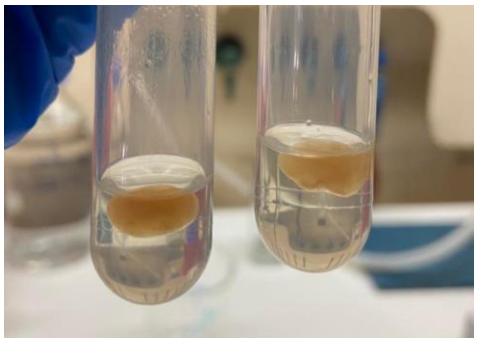

Figure 14. Waxy emulsion layer formed during solvent extraction with DCM.

Chromatograms obtained using this method (Figure 15, top) were dominated by hydrocarbons, in particular, tetradecane (peaks at 29.25 min and 42.03 min), hexadecane (53.82 min) and heptadecane (59.29 min). Although these compounds have all previously been identified in honey, the peak size indicated that they likely originated from the wax. It was clear the method needed to be improved to minimise these compounds.

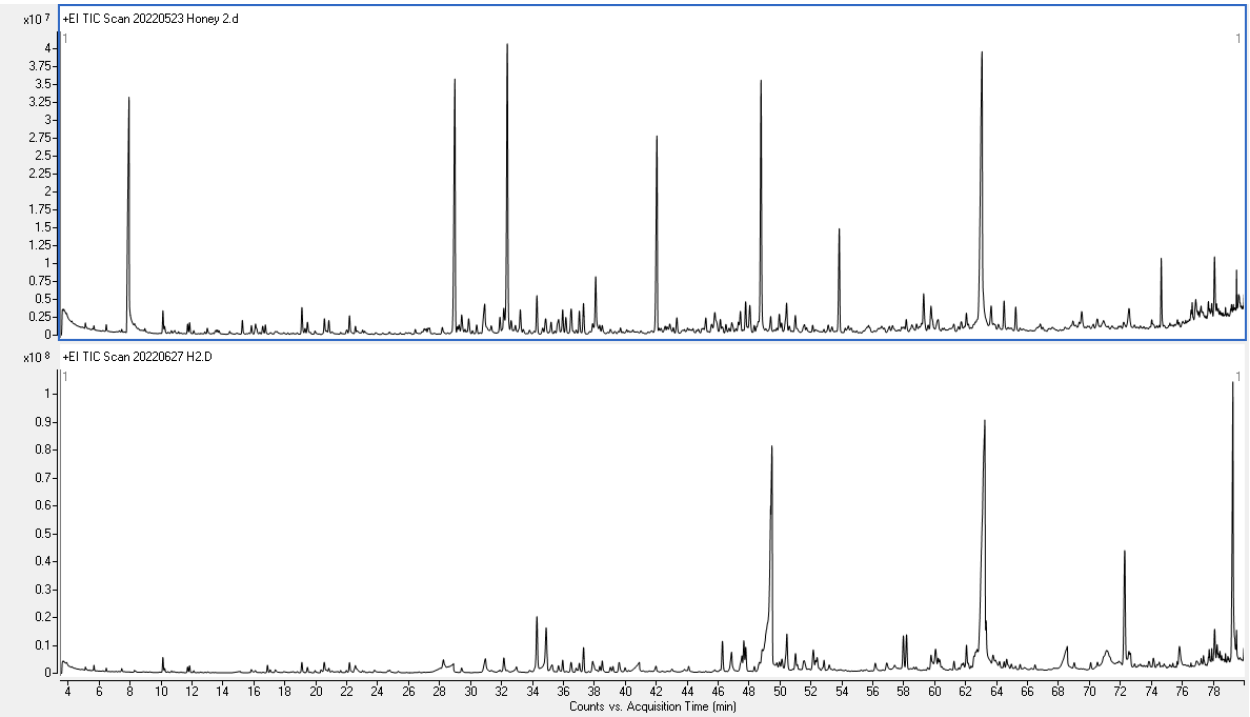

Figure 15. Chromatograms of mānuka honey obtained using the two solvent extraction methods. Top) initial method; Bottom) improved method involving freezing and filtering is shown below. The top chromatogram is dominated by wax hydrocarbons e.g. decane (15.27 min), 2,5-dimethylnonane (16.58 min), 4-methyldecane (16.73), tetradecane (29.25 min) 4-methyldodecane (30.39 min), 1,3-bis(1,1-dimethylethyl)-benzene (32.36 min), and heptadecane (59.29 min). These compounds were absent in the bottom chromatogram, while the aroma compounds limonene (16.88 min), benzyl alcohol (17.38 min), 2,5-furandicarboxaldehyde (20.34 min), 2-ethyl-1,3-cyclopentanedione (23.79 min), benzoic acid ethyl ester (26.68 min), hotrienol (28.24 min), benzeneacetic acid (32.97 min), o-methoxy-α-methylbenzylalcohol (34.28 min), o-methoxyacetophenone (34.87 min), n-decanoic acid (40.89 min), and 2-methoxybenzoic acid (46.85 min) and other compounds 1,3-bis(1,1-dimethylethyl)-benzene (32.36 min), 2,4-di-*tert*-butylphenol (48.76 min), benzoic acid, 4-ethoxy, ethyl ester (49.47 min) and benzoic acid, 4-hydroxy-3,5-dimethoxy-, hydrazide (63.24 min) were newly identified.

In the improved procedure, samples were shaken on an orbital shaker for 30 min during the extraction in flat-bottom centrifuge tubes rather than Falcon tubes, which allowed a better vortex to be established. Samples were centrifuged for a longer time at higher speed (4 °C, 3900 rpm, 30 min) to improve the separation between the aqueous and organic phases. The extracts were frozen (−18 °C) overnight to solidify the wax and any remaining aqueous layer that was unable to be removed by pipette, which were then removed by filtration using glass microfibre filter paper (Figure 16). No alterations were made to the sample concentration step. The full method is described in section 2.3.

Figure 16. Wax and water frozen and filtered from honey extract using glass microfibre filter paper.

Peak areas for the hydrocarbons were smaller in chromatograms obtained using the new procedure (Figure 15, bottom), indicating that the wax was being removed more effectively from the extract by the freezing and filtering procedure. Multiple flavour compounds were identified that were not detected using the first iteration of the method, demonstrating greater extraction efficiency with the alterations to the method. Compounds now detected included limonene (16.88 min), benzyl alcohol (17.38 min), 2,5-furandicarboxaldehyde (20.34 min), benzoic acid ethyl ester (26.68 min), hotrienol (28.24 min), benzeneacetic acid (32.97min), o-methoxyacetophenone (34.87 min), n-decanoic acid (40.89 min), and 2-methoxybenzoic acid (46.85 min), among others. However, some interferences were introduced, including benzoic acid derivatives. The ethyl ester of 4-methoxybenzoic acid (49.47 min) and the hydrazide of 4-hydroxy-3,5-dimethoxybenzoic acid (63.24 min) were the dominating peaks in the chromatogram.

Interferences from the plastic centrifuge tubes were observed in both methods. In the original method, plasticisers such as 1,3-bis(1,1-dimethylethyl)-benzene (32.36 min) and 2,4-di-*tert*-butylphenol (48.76 min) were identified, resulting from the use of 50 mL falcon tubes. In the second method, flat-bottom centrifuge tubes were used and phthalic acid derivatives were identified with RTs >70 min. The late RT of these interferences meant that they did not interfere with analyte peaks, an improvement over the earlier method.

Overall, it was clear that the longer extraction and centrifuge times, the use of a different brand of tubes and the addition of freezing and filtering steps were beneficial to the clean-up of the chromatogram and the extraction efficiency. Further experiments were performed on this improved procedure to optimise recoveries.

3.4.2. Choice of solvent

Most of the volatile compounds in honey are hydrophobic so choosing a non-polar solvent is important in order to obtain the highest recovery of volatiles in the extract. [165] DCM is the most commonly used solvent in the literature, [11,24,27,29,48,53,55,117,119,163,166] so this was chosen for the initial trials. However, other solvents such as ethyl acetate and diethyl ether have also proven useful and their use is commonly reported in the literature. [10,80,89,94,161] Tan *et al.* employed a continuous LLE at elevated temperatures and found that extraction with ethyl acetate, diethyl ether, chloroform and hexane produced similar volatile profiles, but that diethyl ether reduced the amount of HMF produced. [80] However, diethyl ether is also suitable for room-temperature extractions, as shown by Alissandrakis *et al.*, who identified 110 compounds using USE with diethyl ether. [10] D'Arcy *et al.* found that LLE with ethyl acetate provided sufficiently repeatable extracts without the need for excessive heating. [89] 55 compounds were identified this way, while Rowland *et al.* identified 25 using the same solvent.

DCM, ethyl acetate and diethyl ether were investigated as possible choices for solvent extraction. The extraction was carried out as described in section 3.4.1 on mānuka honey. In general, spike recoveries were poor regardless of solvent (Table 9). No compound demonstrated recovery greater than 60%, and a negative recovery was calculated for syringaldehyde in diethyl ether. It is likely that the poor recoveries were due, in part, to the spike concentration being too high. Though a high spike would typically demonstrate good recovery, the spike level of 10 mg kg^{-1} was outside the range of calibration for all compounds, so the linearity of response for the spiked compounds is not guaranteed. Additionally, the poor recoveries are likely due to this being the first of the method optimisation experiments, and they typically improved as each step of the method was optimised.

DCM showed the greatest extraction efficiency for the greatest number of compounds, as benzyl alcohol, 2-phenylethanol, 4-oxoisophorone, *p*-anisaldehyde, octanoic acid, 3-methyl valeric acid, eugenol and syringaldehyde were best extracted by DCM. Benzaldehyde, linalool oxide, linalool, guaiacol, β-ionone, and (*E*)-β-damascenone showed best recoveries when extracted with ethyl acetate, while diethyl ether showed the best recoveries for only two compounds; phenylacetaldehyde and nonanal.

Table 9. Recoveries of aroma compounds when extracted from honeys using DCM, ethyl acetate and diethyl ether (n=3).

	Recovery (%)		
Compound	**DCM**	**Ethyl acetate**	**Diethyl ether**
benzaldehyde	30.2	32.9	24.0
benzyl alcohol	48.2	29.9	43.7
eugenol	52.3	46.4	34.6
linalool	34.8	46.7	37.5
nonanal	25.1	25.8	37.6
octanoic acid	8.0	n.d.	n.d.
3-methylpentanoic acid	5.3	n.d.	n.d.
phenylacetaldehyde	22.7	14.1	32.2
linalool oxide	49.8	53.0	48.9
guaiacol	40.1	46.2	41.1
2-phenylethanol	50.7	37.4	34.4
4-oxoisophorone	56.7	47.9	46.6
p-anisaldehyde	53.2	47.5	51.4
(*E*)-β-damascenone	34.1	46.3	45.4
β-ionone	17.4	55.1	40.7
syringaldehyde	46.1	14.1	-4.80

Chromatograms of samples extracted in each solvent are displayed in Figure 17, and each displayed unique features. RTs were slightly greater for ethyl acetate than the other two solvents. Ethyl acetate has the highest boiling point of the three solvents at 77.1 °C, followed by DCM at 39.6 °C and finally diethyl ether at 34.6 °C. Due to its higher boiling point, compounds spend less time in the gas phase and thus the vapour pressure is low. This caused an increase in RT of ~1 minute for most compounds of interest. The similarity in boiling points between diethyl ether and DCM meant that any difference in RT of compounds extracted in the two solvents was negligible.

Peak area for the compounds of interest was highest for ethyl acetate, followed by diethyl ether and finally DCM. However, this was largely due to the presence of interferences in the ethyl acetate and diethyl ether chromatograms. Both displayed a rising baseline in the 48-52 min region, which is thought to have been caused by octanoic acid. This was observed to a lesser extent in the DCM chromatograms as well. Octanoic acid was removed from the list of compounds of interest due to this response (see section 3.5.2). The diethyl ether chromatogram displayed the highest peaks in the >50 min range, indicating that significant amounts of semi-volatile compounds were being extracted along with the compounds of interest. A large peak at 48 min dominated the chromatogram and was identified as butylated

hydroxytoluene. This compound is an additive used to prevent aging of plastics, and is commonly added to polypropylene. [167] Thus, this contamination was likely from the polypropylene tubes used for the extraction and was extracted with the most efficiency by diethyl ether, causing contamination of the chromatogram.

The DCM chromatogram produced the lowest baseline of the three solvents, and significantly less contaminant peaks were observed. In addition to this, it produced the best recoveries of the three solvents, and so was chosen as the extraction solvent for the rest of the method development experiments.

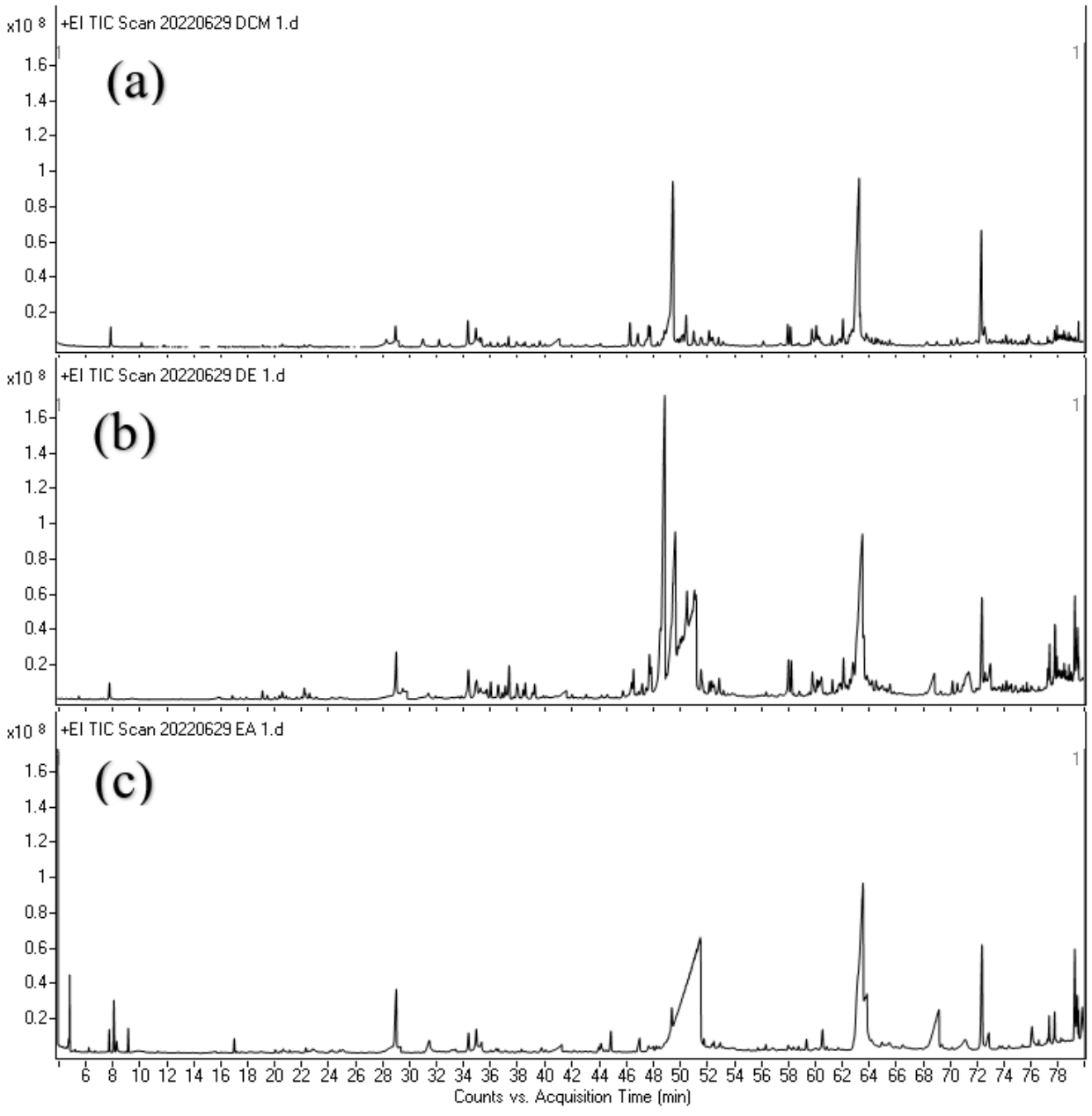

Figure 17. TIC chromatograms of mānuka honey extracted in (a) DCM, (b) diethyl ether, and (c) ethyl acetate.

The experiment was revisited following the optimisation of the method and the development of a SIM method for the GCMS. Because significant analyte loss was shown to occur during solvent evaporation (see section 3.4.4), the recoveries displayed in Table 9 were likely to be inaccurate. Additionally, the results did not show a significant difference between ethyl acetate and DCM, as they each resulted in the best recoveries for six compounds. The experiment was repeated using the final method as outlined in sections 2.3 and 2.4, and the recoveries are given in Table 10.

Table 10. Recoveries of aroma compounds when extracted from honeys using DCM, ethyl acetate and diethyl ether using the final extraction and instrumental method (n = 3). Recoveries in the acceptable range 80 – 120% are indicated in bold.

	Recovery (%)		
Compound	**DCM**	**Ethyl acetate**	**Diethyl ether**
α-pinene	125	149	**95.2**
benzaldehyde	**96.9**	**98.9**	58.3
β-pinene	140	169	**103**
D-limonene	154	184	167
cineole	141	156	**104**
benzyl alcohol	54.5	70.8	35.9
phenylacetaldehyde	125	123	59.1
linalool oxide (isomer 1)	**82.2**	**82.0**	52.1
linalool oxide (isomer 2)	**82.2**	**81.1**	52.5
guaiacol	139	129	**85.4**
linalool	**90.5**	76.1	65.8
nonanal	48.4	45.0	40.3
2-phenylethanol	59.7	52.7	40.0
4-oxoisophorone	**114**	78.6	71.6
anisaldehyde	45.5	36.5	37.5
o-methoxyacetophenone	**115**	74.5	**92.3**
eugenol	**85.2**	63.5	72.7
damascenone	66.9	59.8	74.4

Recoveries were vastly improved compared to the previous iteration of the experiment. Most compounds had recoveries in the acceptable range of 80 – 120% in at least one solvent (indicated in bold), indicating that the improvements to the method improved the recoveries. However, some compounds, such as D-limonene, benzyl alcohol and guaiacol, had recoveries >120%, indicating contamination or interference may have occurred. Additionally, other compounds including nonanal, 2-phenylethanol, (*E*)-β-damascenone and β-ionone had recoveries >80% regardless of solvent.

DCM again demonstrated the best extraction efficiency, having the best recoveries of the three solvents for 9 of 18 compounds. Seven compounds extracted with DCM had recoveries in the acceptable range of 80 – 120%. Interestingly, diethyl ether performed better than ethyl acetate in this experiment, having five compounds in the ideal range of 90 – 110%, whereas ethyl acetate only had one compound in this range. Chromatogram shape and the presence of interferences were less important in this experiment due to the increased selectivity of the SIM method. However, the recoveries confirmed that DCM was the best choice of solvent, so its use was continued.

3.4.3. Sample filtration

Due to the significant amount of wax present in the honey samples, and the difficulty removing it from the organic extract, sample filtration was necessary. Two methods of sample filtration were compared: glass microfibre filter paper used with a funnel, and 0.45 μm PTFE syringe filters. For this experiment, a reduced spike concentration was used (10 μL of 100 mg kg^{-1} stock, final concentration 1 mg kg^{-1}) to better represent the endogenous concentrations found in samples in the previous experiment on solvent choice.

Again, the recoveries were poor for all compounds regardless of filtration method. Recoveries ranged from −181% to 86.6% for the glass microfibre filters, and from −640% to 316% for the PTFE syringe filters. Thus, the choice of filtration method was based on practical considerations. The glass microfibre filter papers were used with a Buchner funnel (not connected to vacuum) and samples were filtered into 20 mL vials. This method was straightforward and due to the large surface area, samples were filtered quickly. However, the funnels had to be rinsed with DCM between samples. The 0.45 μm PTFE syringe filters were used with 10 mL plastic syringes. This method was more difficult as the sample had to be poured into the syringe in aliquots and significant sample loss occurred during the process. For this reason, the glass microfibre filter paper was the chosen method for filtering wax and ice from the extracts.

3.4.4. Sample concentration

Due to the low concentration of aroma compounds in honey, sample concentration by solvent evaporation is a requirement to achieve the required sensitivity. However, the application of heat to samples has the potential to degrade compounds within the sample or evaporate them completely, affecting the volatile composition. Common methods of evaporating solvent include rotary evaporation[80,161] and fractional distillation using a Vigreux column.

[27,29,48,53,55,117,119,122] Rotary evaporation utilises reduced pressure, eliminating the need for heat. However, both these techniques are time consuming and samples must be concentrated individually. An alternative solution that utilises the low boiling point of DCM is evaporation under a stream of nitrogen gas. This method was employed for the initial iterations of the LLE method. A temperature of 30 °C was sufficient to evaporate samples (20 mL) to dryness within 2 hours (lower temperatures resulted in condensation forming on the sample vials, considerably slowing the evaporation process). Multiple samples could be evaporated at once, reducing the time required for sample preparation.

Due to the poor recoveries obtained in the first two experiments, an experiment was conducted to determine if analyte loss was occurring. Evaporation was likely occurring at some point during the sample preparation process owing to the volatility of the compounds of interest, and it was hypothesised that the nitrogen blowdown or sample filtration where the most likely steps for this to occur. Recoveries were poor for all samples (Table 11), indicating that analyte loss was occurring during the solvent evaporation step. Bouseta *et al.* found that as nitrogen flow rate was increased, recoveries of the more volatile components decreased. [130] For example, recoveries of benzaldehyde, camphene and β-pinene decreased from 98, 97 and 97% at 2 mL/min to 78, 18 and 18% at 60 mL/min, respectively.

Table 11. Average recoveries (n=2) of aroma compounds from DCM solutions (20 mg kg^{-1}) after filtration and nitrogen blowdown steps.

Compound	Recovery (%)	
	Nitrogen blowdown only	Filtration and nitrogen blowdown
oxoisophorone	n.d.	8.82*
(*E*)-β-damascenone	4.75*	16.3 ± 7.21
benzaldehyde	n.d.	3.15*
p-anisaldehyde	6.87*	16.7 ± 6.24
syringaldehyde	32.3 ± 0.654	32.5 ± 2.79
phenylacetaldehyde	14.4 ± 2.47	16.7 ± 0.812
benzoic acid	134*	105*
benzyl alcohol	n.d.	10.8*
dodecane	n.d.	2.02*
eugenol	9.01*	21.1 ± 4.96
linalool	2.42*	7.37*
nonanal	n.d.	4.72*
octanoic acid	16.9 ± 4.49	28.3 ± 13.7
2-phenylethanol	n.d.	14.2*
β-ionone	9.37 ± 2.47	20.8 ± 3.43

* Compound was identified in only one replicate, thus standard deviation could not be calculated.

Additionally, results were not consistent, with standard deviations of up to 13.7% for recoveries. Analyte loss was unpredictable, and thus could not be accounted for in recovery calculations. For this reason, an alternative method of improving sensitivity was sought, since solvent evaporation was not feasible. This was carried out by a change in instrumental parameters, rather than a step in the sample preparation. A SIM method was developed which increased sensitivity. This is detailed in section 3.5.4. The solvent evaporation step was removed altogether, and an aliquot of the combined filtered extracts was taken for direct injection.

3.4.5. Solvent volume

Although DCM proved the most effective solvent for extracting aroma compounds from honey, it comes with a myriad of pitfalls. Inhalation and absorbance through the skin are hazardous to human health and DCM has been classified as a "probable human carcinogen" by the United States Environmental Protection Agency. DCM has also been shown to contribute to depletion of the ozone layer. [168] Thus, its use should be minimised. An experiment was carried out to determine whether lower volumes of DCM could be used in the extraction without significantly affecting the recovery of compounds. The following ratios of honey, water and DCM were trialled (Table 12).

Note that the final volume of DCM used is three times that given in the table, as three extractions were carried out. By repeating the extraction procedure multiple times and combining the extracts, additional quantities of analyte are extracted, allowing for a greater response from the instrument. However, multiple extractions are time consuming and allow for additional error to be introduced. Thus, it was necessary to conduct an experiment to determine whether additional extractions significantly increased the recovery of aroma compounds from the sample. Between 1-5 extractions are typically carried out in the literature. [27,48,53,89,122,161,163,169]

Table 12. Ratios of honey, water and solvent trialled in the solvent volume experiment. Three extractions were carried out for one sample.

Sample name	Honey (g)	Water (mL)	DCM aliquot (mL)
1/1/1	1	1	1
1/1/2	1	1	2
2/2/2	2	2	2
2/2/4	2	2	4

The 1/1/1 samples did not produce enough extract after filtering, possibly due to loss of the extract through evaporation or during the pipetting step. The remaining extracts displayed improved recoveries compared to the previous experiments (Table 13), indicating that the removal of the solvent evaporation step was effective in preventing analyte loss. It appeared that the higher ratio of solvent to honey/water was advantageous, as the 2/2/2 extraction performed slightly worse than the 1/1/2 and 2/2/4 extractions. Only two compounds had recoveries between 90-110% from this extraction, which is the ideal range. The 2/2/4 and 1/1/2 extractions had three compounds each with recoveries in that range. The 2/2/4 extraction also had four compounds with recoveries between 80-120%, while the 1/1/2 extraction had two. It was concluded that the 2/2/4 extraction produced the best recoveries and was thus chosen for future extractions.

Table 13. Average recoveries of aroma compounds extracted from honey with different amounts of honey, water and solvent used in the extraction (n = 3).

Compound	Recovery (%)		
	1/1/2	**2/2/2**	**2/2/4**
1-hexanol	**102**	49.6	124
2-phenylethanol	138	141	129
anisaldehyde	146	168	146
benzaldehyde	124	124	**118**
benzyl alcohol	135	137	125
b-ionone	**98.5**	121	**107**
damascenone	**89.6**	**109**	**95.3**
eugenol	162	185	n.d.
guaiacol	**94.5**	**90.3**	**82.8**
linalool	**88.6**	**84.2**	**85.3**
oxoisophorone	128	56.5	**107**
phenylacetaldehyde	79.0	**87.5**	**83.6**

3.4.6. Centrifugation time

The aim of the final experiment was simply to reduce the time required for sample preparation by reducing the length of the centrifugation step. The aqueous and organic phases readily separate upon standing, so a shorter centrifugation step would likely be sufficient to achieve separation of the phases. 10, 20, and 30 minute centrifuge times were compared, at a temperature of 4 °C and speed of 3900 rpm. As demonstrated in Figure 18, sufficient separation was achieved after 10 minutes, hence this was used as the centrifugation time.

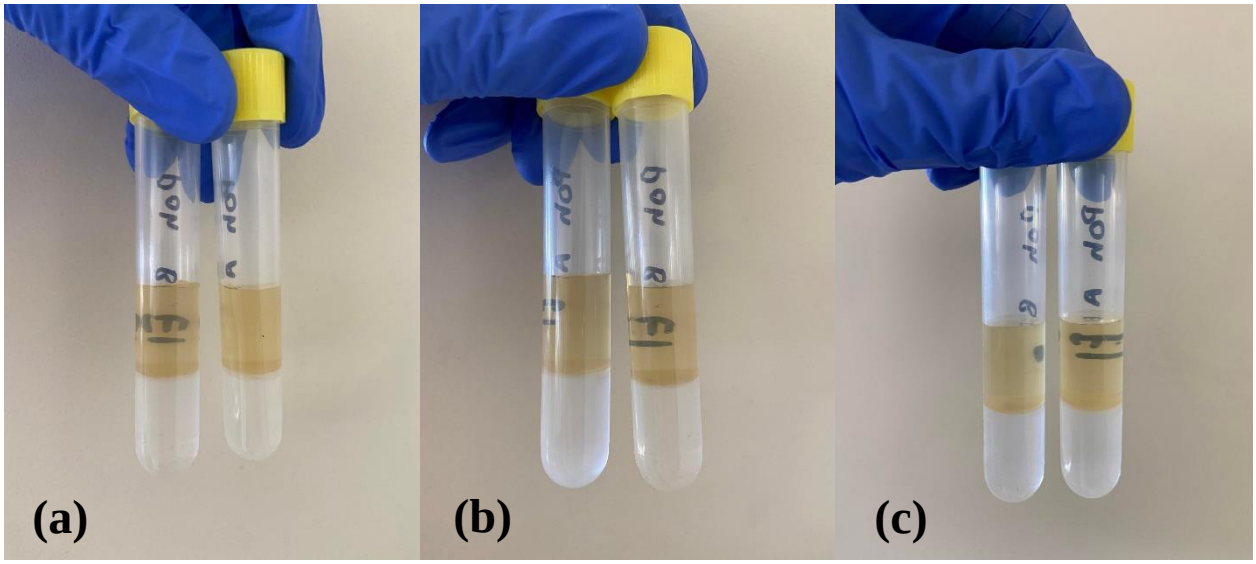

Figure 18. Separation of organic (bottom) and aqueous (top) phases after (a) 10 min, (b) 20 min, and (c) 30 min centrifugation at 3900 rpm at 4 °C.

3.5.GC-MS Method

3.5.1. Instrumental parameters

The analytical column is the primary factor affecting analyte separation. Van der Waals, dipole-dipole, dipole-induced dipole and hydrogen bonding interactions between the stationary phase and the analyte determine how strongly the analyte is retained, and thus, the separation of compounds with slightly different properties. If one were analysing purely non-polar compounds, such as hydrocarbons, a pure PDMS column would be suitable (Figure 19). Conversely, a polyethylene glycol (PEG) "wax" column is suitable for polar analytes as it utilises hydrogen bonding. [170] Of course, the variety of volatiles in honey makes column selection difficult, as the analytes range from nonpolar *n*-alkanes through to polar aromatic compounds. Thus, there is no "ideal" column, as no column will be able to perfectly resolve all the compounds of differing polarities present in these samples. Instead, a suitable column is one that resolves as many of these compounds as possible.

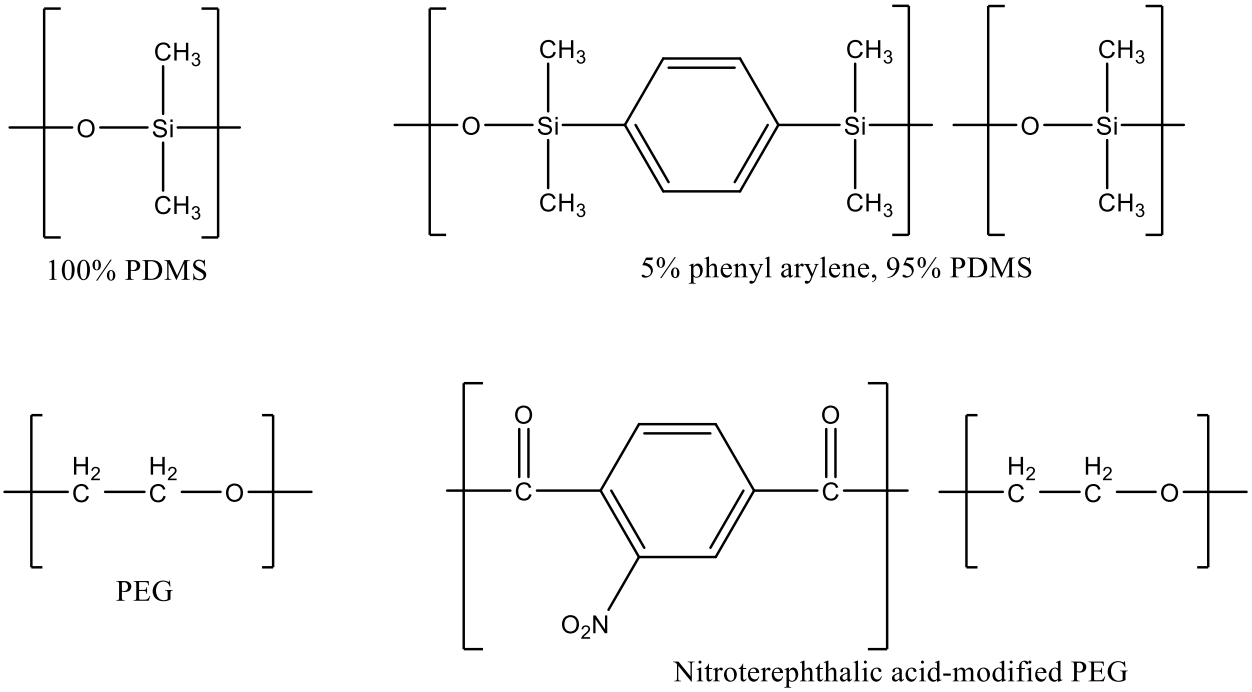

Figure 19. Common stationary phases used for the analysis of volatile compounds in honey by GC-MS.

For the analysis of honey volatiles, common stationary phases are the low polarity 95% PDMS, 5% phenyl arylene and the high polarity PEG (Table 14). Some studies have used both polar and non-polar columns to confirm compound identification and to separate a wider variety of compounds. [9,48,161] Ruisinger (2012) [48] compared the ZB-5, DB-1701 and DB-FFAP columns and other than the acids 3-methylpentanoic acid, 2- and 3-methylbutanoic acid, and 2-methylpropanoic acid, all compounds identified using the wax column were also identified using the non-polar column. Taylor (2015) recommended the use of as low polarity a column as possible, as column bleed increases with polarity. For these reasons, an HP-5MS Ultra Inert (30m x 250 μm x 0.25 μm) column was chosen for the separation of honey volatiles in this method. [170]

An oven temperature programme was employed to elute compounds sequentially according to their boiling point. An initial temperature of 40 °C was held (3 min), before increasing to 180 °C at 2 °C min^{-1}. Finally, the temperature was increased to 250 °C at 10 °C min^{-1}. This temperature programme was sufficient for separating most compounds, and any co-eluting compounds were later separated using the SIM method (see Section 3.5.4). However, the programme was lengthy (80 min), and the last compound of interest, β-ionone, elutes at 46.9 min. The method was shortened to improve the analysis time, while still allowing high-boiling compounds to be eluted from the column to avoid contamination of future samples.

The oven temperature was held at 40 °C for three minutes, before increasing at 2 °C min^{-1} to 154 °C. The temperature was then increased at 20 °C min^{-1} to the final temperature of 250 °C and held for 5 minutes. This decreased the run time by 10 minutes but did not affect the RTs of the analytes, so the results of previous experiments were not compromised. In addition, the detector was disabled at 60 min to avoid the appearance of contaminant peaks on the chromatogram.

Injection volumes between 1-3 μL were commonly used in the literature.[11,24,29,53,89,106,117,119,122,124,125,166] Initially, an injection volume of 1 μL was used. However, this was increased to 2 μL to increase peak height/area, improving LODs.

Table 14. Separation columns used for the analysis of honey volatiles by GC-MS, in order of increasing polarity.

Column	Stationary phase	Length (m)	Inner diameter (mm)	Film thickness (μm)	References
HP-1	100% PDMS	25	0.25	25	[140]
HP5MS, DB5-MS, ZB-5	5% phenyl arylene, 95% PDMS	30	0.25	1	[9,39,151,156,102,130,48]
SE-54	5% Phenyl, 1% Vinyl, 94% PDMS	30	0.32	0.3	[27]
DB-1701/OV-1701	14% cyanopropyl-phenyl, 86% PDMS	30	0.25	0.25	[27,48]
ZB Wax Plus, DB Wax, RTX-WAX	PEG	20	0.18	0.18	[9,50,76,77]
OV-351, DB-FFAP, BP21	Nitroterephthalic acid modified polyethylene glycol	50	0.32	0.2	[11,48,106,161140]

3.5.2. Selection of external standards

A selection of external standards were chosen based on their occurrence in New Zealand honeys and their contribution to honey aroma, as reported in literature (Chapter 1; Table 1, Table 2). To quantify a compound, it needs to produce a linear response on the GC-MS over the typical range of concentrations the compound is seen in honey. Of the total 25 compounds that were evaluated for use as external standards, only 18 were used in the final method. These compounds are listed in section 2.4.1, and are discussed in detail in Chapter 5. Other

compounds (Figure 20) were considered for use but did not meet the above criteria, and so were unsuitable for use. The reasons they were excluded are detailed below.

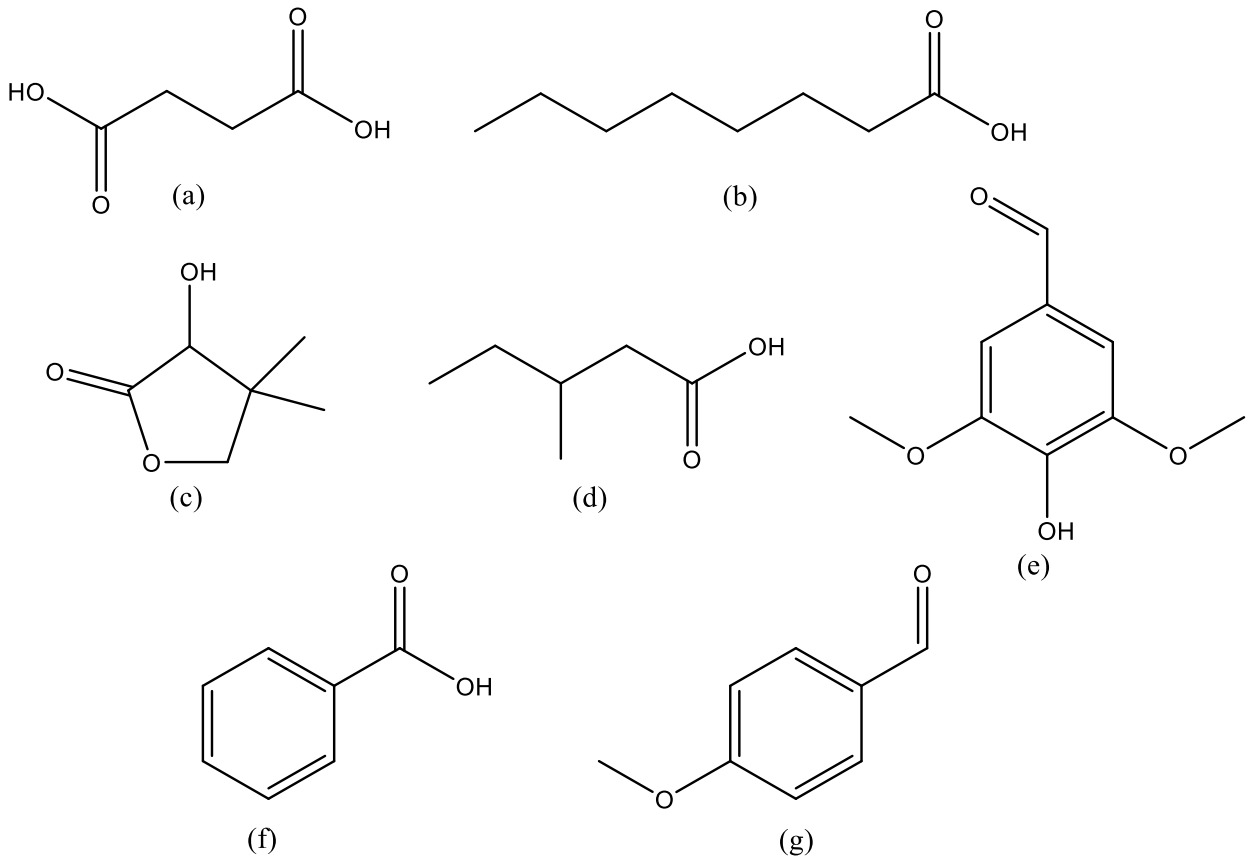

Figure 20. Potential external standards that were removed due to unsuitability. (a) succinic acid, (b) octanoic acid, (c) pantolactone, (d) 3-methylpentanoic acid, (e) syringaldehyde, (f) benzoic acid, (g) *p*-anisaldehyde.

Succinic acid (Figure 20 (**a**)) has been identified in New Zealand clover honey and New Zealand and Tasmanian mānuka honey, as its TMS ester. [94,171] Although it has been identified in longan honey internationally, it is odourless. [156]. However, succinic acid was insoluble in DCM and is only semi-volatile. Thus, succinic acid was not suitable for quanification.

Octanoic acid (Figure 20 (**b**)) has been identified in a range of New Zealand honeys including clover, heather, thyme and mānuka. [80-83,86] Additionally, it contributes a "sweaty, cheese-like" odour to the aroma of haze, [50] astralagus[29] and citrus[53] honeys with an odour threshold of 500 μg kg^{-1}. However, poor peak shape (Figure 17) and occasional co-elution with dodecane were observed. The peak shape, which resembled a rising baseline and then a sharp drop-off, may be caused by an overload of the compound on the chromatographic column (Figure 21).[172] As the odour threshold is so high, analysis of this compound at concentrations

around the odour threshold could potentially damage the column. Due to this, octanoic acid was not suitable for analysis with this method.

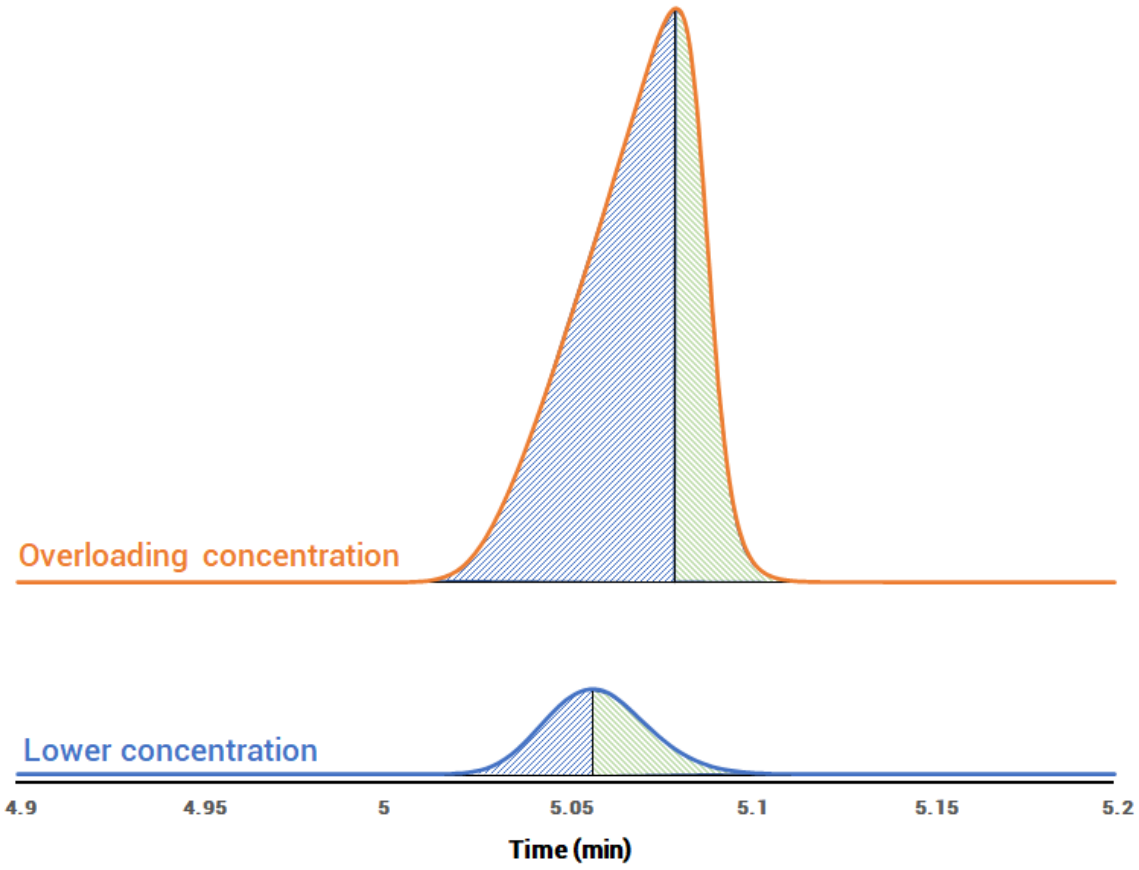

Figure 21. Shape of a fronting peak (top) due to overloading of analyte, vs ideal peak shape obtained at a lower analyte concentration (bottom). ***Reproduced with permission from PerkinElmer (2022).*** [172]

Pantolactone (Figure 20 (**c**)) has a burnt caramel odour. Though its threshold has not been reported, it had an FD of 8 in citrus honey, in which its concentration was 72.1 µg kg^{-1}. [53] Interestingly, in the same study, it was not identified as aroma-active in astralagus honey despite its concentration of 136.5 µg kg^{-1} in this honey. This suggests there may have been additive effects occurring with other aroma compounds in the citrus honey. The boiling point of pantolactone is 224.6 °C, so it was not volatile enough to be detected during the analysis. 3-Methylpentanoic acid (Figure 20 (**d**)) also has a high boiling point of 196-198 °C, and was difficult to detect, appearing in some chromatograms but not others with no apparent cause. 3-Methylpentanoic acid contributes a sweaty aroma, consistent with octanoic acid as the longer chain fatty acids are often reported to possess this aroma. It has been reported as aroma-active in rape honey, and has also been identified in clover, beech honeydew, kāmahi,

mānuka, pōhutukawa, southern rātā, rewarewa, tāwari, thyme and viper's bugloss honey in New Zealand. [15,48,79] As a linear response could not be obtained for this compound, it was removed from the analysis.

Syringic acid has been identified in many New Zealand honeys, including clover, mānuka, heather, thyme, nodding thistle and kāmahi. [80,82,84,86] Typically, aldehydes have lower boiling points than the corresponding carboxylic acids due to the lack of hydrogen bonding, so it was posed that syringaldehyde (Figure 20 (**e**)) may contribute to the aroma of New Zealand monofloral honeys. However, the compound was not volatile enough, and was only detectable at 10 mg kg^{-1} (section 4.2, Table 16).

Benzoic acid (Figure 20 (**f**)) was also detected at a minimum concentration of 10 mg kg^{-1}. With a boiling point of 249 °C, it is only semi-volatile, which explains this observation; yet it has been identified as aroma-active in cambará, cashew, marmeleiro, raspberry, rape, heather, alder buckthorn honeys. [49,52,58] Derivatisation should be a consideration for future work, as methyl benzoate has a boiling point of 199 °C. Most New Zealand studies have detected benzoic acid as its methyl ester by derivatisation, using etheral diazomethane or BSTFA. [80-86,94]

p-Anisaldehyde (Figure 20 (**g**)) has a mint-like and aniseed-like aroma, and has a low odour threshold of 27-33 μg kg^{-1}. [40,65] This explains why it has been identified as aroma-active in a wide variety of honeys internationally, including linden, acacia, heath, buckwheat, cloudberry-bog, lingonberry, sweet clover, willowherb, rape, heather, alder buckthorn and haze. [27,48-51,55] However, a linear calibration curve could not be established for *p*-anisaldehyde, so it was not included as an external standard. *p*-Anisaldehyde has previously been found in New Zealand honeys including heather, thyme, nodding thistle, mānuka, nodding thistle, viper's bugloss and kānuka. [81-86,90,93]

Finally, this left the group of compounds that were used in the final method as external standards for quantification. Their structures and more detailed information about them can be found in Chapter 5. The external standards were analysed to determine their RTs (Table 15). Calibration curves were produced for each compound as described in section 2.2, which were used for quantification and calculation of recoveries during method development.

Table 15. Retention times of external standards.

Compound	RT (min)	Compound	RT (min)
1-hexanol	7.8	Linalool	21.9
α-pinene	11.0	Nonanal	22.3
Benzaldehyde	12.5	2-phenylethanol	22.7
β-pinene	13.5	Oxoisophorone	24.7
D-limonene	16.9	Dodecane	28.9
Cineole	17.0	Anisaldehyde	32.1
Benzyl alcohol	17.4	*o*-methoxyacetophenone	34.8
Phenylacetaldehyde	17.9	Eugenol	39.0
Linalool oxide	19.9/21.0[a]	β-damascenone	40.7
Guaiacol	21.0	β-ionone	46.9

a. The linalool oxide standard contained two isomers, *S*-(+)-linalool oxide and *R*-(−)-linalool oxide, which produced separate peaks in the chromatogram.

3.5.3. Internal standard

Typically, an internal standard is used in conjunction with the external standards described above to account for any fluctuations in the instrumental analysis, allowing quantification of compounds. This is especially important for GC-FID, where fluctuations in flame can significantly affect results. Internal standard is added to the sample immediately prior to instrumental analysis. Peak area is monitored to determine fluctuations in detector response, which may occur over time due to instrument tuning or performance. A ratio is taken of the peak area of the compound and the peak area of the internal standard. This ratio is then used for quantification. The internal standard must behave similarly to the analyte compounds but must not itself be present in the sample. Stable isotope-labelled internal standards are the ideal choice, as they behave identically to the analyte but are easily distinguished from the analyte by mass spectrometry. This is especially useful in such a complex mixture as honey, as it is difficult to guarantee that a particular compound will not occur in any of the samples. However, stable-isotope labelled compounds are prohibitively expensive.

In addition to the internal standard, system monitoring compounds (SMCs, also known as surrogates) are added to the sample at the beginning of sample preparation, and peak areas of target compounds are taken as ratios to that of the SMC. SMCs allow for analyte loss occurring during sample preparation to be accounted for, as it is assumed that any loss of the two compounds occurs at the same rate.

Numerous studies have used linear alcohols[3,4,29,53,117,161] and alkanes[80,94] as internal standards. *n*-dodecane and 1-hexanol performed well in initial studies on solutions of external standards.

However, both compounds were later identified in some of the honey samples to be analysed, so were not deemed suitable as internal standard or SMC. Due to the range of analytes with different functional groups present, which behave differently and have different response factors, multiple internal standards would likely be required for reliable quantification. Additionally, method validation (section 0) showed that sufficient precision was achieved without the need for correction using an internal standard. It was decided that quantification would proceed without an internal standard (for those compounds that had an external standard) and additional compounds would be tentatively identified using mass spectral libraries and their abundance estimated as a percentage of the total peak area.

3.5.4. Selected ion monitoring

Initial analysis by TIC of the standard solutions produced linear calibration curves with $R^2 >$ 0.99 for most compounds (Table 16). However, the limits of detection (LOD) for most compounds were poor. These compounds are typically found in low parts per billion (ppb) levels in honey, so the sensitivity achieved using the TIC mode was not sufficient to quantify these compounds.

Table 16. Calibration curve details and lowest detected concentrations for the external standards using TIC and SIM methods.

Compound	TIC			SIM		
	R^2	Gradient	Lowest detected concentration (mg kg^{-1})	R^2	Gradient	Limit of detection (mg kg^{-1})
1-Hexanol	0.9939	2597357	1		n.a.	
4-oxoisophorone	0.9954	3595691	0.1	0.9784	249041	0.012
(*E*)-β-damascenone	0.9937	10383799	0.05	0.9726	120031	0.0079
Benzaldehyde	0.9975	8217902	0.2	0.9942	1336536	0.32
p-anisaldehyde	0.9890	10024859	0.5		n.a.	
syringaldehyde	0.9478	3845127	2		n.a.	
phenylacetaldehyde	0.9924	2344930	2	0.9783	1771040	0.231
Benzoic acid	N/A	989480	10		n.a.	
Benzyl alcohol	0.9873	13772660	0.5	0.9847	787045	0.36
Dodecane	0.9973	6645049	0.05		n.a.	
Eugenol	0.9862	8568896	0.2	0.9424	98323	0.39
Linalool	0.9941	6960684	0.05	0.9536	137752	0.011
Nonanal	0.9906	3112866	0.1	0.9693	38587	0.23
Octanoic acid	0.9814	1284689	1		n.a.	
3-methylvaleric acid	N/A	799280	10		n.a.	
guaiacol	0.9919	10133932	0.2	0.9468	661558	0.30
2-phenylethanol	0.9868	9210604	0.5	0.9775	536025	0.37
β-ionone	0.9926	11791799	0.05	0.9430	168598	0.0072
linalool oxide (isomer 1)	0.9915	3612792	0.05	0.9684	70722	0.0027
linalool oxide (isomer 2)		n.a.		0.9920	127294	0.0045
α-pinene		n.a.		0.9942	585470	0.014
β-pinene		n.a.		0.9953	383097	0.011
ɒ-limonene		n.a.		0.9855	668055	0.015
cineole		n.a.		0.9771	229322	0.0091
o-methoxyacetophenone		n.a.		0.9586	1430822	0.40

n.a.: not analysed using given method

To improve sensitivity, SIM was used. In this technique, the MS is set to monitor the intensity of selected *m/z* values, rather than scanning over a wide range. Typical ions for each compound of interest are selected and scanned in time segments around the expected RT of the compound. As the scan time for each ion is much longer than in TIC mode, sensitivity is significantly increased. Additionally, selectivity is increased using a SIM method. The TIC chromatograms were very complex due to the nature of the matrix, containing interferences from semi-volatiles and waxy compounds. By monitoring ions only relating to the compounds of interest, interference signals were reduced, improving peak shape, signal-to-noise ratio and allowing for reliable quantification.

Initially, 5-10 ions were selected for each compound from their mass spectra obtained from the TIC. Spiked honey samples were analysed at low resolution to determine which of these ions were most suitable for the identification and quantification of each compound. The most suitable ions did not produce peaks elsewhere in the time segment and gave a linear response for the analyte peak. Once ions had been selected, a dynamic SIM calibration was performed to increase resolution. The *m/z* tolerance was decreased and incremental values around the *m/z* of interest were analysed to determine the optimal value for each ion. The final SIM method is detailed in Table 5 in section 2.4. One ion was chosen for quantification of each compound (typically that with the highest abundance) and two others were used to confirm the peak identity.

As shown in Table 16, the slope of the SIM calibration curve was lower for all compounds, typically by at least one order of magnitude. While this may indicate lower sensitivity, it is important to remember that the peak areas used in the TIC calibration curves were combined responses from all monitored ions, whereas in the SIM calibration curves peak areas for only the quantifier ion were used. This means the peak areas were smaller and thus the slope would be as well. Rather, attention should be focussed on the detection limits of each method to indicate the sensitivity of each method. The lowest detected concentration for each compound using the TIC is given in Table 16: this means the compound was detected at that concentration at least once, but not necessarily consistently. For the SIM method, the limit of detection (LOD) was calculated (see section 4.3) and reported. LOD describes the minimum concentration at which a peak can be confidently distinguished from background noise, however, each analyte was detected at least once at the lowest calibration point; that is, 0.10 mg kg^{-1} for the Group A compounds, and 0.5 mg kg^{-1} for the Group B compounds. For

almost all compounds, the LOD using SIM was much lower than that of the TIC, indicating the increased sensitivity.

The SIM method also allowed for quantification of compounds that were poorly resolved. D-limonene and cineole elute at 16.9 min and 17.0 min respectively, and in TIC mode their peaks overlap. The second isomer of linalool oxide co-elutes with guaiacol at 21.0 min, producing completely overlapping peaks (Figure 22). Careful selection of ions characteristic of each compound meant that the peaks for these compounds could be separated based on *m/z* of their fragments, rather than RT.

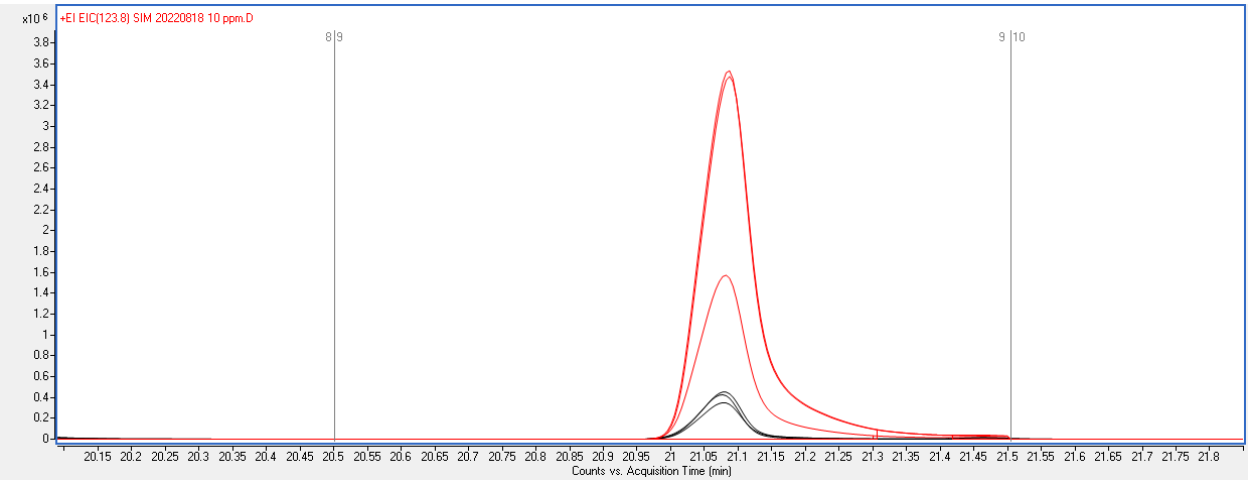

Figure 22. Section of the SIM chromatogram for a spiked honey sample, showing the separation of the co-eluting guaiacol (red) and linalool oxide isomer 2 (black) based on characteristic ions of each compound.

3.5.5. Summary

In conclusion, the development of a SIM method for the analysis of volatile compounds in honey by GC-MS allowed for much more sensitive and selective analysis. The SIM method was validated (Chapter 4) and used for the analysis of honey samples (Chapter 5). One disadvantage of using the SIM method was that it did not allow for screening of other volatiles. For this reason, a TIC injection was run for each sample for the purpose of identifying other volatiles present in the sample, although the sensitivity was lower and they could not be quantified.

4. Chapter Four: Method Validation

After the method was developed and optimised, its performance was tested to ensure that it was adequate for the purpose of quantifying aroma compounds in honey. This process is called method validation. [173] This chapter outlines the procedures that were undertaken to validate the method and the results of these.

4.1. Methods

For method validation, four batches of samples were prepared and analysed over the course of two weeks. Each batch contained:

- Calibration standards (see section 2.2)
- Instrument blank – a vial containing only DCM. This was used to monitor instrumental fluctuations and contamination that may be carried over from previous samples.
- Method blank – containing no sample but carried through the entire extraction process. This was used to monitor any contamination arising during sample extraction.
- Method blank spike – identical to the method blank but spiked with external standards. A low spike and high spike were analysed, which are further detailed in section 4.4. These were used to calculate recoveries to assess the accuracy of the method.
- Mānuka honey sample – the sample NZ018 was analysed in each batch. This was used for precision calculations.
- Additional honey sample – samples NZ270 (pōhutukawa), NZ271 (rewarewa), NZ272 (kāmahi) and NZ273 (southern rātā) were each analysed in one batch. These were analysed to assess the method's performance over different honey types.
- Sample spike – for each honey sample analysed, a low spike and high spike were also analysed. These were used to calculate recoveries to assess the accuracy of the method.

All samples were prepared in triplicate to assess intra-day precision. Samples were repeated over four days to assess inter-day precision. Spikes were added directly to the honey samples and equilibrated for 30 min prior to dilution.

4.2. Range of linearity and treatment of calibration curves

The range of linearity is the concentration range over which the instrument response (peak area) is directly proportional to the concentration of the analyte in the sample. The use of an accurate calibration curve is essential to accurate quantification. As the exact concentration of each compound of interest varied between samples, especially between floral types, the calibration curve needed to cover a wide range of concentrations. It is generally accepted that a minimum of five concentrations must be included in the calibration curve.

Range of linearity was determined in parallel with LOD and LOQ (see section 4.3). The three parameters are linked directly to the calibration curves, so any change in the curves or in one parameter affected the others as well. For simplicity of explanation and reporting, this section discusses the processing of calibration data and the resulting range of linearity, while the LOD and LOQ calculations are presented in the next section.

Initially, a range of concentrations between 10 μg kg^{-1} and 10 mg kg^{-1} was used for calibration curves. For all compounds, a sharp increase in the slope of the regression line occurred around 1 mg kg^{-1} (Figure 23). For some compounds, the lower end of the curve appeared linear, but above 1 mg kg^{-1} the instrument response increased exponentially with increasing concentration. This indicated that a linear response could not be achieved at the higher concentrations. These compounds were assigned to Group A. Other compounds showed better linearity above 1 mg kg^{-1} and had higher LOD (see section 4.3). These compounds were assigned to Group B. Hence, the two sets of calibration standards were made up as detailed in section 2.2. These sets each had eight calibration points spanning a 0.010-1.0 mg kg^{-1} for Group A and 0.5-3.0 mg kg^{-1} for Group B.

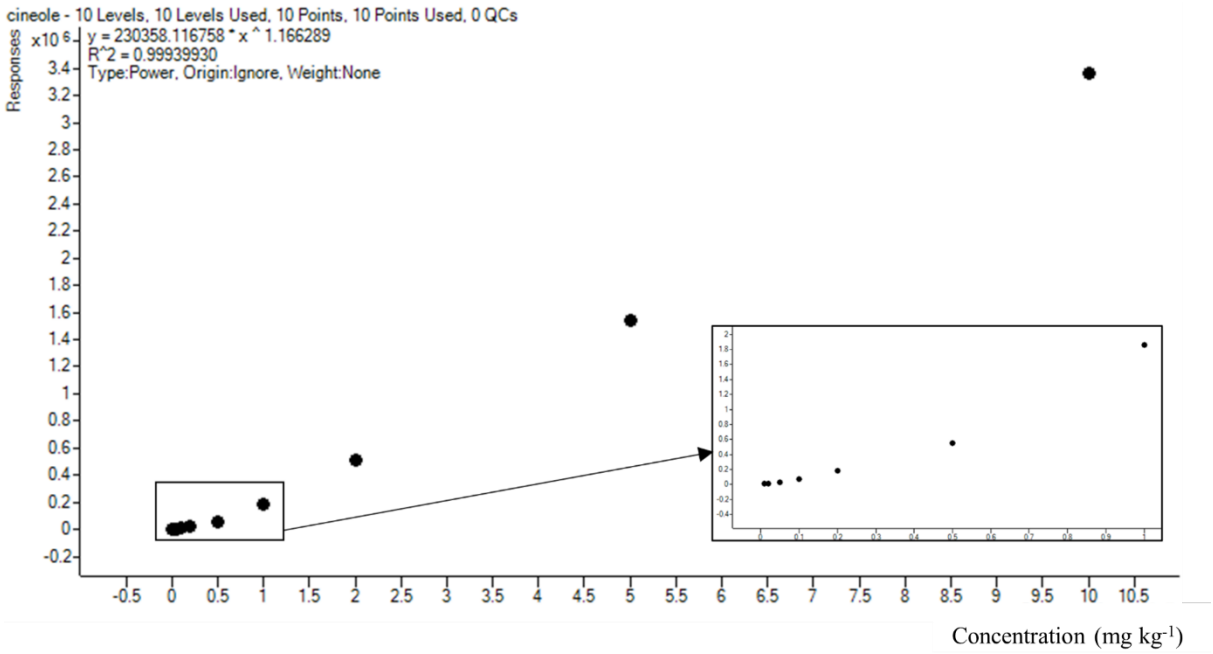

Figure 23. Calibration curve of cineole from 10 µg kg^{-1} to 10 mg kg^{-1}. Over such a wide concentration range, a linear relationship between concentration and instrument response could not be achieved and the slope of the graph significantly changed above 1 mg kg^{-1}.

The actual range of linearity was slightly different for each compound. The lower limit was defined as the LOQ (see section 4.3), however, peaks were also inspected to ensure that the correct peak was detected by the software and that the peak shape was satisfactory. For example, the LOQ of D-limonene was 0.050 mg kg^{-1}. At the lowest calibration point, which was 0.010 mg kg^{-1}, the peak shape was inconsistent and could not be integrated properly. Hence, this concentration was too low to be included in the range of linearity. Any peaks that were not present or did not fit the linear regression model were removed from the curve. The final linearity ranges for each compound are listed in Table 17.

Table 17. Range of linearity for the compounds of interest.

Compound of interest	Lower linearity limit (mg kg^{-1})	Upper linearity limit (mg kg^{-1})
α-pinene	0.0460	1.00
benzaldehyde	1.10	3.50
β-pinene	0.0360	1.00
D-limonene	0.0500	1.00
cineole	0.0300	1.00
benzyl alcohol	1.20	3.50
phenylacetaldehyde	0.770	3.50
linalool oxide (isomer 1)	0.00910	0.500
linalool oxide (isomer 2)	0.0150	0.500

Compound of interest	Lower linearity limit (mg kg^{-1})	Upper linearity limit (mg kg^{-1})
guaiacol	0.980	3.50
linalool	0.0360	1.00
nonanal	0.780	3.50
2-phenylethanol	1.20	3.50
oxoisophorone	0.0390	0.500
o-methoxyacetophenone	1.30	3.50
eugenol	1.30	3.50
(*E*)-β-damascenone	0.0260	1.00
β-ionone	0.0240	1.00

The Food and Drugs Administration guideline on the establishment of calibration curves states that "the simplest model that adequately describes the concentration-response relationship should be used and selection of weighting and use of a complex regression equation should be justified." [114]

Initially, a non-weighted linear regression curve was applied to the calibration standards for each compound as this is the simplest model. An example of the cineole curve is given in Figure 24. These typically produced curves with high coefficients of determination (R^2), indicating that the data fit the regression model. However, the accuracy of the curve was poor, especially at the low end of the curve. For cineole, percentage relative errors as high as 477% were observed at the lowest concentration (Table 18). Quantification is not possible with error this high, as the reported concentration would be wildly different from the actual concentration of the compound in the honey extract.

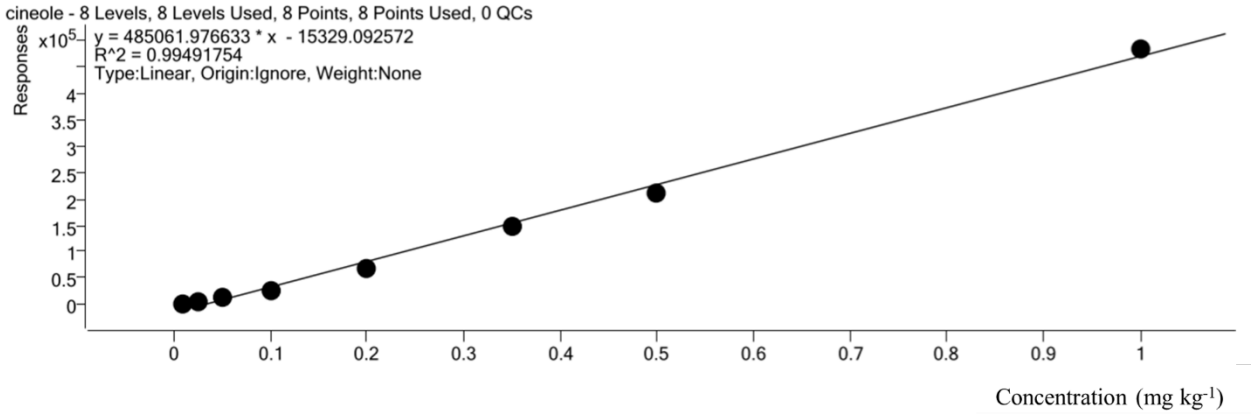

Figure 24. Unweighted linear regression curve for cineole (n=1).

Table 18. Average calculated percentage relative error (%RE) of each point in the cineole calibration curve (n=4)

Standard concentration (mg kg^{-1})	Peak area	Calculated concentration (mg kg^{-1})	%RE
0.01	2901	0.0488	388
0.025	5626	0.0554	122
0.05	10887	0.0682	36.3
0.1	23444	0.0990	1.03
0.2	52936	0.170	14.8
0.35	106179	0.298	14.9
0.5	166028	0.446	10.7
1	415480	1.05	4.91

This occurred because the use of a non-weighted curve assumes that variance is equal over the entire calibration range, independent of analyte concentration. This is rarely the case; rather, variance tends to increase with concentration, which "weights" the curve, resulting in low accuracy on the low concentrations (Figure 25).[174,175] This is especially common when the calibration curve spans a wide concentration range – typically, if the calibration range spans more than one order of magnitude, the variance can be expected to differ between concentrations. Although the overall variance increases with concentration, the relative standard deviation (%RSD) remains reasonably constant. Curve weighting should be considered when the %RSD is fairly constant throughout the calibration curve. [175]

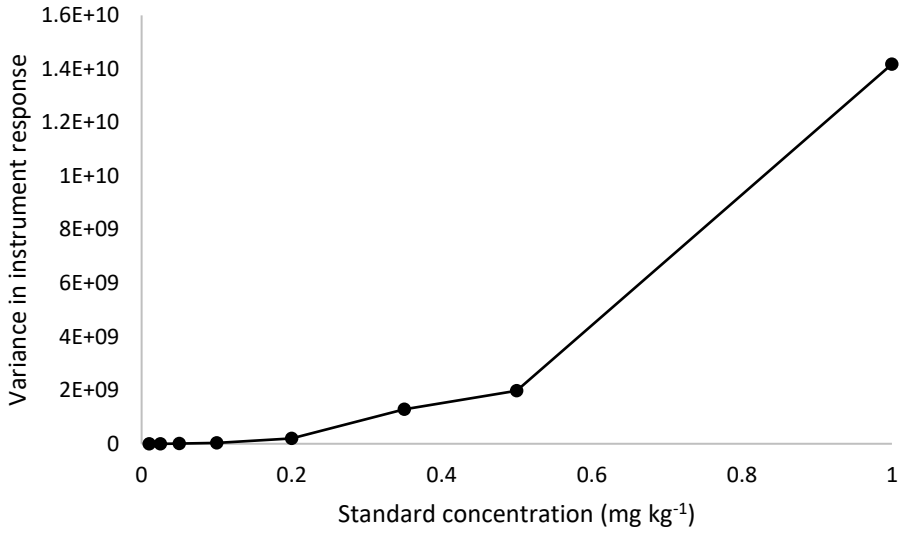

Figure 25. Relationship between concentration and variance of instrument response for cineole, showing that variance increases at higher concentrations.

For these reasons, weighting was applied to the regression curve. The purpose of weighting is to ensure that each calibration point has the appropriate influence on the curve. To choose the best weighting factor, the sum of the absolute value of the percentage relative error (%RE) at all calibration points is taken, and compared between the various weighting factors. An example calculation of this is carried out in Table 19 for cineole, with the curves displayed in Figure 26.

Table 19. Assessment of various weighted linear regression models for the calibration curve of cineole (n=4).

Weighting	Slope	Intercept	R^2	Σ%RE
Unweighted	412340	−17262	0.8992	2560
$1/x$	362814	−3425	0.9081	909
$1/x^2$	295557	−450	0.8711	806
$1/y$	330553	−3037	0.9009	903
$1/y^2$	242405	−270	0.8688	1050

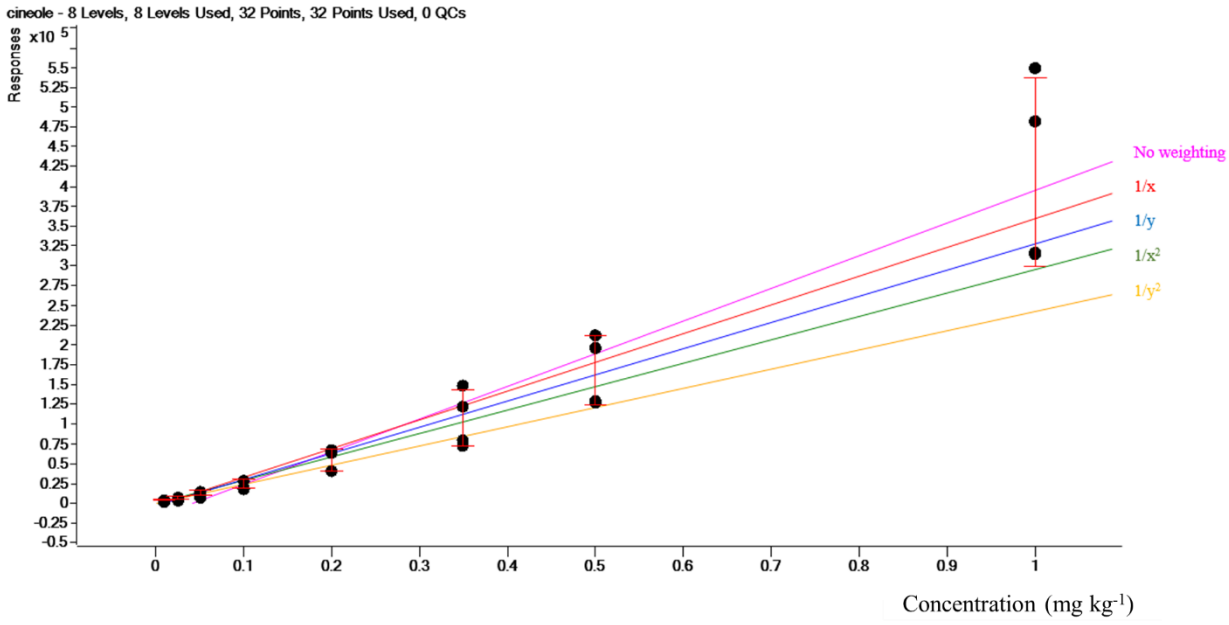

Figure 26. Calibration curves for cineole using various weighting factors.

After comparing the data, the appropriate weighting factor was selected for each compound. For cineole, $1/x^2$ was chosen due to the lowest sum of %RE across the concentration range. $1/x^2$ weighting was also used for the two linalool oxide isomers, linalool, 4-oxoisophorone, (*E*)-β-damascenone and β-ionone, while $1/y$ weighting was used for nonanal. All other compounds displayed the lowest Σ%RE with $1/x$ weighting. Once a weighting scheme was decided upon for each compound, this was used for each subsequent calibration curve to

ensure the curves were consistent. Although the equation of the calibration curve changed between batches, an example for each compound is given in Table 20.

Table 20. Summary of calibration curve details.

Compound	Regression Equation	Weighting factor	R^2
α-pinene	585470*x* + 5399	1/*x*	0.994
Benzaldehyde	1336536*x* – 564090	1/*x*	0.994
β-pinene	383097*x* + 721	1/*x*	0.995
ɒ-limonene	668055*x* – 16860	1/*x*	0.986
Cineole	229322*x* – 1067	$1/x^2$	0.977
Benzyl alcohol	787045*x* – 642403	1/*x*	0.984
Phenylacetaldehyde	1771040*x* – 864075	1/*x*	0.978
Linalool oxide (isomer 1)	70722*x* – 127	$1/x^2$	0.968
Linalool oxide (isomer 2)	127294*x* – 352	$1/x^2$	0.992
Guaiacol	661558*x* – 456286	1/*x*	0.947
Linalool	137752*x* – 1367	$1/x^2$	0.954
Nonanal	38587*x* – 18706	1/*y*	0.969
2-phenylethanol	536025*x* – 403663	1/*x*	0.977
Oxoisophorone	249041*x* – 835	$1/x^2$	0.978
o-methoxyacetophenone	1430822*x* – 1159115	1/*x*	0.959
Eugenol	98323*x* – 89312	1/*x*	0.942
(*E*)-β-damascenone	120031*x* – 842	$1/x^2$	0.973
β-ionone	135643*x* – 555	$1/x^2$	0.969

4.3. Limits of detection and quantification

Various descriptions exist for the terms limit of detection (LOD) and limit of quantification (LOQ). In general, LOD is defined as the lowest concentration at which the analyte can be detected, but not necessarily quantified, under the standard conditions of the method. [176] It describes the lowest concentration at which the analyte signal can be distinguished from instrumental noise. LOQ is defined as the lowest concentration at which the analyte can be quantified within the accepted levels of accuracy and precision for the method. [176]

As the definitions of the term vary between institutions and authorities, so too do the methods of determining LOD and LOQ. A common method involves calculating the signal-to-noise ratio (SNR) for each peak, which can be determined visually or by peak height (Figure 27).

An SNR of 3 is typically used for LOD, and 10 for LOQ. Although this method is useful for methods that exhibit baseline noise, it is only useful for peak height measurements, so is not suitable for this method as peak area was used for quantitation.

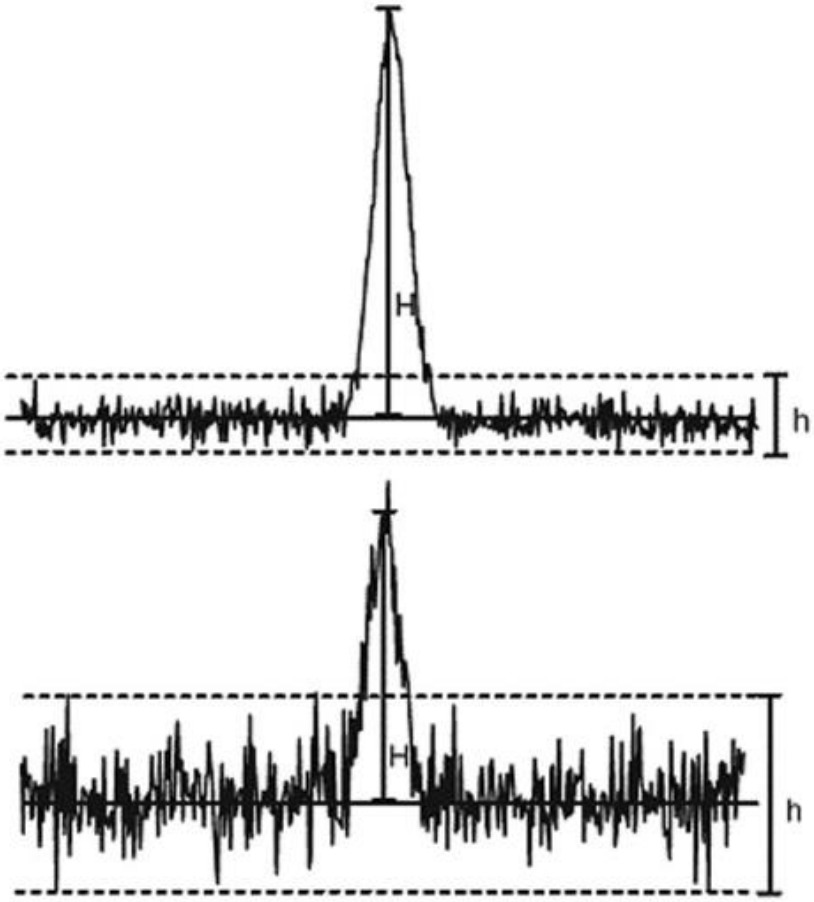

Figure 27. Signal-to-noise examples of 10:1 (top) and 3:1 (bottom), using the method of the European Pharmacopoeia. SNR=2*H*/*h*, where *H* is the peak height, measured from the top of the peak to the baseline, as extrapolated over a distance equal to 20 times the peak width at half height. *h* is the peak height of the baseline noise observed in this region. *Reproduced from Alankar & Vipin (2011)* [176] *(open access).*

The LOD and LOQ can also be calculated from the linear regression curve. LOD/LOQ calculations determined using the calibration curve are less subjective than the SNR method or visual determination, and are less susceptible to operator bias. [173] They are calculated using Equation 2, where σ is the standard deviation of either the y-intercept, the response of a blank sample with no analyte present, or the residual standard deviation of the linear regression, and *s* is the slope of the curve.:

$$LOD = {}^{3\sigma}/_{S}$$

$$LOQ = {}^{10\sigma}/_{S}$$

Equation 2. Calculation of LOD and LOQ.

Swartz and Krull recommend calculating σ from the standard error of the y-intercept, as it is a better fit for data at the low concentration end of the curve, rather than using the residual standard deviation of the curve which is more accurate at higher concentrations. [173] This approach was used to calculate the LOD and LOQ for each compound of interest. These are listed in Table 21, and an example calculation for cineole is given in Table 22.

Table 21. Limits of detection and quantification for the compounds of interest.

Compound of interest	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
α-pinene	0.014	0.046
Benzaldehyde	0.32	1.1
β-pinene	0.011	0.036
D-limonene	0.015	0.050
Cineole	0.0091	0.030
Benzyl alcohol	0.36	1.2
Phenylacetaldehyde	0.23	0.77
Linalool oxide (isomer 1)	0.0027	0.0091
Linalool oxide (isomer 2)	0.0045	0.015
Guaiacol	0.30	0.98
Linalool	0.011	0.036
Nonanal	0.23	0.78
2-phenylethanol	0.37	1.2
Oxoisophorone	0.012	0.039
o-methoxyacetophenone	0.40	1.3
Eugenol	0.39	1.3
β-damascenone	0.0079	0.026
β-ionone	0.0072	0.024

Table 22. Calibration data used for the calculation of LOD and LOQ for cineole.

Batch	Slope	Intercept	Average slope (*s*)	Standard error of intercept (σ)
1	361816	−943	295557	899
2	376052	−1172		
3	218142	1088		
4	226217	−775		

The values of s and σ above were used in Equation 3 to calculate the LOD and LOQ for cineole.

$$LOD = {}^{3\sigma}/_{s} = {}^{3 \times 899}/_{295557} = 0.0091$$

$$LOQ = {}^{10\sigma}/_{s} = {}^{10 \times 899}/_{295557} = 0.030$$

Equation 3. Example calculation of LOD and LOQ for cineole.

The limits of detection and quantification were sufficiently low for the method. For most compounds, the smallest peak that could be visually detected with confidence was of the same order of magnitude as the LOD and LOQ, providing confidence that the detected peaks could be quantified.

4.4. Accuracy

The accuracy of the method describes the closeness of the reported result to the actual value; in the case of this method, the closeness of the reported concentration of the analyte in the honey sample to its actual concentration. [173] Accuracy is measured by spiking a sample with analyte at a known concentration, and measuring the recovery of the analyte.

Unspiked samples of mānuka (NZ018), pōhutukawa (NZ270), rewarewa (NZ271), kāmahi (NZ272) and rātā (NZ273) honey were analysed to estimate endogenous concentrations of each compound of interest, and the spike concentrations chosen for each compound are summarised in Table 23. The "low spike" concentration was determined from 50% of the average endogenous concentration of the compound, and the "high spike" concentration was chosen so that the spiked honey should fall in the middle of the calibration range.

Table 23. Spike concentrations for accuracy studies, based on estimated endogenous levels of analytes in honey.

Compound of interest	Spike concentrations (mg kg^{-1})	
	Low spike	High spike
α-pinene	0.03	0.2
benzaldehyde	0.02	0.2
β-pinene	0.02	1.5
D-limonene	0.03	0.1
cineole	0.05	0.1
benzyl alcohol	0.02	1.5
phenylacetaldehyde	0.25	1.5
linalool oxide (isomer 1)	0.005	0.05
linalool oxide (isomer 2)	0.005	0.05
guaiacol	0.02	1.5
linalool	0.01	0.1

Compound of interest	Spike concentrations (mg kg^{-1})	
	Low spike	High spike
nonanal	1	2
2-phenylethanol	0.5	1.5
4-oxoisophorone	0.2	0.4
o-methoxyacetophenone	0.25	1.5
eugenol	0.5	1.5
(*E*)-β-damascenone	0.5	1.5
β-ionone	0.01	0.2

Guaiacol, 2-phenylethanol, benzaldehyde, benzyl alcohol, nonanal, eugenol and (*E*)-β-damascenone were not detected in the preliminary analysis. Low spikes for these samples were chosen as the low end of the calibration range.

The calculated recovery of the compounds of interest is given in Table 24 for mānuka honey, and Table 25 and Table 26 for the other honey types. Unfortunately, despite the efforts to improve recoveries through improvements to the method during method development (Chapter 3), recoveries for some compounds did not fall in the ideal 80 – 120% range. Some recovery values could not be included in the table, these are discussed below.

Table 24. Average recovery of compounds of interest in mānuka honey (n=4). Recoveries in the acceptable range are highlighted in bold.

Compound of interest	Recovery (%)	
	Low spike	High spike
α-pinene	N/A†	N/A†
benzaldehyde	N/A*§	N/A*§
β-pinene	N/A†	N/A*†
D-limonene	N/A¶	320 ± 100§
cineole	**120 ± 42**	160 ± 21
benzyl alcohol	N/A*§	**110 ± 17**§
phenylacetaldehyde	N/A*§	59 ± 8.2§
linalool oxide (isomer 1)	59 ± 12	140 ± 22
linalool oxide (isomer 2)	N/A*	210 ± 9.4
guaiacol	N/A*§	**110 ± 14**§
linalool	N/A*	140 ± 28
nonanal	**94 ± 11**	**120 ± 20**
2-phenylethanol	N/A*	130 ± 9.1
4-oxoisophorone	N/A*	N/A*
o-methoxyacetophenone	300 ± 56	N/A*
eugenol	N/A*	150 ± 17
(*E*)-β-damascenone	150 ± 35	N/A*
β-ionone	190 ± 29	260 ± 25

* value not included due to spiked sample lying outside of calibration range, † value not included due to contamination of the method blank, ¶spike concentration overwhelmed by endogenous concentration, §recovery may be affected by poor peak integration.

A reasonable proportion of the omitted recoveries could not be included due to the spiked samples falling outside the range of calibration. These instances are denoted in Table 24 by an asterisk. When the response for the spiked sample is too low (for example, the low spike of benzyl alcohol) or too high (for example, the low spike of benzaldehyde), the value is not reliable as the linearity of the instrument response does not hold outside the range established in section 4.2. Therefore, the calculated recovery could not be considered reliable.

Some of these instances were caused by poor choice of spike concentration For example, (*E*)-β-damascenone was not detected during the preliminary analysis, yet a high spike concentration of 1.5 mg kg^{-1} was chosen based upon mistaking which calibration group (*E*)-β-damascenone was in. The high spike of 1.5 mg kg^{-1} was outside the calibration range for this compound. The intended spike levels for benzaldehyde and β-pinene were accidentally swapped during preparation of the spike solutions – the actual concentrations used are what is listed in Table 23. The result of this was that the high spike for β-pinene was out of the calibration range, and therefore this result cannot be accurately quantified. Likewise, the high spike for benzaldehyde was lower than intended, although the recovery was not significantly impacted by this. These errors in judgement caused a lack in reliable recovery data, and the values could not be included.

Due to the endogenous levels of guaiacol, 2-phenylethanol, benzaldehyde, benzyl alcohol, nonanal, eugenol and (*E*)-β-damascenone being either below the range of linearity or not detected at all, the accuracy of the reported concentrations for the unspiked honeys is likely compromised for these honeys. Due to the slope of the calibration curve, eugenol, for example, shows a concentration of 0.74 mg kg^{-1} in method blanks and unspiked honey, when there is no peak present. This affects the recovery, as the unspiked concentration cannot reliably be used for the calculation.

A variety of factors also affected the peak integration, which had varied effects on the recoveries. Some compounds could not be properly integrated due to significant peak tailing. Benzaldehyde, benzyl alcohol (Figure 28), phenylacetaldehyde, and guaiacol all displayed peak tailing and RT shift over the calibration range. This was possibly caused by their high boiling points – 178 °C, 205 °C, 195 °C, and 205 °C, respectively. The higher boiling compounds tend to move more slowly through the column and as a result their peaks are broadened. Although peak tailing was not observed for *o*-methoxyacetophenone or eugenol, which have boiling points of 258 °C and 254 °C respectively, it is certainly a possibility. The

sample inlet was regularly cleaned and the inlet liner replaced weekly to minimise the effect of this, but unfortunately the peak shape for these compounds was still affected. The tails of these peaks were often cut off when the detector switched to the next set of ions to be scanned, so accurate quantitation of these compounds, both in the calibration curves and samples, was not guaranteed. However, the tail area of the peaks was quite small in comparison to the rest of the peak, so the cutting off of this area likely had little effect on the reported recoveries. The effect was more significant at lower concentrations, as the cut off area was a higher portion of the total area compared to higher concentrations. The high spike recoveries for benzaldehyde, benzyl alcohol and guaiacol were satisfactory despite this error.

Table 25. Percentage recoveries of compounds of interest in pōhutukawa and kāmahi honeys (n=1). Recoveries in the acceptable range are highlighted in bold.

Compound	Pōhutukawa			Kāmahi		
	Endogenous concentration (mg kg^{-1})	Low spike	High spike	Endogenous concentration (mg kg^{-1})	Low spike	High spike
		% Recovery			% Recovery	
α-pinene	0.072	N/A†	N/A†	0.049	N/A†	N/A†
Benzaldehyde	0.51	N/A*	N/A*	0.52	N/A*	N/A*
β-pinene	0.047	N/A†	N/A*†	0.0074	N/A†	N/A*
D-limonene	0.039	210	250	0.87	N/A¶	N/A¶
Cineole	n.d.	130	150	0.087	150	130
Benzyl alcohol	0.52	N/A*	**110**	0.66	N/A*	**110**
Phenylacetaldehyde	0.47	N/A*	59	0.53	N/A*	55
Linalool oxide (isomer 1)	0.024	19	**110**	0.025	**110**	150
Linalool oxide (isomer 2)	0.007	N/A*	190	0.0015	N/A*	170
Guaiacol	0.64	N/A*	130	0.64	N/A*	**100**
Linalool	0.015	N/A*	130	0.021	N/A*	160
Nonanal	0.99	**91**	**120**	0.94	**88**	**120**
2-phenylethanol	0.54	N/A*	130	0.6	N/A*	**120**
Oxoisophorone	1.3	N/A*	N/A*	6.9	N/A*¶	N/A*¶
o-methoxyacetophenone	0.59	N/A*	N/A*	0.94	N/A*	N/A*
Eugenol	0.75	N/A*	150	0.75	N/A*	130
β-damascenone	0.015	**110**	N/A*	0.04	N/A*	N/A*
β-ionone	0.013	130	210	0.021	1200	390

*value not included due to spiked sample lying outside of calibration range, † value not included due to contamination of the method blank, ¶spike concentration overwhelmed by endogenous concentration.

Table 26. Percentage recoveries of compounds of interest in rātā and rewarewa honeys (n=1). Recoveries in the acceptable range are highlighted in bold.

Compound	Rātā			Rewarewa		
	Endogenous concentration (mg kg^{-1})	Low spike	High spike	Endogenous concentration (mg kg^{-1})	Low spike	High spike
		% Recovery			% Recovery	
α-pinene	0.057	N/A†	N/A†	0.07	N/A†	N/A†
Benzaldehyde	0.39	N/A*	N/A*	0.43	N/A*	N/A*
β-pinene	0.03	N/A†	N/A*†	0.043	N/A†	N/A*†
D-limonene	0.57	N/A¶	N/A¶	0.034	200	250
Cineole	0.032	180	170	n.d.	150	180
Benzyl alcohol	0.51	N/A*	**110**	0.53	N/A*	140
Phenylacetaldehyde	0.41	N/A*	63	0.48	N/A*	**98**
Linalool oxide (isomer 1)	0.048	**83**	**120**	0.018	21	160
Linalool oxide (isomer 2)	0.007	N/A*	220	0.007	N/A*	220
Guaiacol	0.49	N/A*	**120**	0.48	N/A*	**110**
Linalool	0.017	N/A*	150	0.016	N/A*	**120**
Nonanal	1	140	150	1.5	**110**	130
2-phenylethanol	0.51	N/A*	**120**	0.49	N/A*	**120**
Oxoisophorone	0.47	190	N/A*	0.27	330	N/A*
o-methoxyacetophenone	0.91	N/A*	N/A*	0.61	N/A*	N/A*
Eugenol	0.61	N/A*	150	0.66	N/A*	150
β-damascenone	0.031	**100**	N/A*	0.015	160	N/A*
β-ionone	0.057	N/A†	N/A†	0.07	N/A†	N/A†

* value not included due to spiked sample lying outside of calibration range, † value not included due to contamination of the method blank, ¶spike concentration overwhelmed by endogenous concentration.

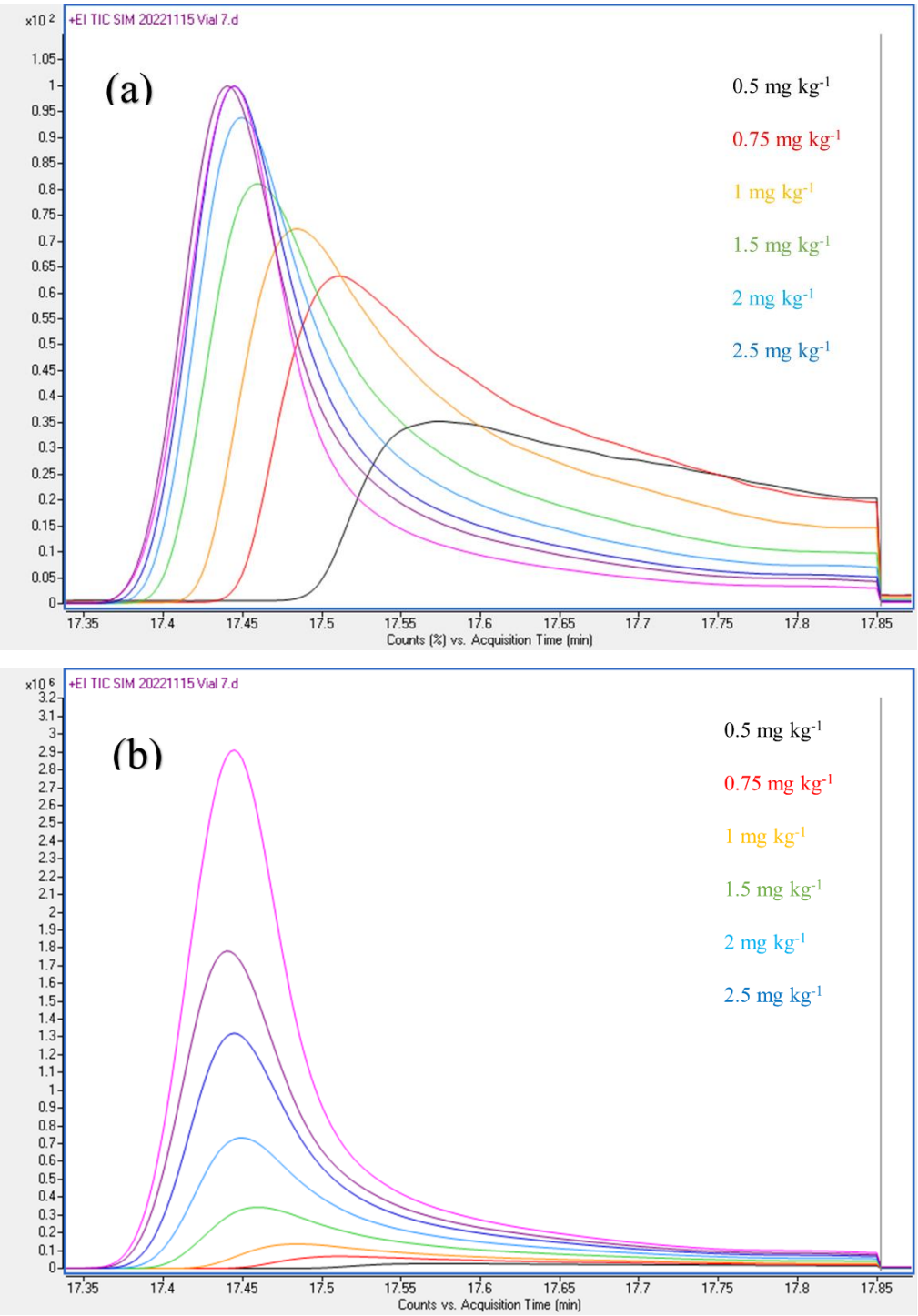

Figure 28. The peak shape and RT of benzyl alcohol peaks varied with concentration. RT decreased with increasing concentration. The peaks also displayed significant tailing, which resulted in a portion of the peak being cut off at the end of the SIM segment (17.85 min). a) Peak height is not to scale in this figure to better display the peak shapes, b) peak height to scale.

Other peaks could not be properly integrated due to interference from other compounds. For example, D-limonene (16.9 min) and cineole (17.0 min) closely co-elute (see section 3.5.4).

For cineole, unique ions could be chosen such that the D-limonene peak did not interfere and the low spike recovery was satisfactory. However, D-limonene does not contain any major ions that are not also present in cineole, meaning that complete baseline separation could not be achieved for D-limonene as both compounds appeared in its segment of the chromatogram. Additionally, in the honey samples another interfering peak was observed, eluting slightly earlier than D-limonene. In spiked samples, the effect of this interference increased, causing much higher recoveries than is acceptable. However, in unspiked samples, the low concentration of these compounds mean that the effect of the interference is low compared to the spiked samples.

Lastly, some peaks could not be properly integrated due to poor shape. Examples include benzaldehyde and linalool oxide. Peak integration for these compounds was reviewed across all samples to ensure consistency as much as possible.

Peaks for α-pinene and β-pinene were present in the method blanks, at levels comparable to those in the samples (Figure 29). The main cause of peaks in method blanks is contamination, for which there are a few possible causes. The first is carry-over of the compounds between injections. This is quite unlikely given the low boiling points of the compounds (150-170 °C) and the wash solvents (DCM and acetone) used to clean the injection needle before and after injection. Additionally, the method blanks are run directly after the instrument blank, which contains no analyte and did not display a peak for either of these compounds. The peak shape of α-pinene and β-pinene peaks was good in all samples; no fronting was observed which rules out the possibility of the detector being overloaded by these compounds. The other possibility is contamination occurring during sample extraction. It is possible that there was some contamination of the method blank samples by α-pinene and β-pinene, perhaps from improperly cleaned equipment. However, if this were the case, contamination of the other compounds of interest would also be likely. This was not observed. Additionally, it is unlikely that the same level of contamination would occur in every replicate.

This leads to the possibility that the quantification method was set up incorrectly in the MassHunter software. The TIC chromatogram showed no peaks at 11.12 min (RT of α-pinene) for method blanks, samples or spiked samples. The most significant ions in the mass spectrum at this RT were *m/z* 43.9, 31.9, and 39.9. This supports the hypothesis that the "peak" isolated in the SIM method is not actually α-pinene, as the principal ions present in the TIC at this time are not those used to quantify α-pinene. The signal for the quantifier ion

m/z 92.8 was buried in the noise, and therefore the peak can be disregarded as noise caused by the solvent. Unfortunately, this means quantification cannot be performed for α-pinene using this method.

Unlike α-pinene, a peak was observed for β-pinene (13.47 min) in the TIC chromatograms of spiked samples. As mentioned earlier, the spike level of β-pinene was accidentally swapped with benzaldehyde, so the observed peak was caused by a spike level of 1.5 mg kg^{-1}. This suggests that the noise observed in the method blanks, and the levels in the unspiked and spiked (at the α-pinene level of 0.2 mg kg^{-1}) samples were below the actual LOD for both α- and β-pinene.

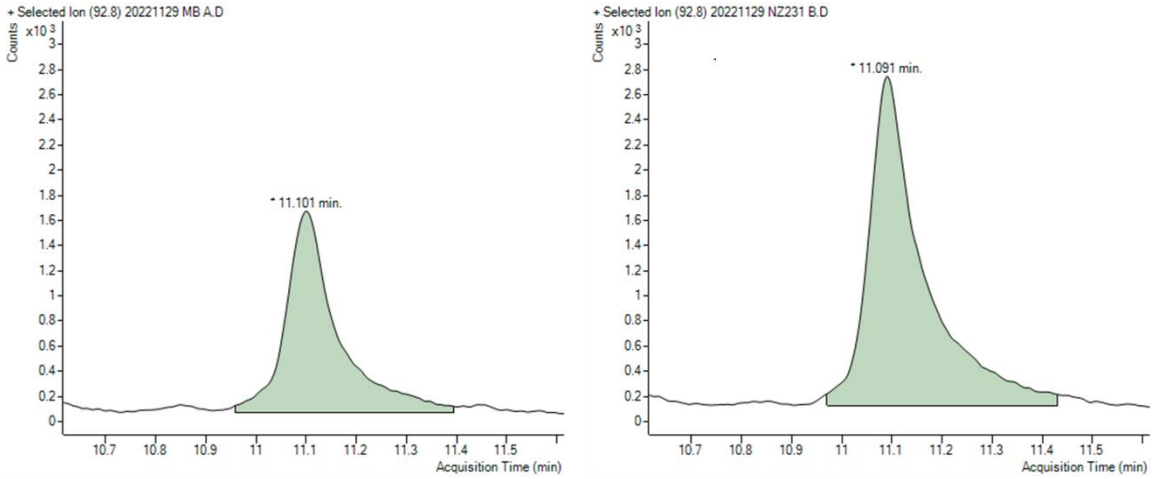

Figure 29. α-pinene peaks in a) method blank and b) mānuka honey samples. Peak shape is symmetrical in both instances, and the peak size is similar indicating probable contamination.

Ultimately, a myriad of factors affected the accuracy of the quantification, and the performance of the method in this regard was unsatisfactory for most compounds, with recoveries outside the acceptable range of 80-120% or that could not be reported due to the reasons discussed. Despite this, some compounds displayed acceptable recoveries and could be quantified accurately. This included nonanal, which could be quantified at all concentrations within the range of linearity due to acceptable recoveries at both low and high spikes, as well as cineole, benzyl alcohol and guaiacol. Cineole displayed acceptable recovery for the low spike, which meant that samples falling in the lower end of the calibration range could be quantified, but that the accuracy of response fell as concentration increased. Conversely, benzyl alcohol and guaiacol could only be quantified towards the higher end of their respective calibration ranges as only their high spike recoveries were acceptable. For all other compounds, a semi-quantitative approach was the most suitable way to compare relative differences in the levels of compounds present in different samples.

While this meant the exact contribution of each compound to the aroma of the honey could not always be calculated, the honeys could still be compared and the importance of each compound to the aroma was discussed.

4.5. Precision

Precision describes the ability of the method to produce consistent results over multiple analyses of the same homogenous sample. [173] There are multiple levels of precision that can be evaluated.

4.5.1. Repeatability

Repeatability is the measure of closeness between results of analyses of the same homogeneous sample performed over a short time interval, with identical conditions. Repeatability was determined for each compound of interest at three concentration levels, which were extracted and analysed in triplicate, giving nine replicates total. This data is given in Table 27, with an example calculation for cineole in Table 28. Repeatability was generally excellent, with 83% of the reported values being under 5% RSD. This is interesting considering the low accuracy reported in section 4.4, and suggests that although the reported concentration was incorrect, the result was consistent. This points to a problem in the quantification being the cause, rather than any analyte loss during sample extraction which would be less consistent.

Table 27. Repeatability of measurement of compounds of interest at three concentration levels in mānuka honey (n=3).

Compound	% RSD		
	Unspiked	**High Spike**	**Low Spike**
α-pinene	1.84	1.74	3.18
benzaldehyde	0.24	0.76	0.64
β-pinene	0.63	2.78	3.06
ᴅ-limonene	7.04	2.64	7.28
cineole	n.d.	2.43	5.17
benzyl alcohol	0.07	1.87	2.78
phenylacetaldehyde	0.10	2.54	1.44
linalool oxide (isomer 1)	2.84	5.43	3.39
linalool oxide (isomer 2)	10.02	2.86	3.05
guaiacol	0.22	2.75	0.36
linalool	2.19	1.89	2.40
nonanal	9.76	3.05	2.09
2-phenylethanol	0.45	2.66	1.03
4-oxoisophorone	3.04	2.94	1.97
o-methoxyacetophenone	2.52	3.16	0.25
eugenol	0.12	8.16	4.51

Compound	% RSD		
	Unspiked	High Spike	Low Spike
(*E*)-β-damascenone	3.14	8.50	6.13
β-ionone	2.03	3.47	3.58

Table 28. Example calculation of repeatability for cineole at the high spike concentration.

Replicate	Concentration (mg kg^{-1})
1	0.17
2	0.18
3	0.16
Mean	0.17
Standard deviation	0.01
%RSD	3.7

4.5.2. Intermediate precision

Intermediate precision describes the precision of the method when various within-laboratory variations are applied, such as different analysts, instruments, or days. The intermediate precision of the method was tested by comparing the results of the unspiked mānuka honey samples over the four batches, which were run over the course of two weeks. The main factor that may have affected the analysis was the weekly tuning of the GCMS, which occurred between the analysis of batches 2 and 3. No other changes could be made, as training another analyst to carry out the method or running it on another instrument would have been time prohibitive. However, with the time variation, the intermediate precision (Table 29) was still very good, although slightly worse than the repeatability measurement. An example of the intermediate precision calculation for phenylacetaldehyde is given below (Table 30).

Table 29. Intermediate precision of measurement of compounds of interest over four batches, with analysis spanning two weeks.

Compound	%RSD
α-pinene	12.0
benzaldehyde	11.9
β-pinene	30.8
ɒ-limonene	5.2
cineole	31.8
benzyl alcohol	1.7
phenylacetaldehyde	7.2
linalool oxide 1	11.5
linalool oxide 2	3.5
guaiacol	14.1
linalool	12.6
nonanal	13.4

Compound	%RSD
2-phenylethanol	8.0
4-oxoisophorone	3.0
o-methoxyacetophenone	4.1
eugenol	9.2
(*E*)-β-damascenone	30.1
β-ionone	12.0

Table 30. Example calculation of intermediate precision for phenylacetaldehyde.

Batch Mean (n=4)	Concentration (mg kg^{-1})
Batch 1	0.5255
Batch 2	0.5139
Batch 3	0.5811
Batch 4	0.4763
Mean	0.5242
Standard deviation	0.04
%RSD	7.2

4.6. Conclusion

Validation of the method showed that its performance is satisfactory in most respects. Limits of detection and quantification were sufficiently low for all the Group A compounds, and the instrument response was linear over a wide range to account for differences in endogenous concentrations between honey types. The instrument response was also precise, with excellent results achieved for repeatability and intermediate precision. Unfortunately, the accuracy of the method was poor, with multiple issues preventing the possibility of quantification. For this reason, the results reported in the following chapter focus on comparing relative differences between samples, and peak area will be used for this purpose rather than a reported concentration.

5. Chapter Five: Analysis of New Zealand Monofloral Honeys

5.1. Introduction

A selection of 44 New Zealand monofloral honeys were analysed using the chosen method, spanning seven native and four non-native floral types. The samples were analysed in duplicate using both the SIM and TIC methods. In this chapter, the volatile compositions of each honey type will be discussed, as well as differences between the honey types. Chemometric methods were used to explore the variation between samples and floral types.

As discussed in Chapter 4, accurate quantification could not be achieved for all compounds. Concentrations for nonanal are reported, as well as concentrations for cineole which fell within the appropriate range of the calibration curve for accurate quantification. Benzyl alcohol and guaiacol were able to be quantified within a limited range of the calibration curve, however, all samples displayed concentrations outside these ranges. For all other compounds, relative differences between the volatile compositions of the honey types are discussed, using peak areas from the SIM chromatograms and complemented by the TIC chromatograms. It is important to note that peak sizes between compounds cannot be compared, only peak sizes of the same compound between different samples. This is because the MS detector showed different sensitivity for each compound, as evidenced by the slopes of their calibration curves.

As no internal standard was used, fluctuations in instrument response could not be accounted for and thus the error in peak areas may be slightly greater, however, the precision of the measurement was acceptable even without internal standard (Chapter 4, section 0). The other option available was to report concentrations as calculated using the calibration curves which were run in each batch of samples. This would have allowed some correction for instrument fluctuations. However, reporting peak areas is the most common method of discussing results when an internal standard is not used. [15,79,119,125,166] These are commonly reported as a percentage of the total peak area, however a more accurate comparison could be made by directly comparing peak areas in this case due to the use of SIM rather than TIC chromatograms.

Using SIM allowed for the 18 external standards (Figure 30) to be identified in almost all honey types, even if no peak was identified in the TIC chromatogram. This confirms that the

increased sensitivity of the SIM method was useful for separation of the selected external standards from the complex extract, even if it could not be used for quantification in this instance. The average peak areas for each compound are summarised for each honey type in Table 31, with the concentrations of selected compounds summarised in Table 33 and Table 32. The peak areas of the two linalool oxide isomers were combined as the identity of each peak as the *cis*- or *trans*- isomer could not be identified by mass spectrometry.

In this chapter, the volatile profiles of each honey type are discussed in turn, with the discussion centred around the honey types with the highest peak area or concentration for each compound. The dominant peaks in each chromatogram are explained, though as noted, these do not necessarily indicate the compounds with the highest concentrations, rather those with the highest average peak area. In section 5.11, the chemometric methods used to separate and classify the honey samples are discussed.

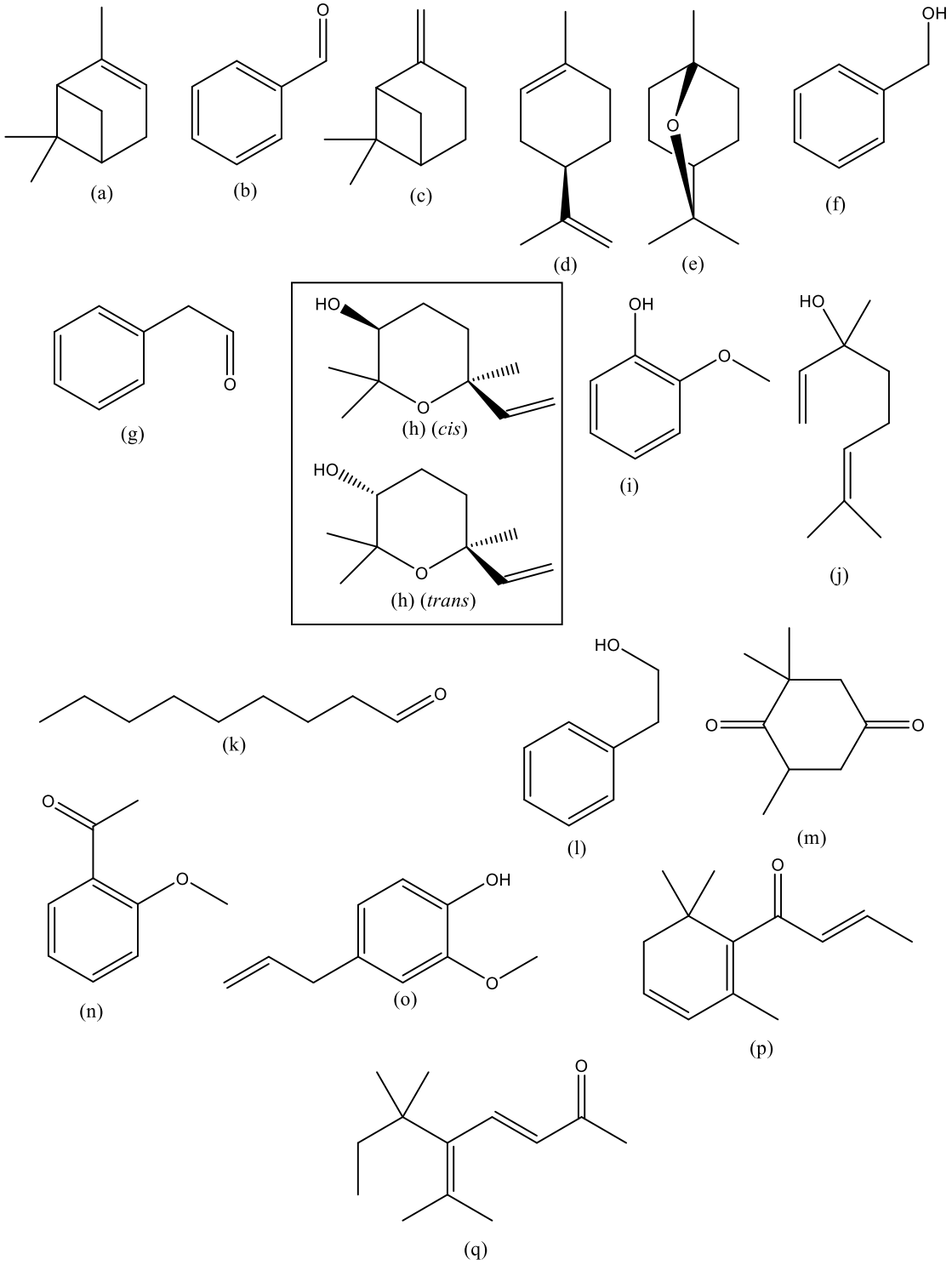

Figure 30. Chemical structures of the 18 external standards analysed using the GC-MS SIM method. A) α-pinene, b) benzaldehyde, c) β-pinene, d) ɒ-limonene, e) cineole, f) benzyl alcohol, g) phenylacetaldehyde, h) linalool oxide (*cis*- and *trans*- isomers), i) guaiacol, j) linalool, k) nonanal, l) 2-phenylethanol, m) 4-oxoisophorone, n) *o*-methoxyacetophenone, o) eugenol, p) (*E*)-β-damascenone, q) β-ionone.

Table 31. Average peak areas for clover, honeydew, kāmahi, kānuka, ling and lotus/blackberry honeys of the 17 external standards analysed using the SIM method. The largest peak area for each compound is highlighted in bold.

Compound	Average Peak Area					
	Clover (n=6)	Honeydew (n=6)	Kāmahi (n=5)	Kānuka (n=4)	Ling (n=1)	Lotus/ Blackberry (n=1)
α-pinene	29300 ± 15900	18900 ± 12800	13300 ± 10000	29800 ± 28500	9520	9250
benzaldehyde	11500 ± 7620	13900 ± 14500	6710 ± 2640	23800 ± 49200	2620	777
β-pinene	17100 ± 16100	13200 ± 14200	6660 ± 4140	14200 ± 10200	9100	9180
D-limonene	**524000*** ± 335000	405000* ± 369000	293000* ± 343000	514000* ± 449000	47300*	143000*
cineole	24300* ± 17400	17800* ± 16300	14000* ± 17600	**26100*** ± 7900	1380*	3430*
benzyl alcohol	2000 ± 1320	208 ± 219	2570 ± 2400	4490 ± 7900	3450	3280
phenylacetaldehyde	92400 ± 56600	32300 ± 35200	57500 ± 68700	**957000*** ± 244000	20100	10300
linalool oxide	18100 ± 8730	11300 ± 11500	12100 ± 10700	**22900** ± 27900	1890	6450
guaiacol	**56500** ± 136000	1800 ± 1300	1260 ± 840	2000 ± 1480	1090	760
linalool	8230 ± 7510	**16900** ± 13300	4670 ± 2550	9540 ± 8590	1680	1430
nonanal	35000 ± 13300	32600 ± 10400	**58600** ± 41400	52500* ± 23900	14700	19500
2-phenylethanol	7560 ± 2850	5320 ± 2740	**12400** ± 11300	9250 ± 5080	6520	6660
4-oxoisophorone	123000 ± 182000	41000 ± 40200	**2370000** ± 2280000	847000 ± 232000	48500	21800
***o*-methoxyacetophenone**	22300 ± 189000	95600 ± 105000	142000 ± 215000	217000 ± 218000	2290	1930
eugenol	423 ± 342	423 ± 525	2250 ± 3000	1280 ± 663	634	315
(*E*)-β-damascenone	2070 ± 2568	2540 ± 3140	1590 ± 2100	1450 ± 876	824	907
β-ionone	2260* ± 5090	1030* ± 951	1380* ± 2660	1330* ± 769	1860*	795*

Table 32. Average peak areas for mānuka, pōhutukawa, rātā, rewarewa and tāwari honeys of the 17 external standards analysed using the SIM method. The largest peak area for each compound is highlighted in bold.

Compound	Average Peak Area				
	Mānuka (n=5)	**Pōhutukawa (n=5)**	**Rātā (n=1)**	**Rewarewa (n=5)**	**Tāwari (n=5)**
α-pinene	29500 ± 28300	25100 ± 24650	**30600**	21800 ± 8910	30400 ± 22400
benzaldehyde	**40200** ± 47600	20800 ± 29000	4250	12900 ± 35800	11000 ± 10300
β-pinene	**22800** ± 29500	18000 ± 28800	19800	14700 ± 8010	17800 ± 16300
ɒ-limonene	276000* ± 275000	283000* ± 168000	196000*	189000* ± 164000	359000*± 303000
cineole	10900* ± 13200	11400* ± 9760	5540*	7960* ± 11800	17200*± 16200
benzyl alcohol	5110 ± 1320	4590 ± 6900	2990	**7970** ± 10900	2490 ± 1480
phenylacetaldehyde	405000 ± 557000	135000 ± 132000	20300	196000 ± 617000	107000 ± 73000
Linalool oxide	8720 ± 4950	8720 ± 6470	7450	4800 ± 5690	9000 ± 6650
guaiacol	1920 ± 1320	39300 ± 96000	860	1440 ± 1150	2500 ± 1690
linalool	5420 ± 6520	4850 ± 4100	4460	3560 ± 2500	7340 ± 5140
nonanal	35700 ± 23100	37000 ± 20700	29400	22000 ± 5750	31700 ± 14900
2-phenylethanol	8250 ± 1950	7530 ± 3000	5410	6080 ± 2960	7710 ± 3240
4-oxoisophorone	43700 ± 16100	33600 ± 31600	804000	153000 ± 14100	128000 ± 116000
***o*-methoxyacetophenone**	**10900000** ± 11200000	1850000 ± 70500	12300	19600 ± 22700	22300 ± 21900
eugenol	2050 ± 1410	1020 ± 423	**3720**	1800 ± 672	522 ± 140
(*E*)-β-damascenone	2500 ± 2490	1210 ± 640	**6380**	2200 ± 757	1910 ± 1460
β-ionone	1910* ± 3490	1240* ± 806	**8550***	2090* ± 479	4950* ± 13400

Table 33. Average concentrations (mg kg^{-1} in honey) for each honey type of the cineole, benzyl alcohol, guaiacol and nonanal. The highest concentration for each compound is highlighted in bold.

Compound	Average Concentration (mg kg $^{-1}$)	
	cineole	**nonanal**
Clover (n=6)	1.46*† ± 0.98	10.7† ± 2.0
Honeydew (n=6)	1.09*† ± 0.98	10.3† ± 1.5
Kāmahi (n=5)	0.740*† ± 1.05	**15.8† ± 4.7**
Kānuka (n=4)	**1.47***† ± 1.3	12.5*† ± 3.1
Ling (n=1)	<LOQ	7.06†
Lotus/ Blackberry (n=1)	<LOQ	8.08†
Mānuka (n=5)	0.745*† ± 0.81	11.3† ± 3.6
Pōhutukawa (n=5)	0.693*† ± 0.71	11.1† ± 3.6
Rātā (n=1)	<LOQ	10.2†
Rewarewa (n=5)	0.458*† ± 0.54	8.57† ± 0.93
Tāwari (n=5)	1.15*† ± 0.85	10.4† ± 2.4

*compound identified in this honey type for the first time, †compound identified as aroma-active in this honey type for the first time

5.2. Mānuka honey

Despite mānuka honey typically containing higher levels of volatiles than most other New Zealand honeys, the levels of the analysed compounds were generally comparable with the other honey types.

5.2.1. o-Methoxyacetophenone

The distinguishing feature of mānuka honey was the high concentration of *o*-methoxyacetophenone (Figure 31). The average peak area for this compound in the SIM chromatogram was 10900000 ± 11200000, almost 60 times greater than any other honey type. This was reflected in the TIC chromatogram (Figure 32) as a large peak was observed at RT 34.7 min. This peak was not observed in the TIC of any other honey type.

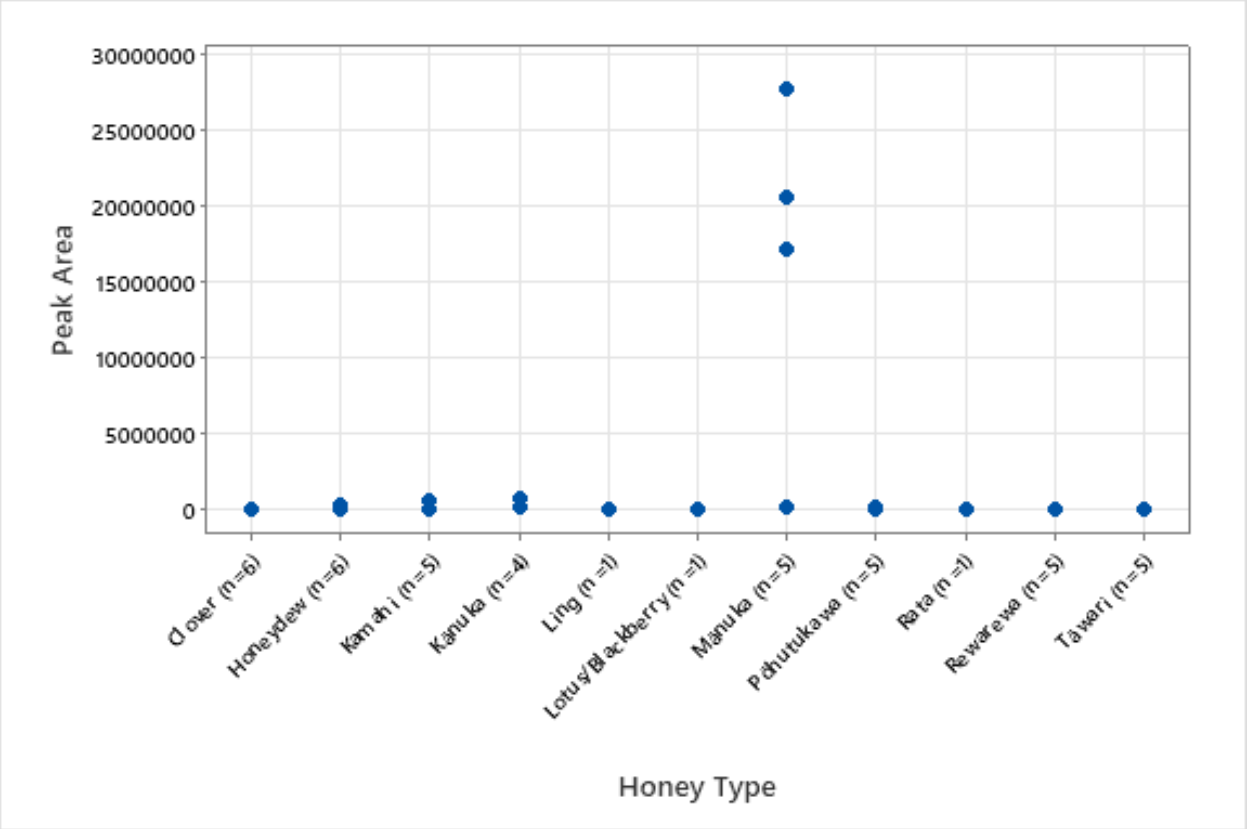

Figure 31. Individual value distribution plot of *o*-methoxyacetophenone peak area by honey type.

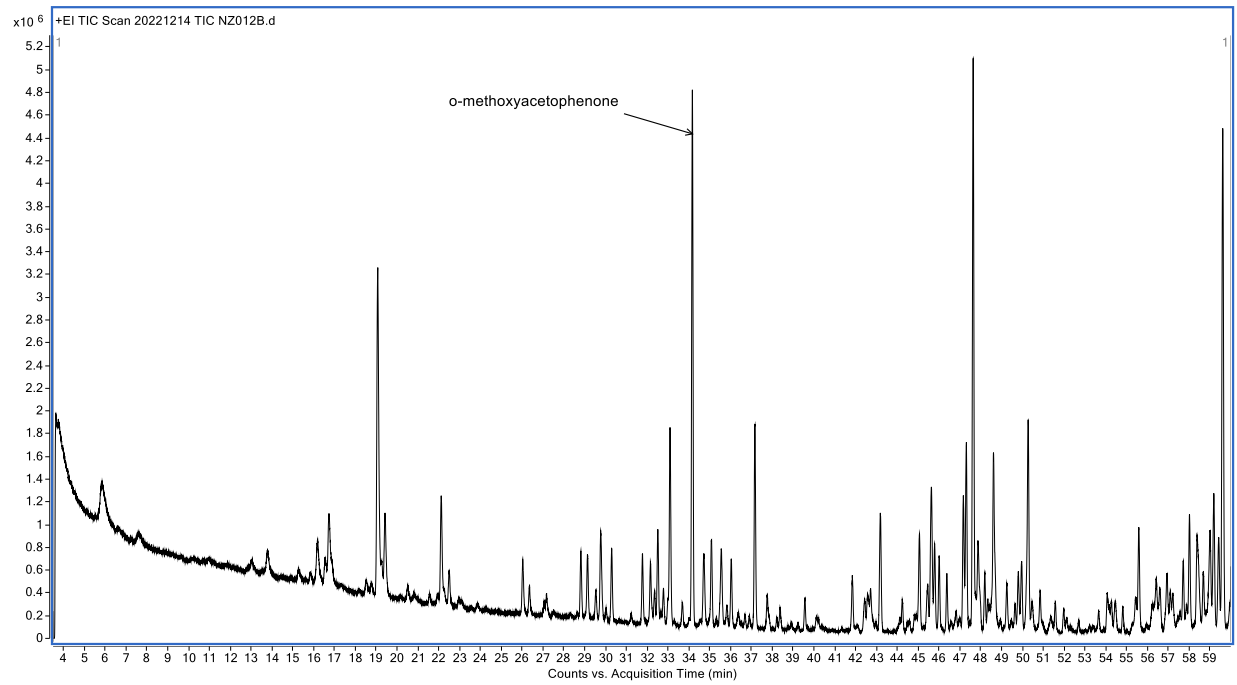

Figure 32. TIC chromatogram of a typical mānuka honey (NZ012). Labelled peak is *o*-methoxyacetophenone (34.7 min).

o-methoxyacetophenone (Figure 30 (**n**)) has been identified in the mānuka plant, and no other plants to date. [21] However, it has previously been identified in New Zealand monofloral honeys including clover, [15,79,80,86] beech honeydew, kāmahi, mānuka, pōhutukawa, rātā, rewarewa, tāwari, thyme and viper's bugloss. [4,15,79,82,83,86,93,94] This suggests that the presence of the compound in other floral honeys is due to contribution of mānuka nectar. Its abundance in mānuka honey is much higher than in the other honeys, and it has been identified as one of the dominant volatiles in mānuka honey. [21,86,93,150] Tan (1989) reported a concentration of 10 mg kg^{-1}, which was higher than the calibration range used in this method. [86] Due to this high abundance, *o*-methoxyacetophenone is used as a marker compound to distinguish mānuka honey from other honey types. To be classed as mānuka honey, honey must contain ≥ 5 mg kg^{-1} *o*-methoxyacetophenone, although this compound cannot be used to distinguish between monofloral and multifloral mānuka. [21] This value was increased from 1 mg kg^{-1} in 2017, to make it more difficult for other honey types blended with mānuka to meet the required concentration. [177] The abundance of *o*-methoxyacetophenone in mānuka honey compared to other floral types is particularly useful for distinguishing mānuka honey from kānuka, which is difficult because the pollen grains of the two species appear almost identical (Figure 33). Indeed, kānuka honeys, as well as kāmahi, displayed slightly higher peak areas for *o*-methoxyacetophenone than the other honeys in the SIM chromatograms (Table 31). However, these peak areas were negligible compared to the mānuka honeys, as expected.

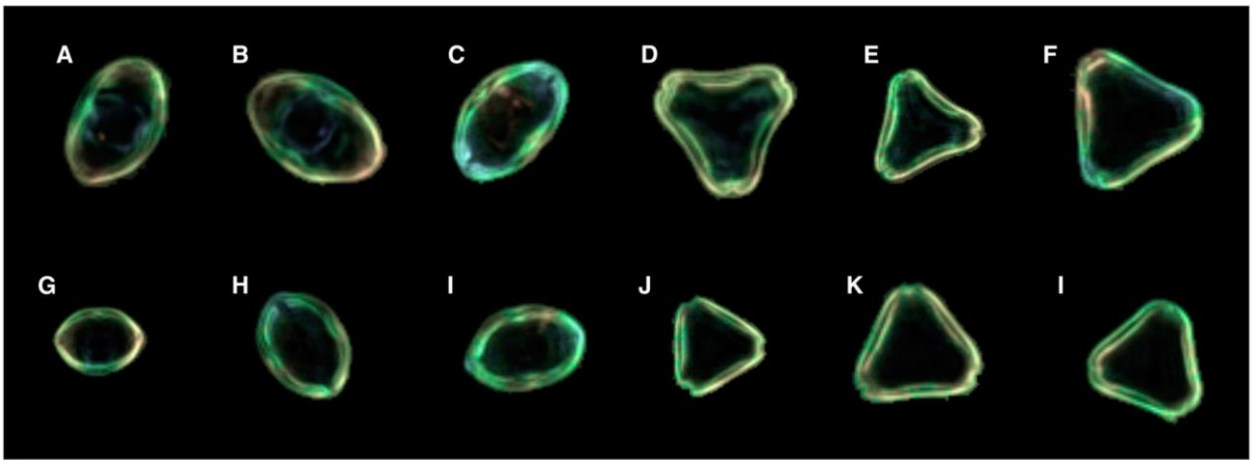

Figure 33. Pollen grains of mānuka (*Leptospermum scoparium*) and kānuka (*Kunzea ericoides*). (A-C) *L. scoparium*, equatorially oriented images; (D–F) *L. scoparium*, polar-oriented images; (G–I) *K. ericoides*, equatorially oriented images; (J–L) *K. ericoides*, polar-oriented images. *Reproduced from Holt & Bebbington (2014)* [16] *(open access).*

High peak areas for *o*-methoxyacetophenone were only observed in three of five mānuka honey samples (NZ017, NZ298, NZ299) while the other two samples (NZ012, NZ037) displayed levels similar to the other honey types. A t-test at the 95% confidence level confirmed that the high and low concentration samples were statistically different (p=0.020). A study by Oelschlaegel *et al.* (2012) found that pure mānuka honeys were characterised by high concentrations of *o*-methoxyacetophenone compared to mānuka honeys with significant contributions from other floral types. [97] However, MPI (2017) reported that the compound cannot be used to distinguish between monofloral and multifloral mānuka honeys, and the same concentration is required to class honey as either type. [21] It is possible that NZ012 and NZ037 contain lower levels of *o*-methoxyacetophenone due to increased contribution from other nectar sources, though pollen analysis would be required for a further investigation. Previous testing on samples NZ298 and NZ299 confirms that they are high quality mānuka samples due to high dihydroxyacetone content (1780 mg kg^{-1} and 1980 mg kg^{-1}, respectively). [178] Dihydroxyacetone is the precursor to methylglyoxal, which is the compound responsible for the non-peroxide antibacterial of mānuka honey. [6,179] Therefore, it is used as an indicator of mānuka quality. The high dihydroxyacetone content of these two samples suggests that they likely have a high contribution of nectar from mānuka.

The odour of *o*-methoxyacetophenone is described as powdery, anisic, almond-like and phenolic. [180] Though its odour threshold is not reported in literature, it is a likely contributor to the aroma of mānuka honey due to its exceptionally high contribution to the volatile profile. As mentioned in Chapter 1, mānuka honey has a distinctive earthy, mineral aroma.

The description *mineral* does not appear on the UC Davis honey flavour wheel, but *earthy* does, and it is positioned close to *anisic* and *almond* on the wheel, indicating that the aromas are similar in quality. [181] This suggests that *o*-methoxyacetophenone is an aroma contributor.

5.2.2. Benzaldehyde and β-pinene

Mānuka honey also had the highest average peak area (and total peak area) of all the honey types for benzaldehyde (Figure 34) and β-pinene, however, the difference between these and the other honey types was less pronounced than for *o*-methoxyacetophenone – t-tests at the 95% confidence interval showed that the difference was not significant ($p>0.05$). Benzaldehyde (Figure 30 (**b**)) is the simplest aromatic aldehyde and has a characteristic almond-like aroma. It is used in synthetic almond extracts and is found in honeys worldwide regardless of botanical origin. The odour threshold of benzaldehyde is 150 μg kg^{-1}, and it has been reported as an aroma contributor in citrus, cambará, black mangrove, rape and haze honeys. [11,39,48,50,52] Benzaldehyde has been identified in all New Zealand monofloral honeys that have been studied. [40] With a low odour threshold, and reasonably similar average peak areas among all honey types (within the same order of magnitude), it is likely that benzaldehyde at least contributes to the aroma of the mānuka and kānuka honeys, if not all the honey types analysed.

Peak areas for β-pinene (Figure 30 (**c**)) were very low for all compounds studied, and though the peak areas in mānuka honey were the highest of these, none were significantly different from the average peak area of the method blanks ($p>0.05$). As discussed in Chapter 4 (section 4.4) it is unlikely that β-pinene was actually detected in the honey samples at such a low level, thus this compound will not be discussed.

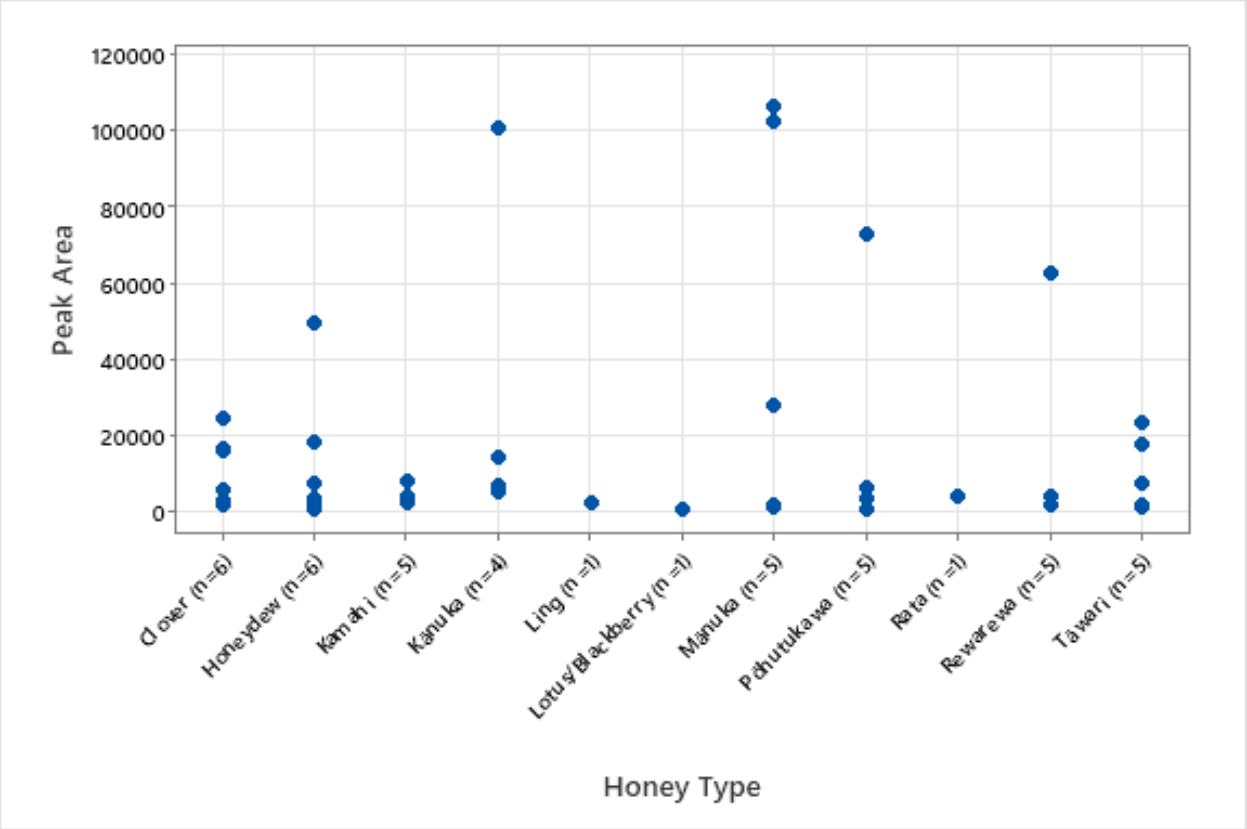

Figure 34. Individual value distribution plot of benzaldehyde peak area by honey type.

5.3. Clover honey

The chromatograms of clover honey did not contain many distinguishing features. There were no compounds with particularly high peak areas compared to the other honeys, especially when considering the peak area of *o*-methoxyacetophenone in mānuka honey as described previously. Even the most prominent compounds in the chromatogram, D-limonene and 4-oxoisophorone, had comparable peak areas to the other honeys. These observations agree with the previous reports of low levels of volatile compounds in clover honey. [86] Clover honeys did have the highest average peak areas for D-limonene (Figure 35) and guaiacol (Figure 36), though t-tests at the 95% confidence interval showed that these peak areas were not statistically different from those of other honey types ($p>0.05$). Guaiacol was the dominant peak in the chromatogram, with an average peak area of 56500 ± 136000.

Internationally, white clover (*Trifolium repens*) honey is mainly characterised by benzene derivatives such as *p*-anisaldehyde, benzyl alcohol, phenylacetaldehyde, benzaldehyde, methyl benzoate and benzoic acid. [163] In contrast, red clover (*Trifolium pratens*) is characterised by the lilac aldehydes. [123] The peak areas of the benzene derivatives analysed

using the SIM method were low compared to the other honey types, which contrasts the previous research.

5.3.1. ᴅ-limonene

ᴅ-limonene (Figure 30 (**d**)) is a cyclic monoterpene. Monoterpenes are a class of terpenes that consist of two isoprene units and have the formula $C_{10}H_{16}$. They are a key component of plant essential oils and are derived from geranyl pyrophosphate. [104,105,182] As monoterpenes are found in the essential oils of many plants, and have been confirmed not to originate from the honey bee, it is suggested that the terpenes in the volatile fraction of honey are of botanical origin. [89,133] Many of these terpenes are not unique to a single floral type, but they may contribute to the aroma of the honey. They typically have floral scents that are reminiscent of the botanical source. [53]

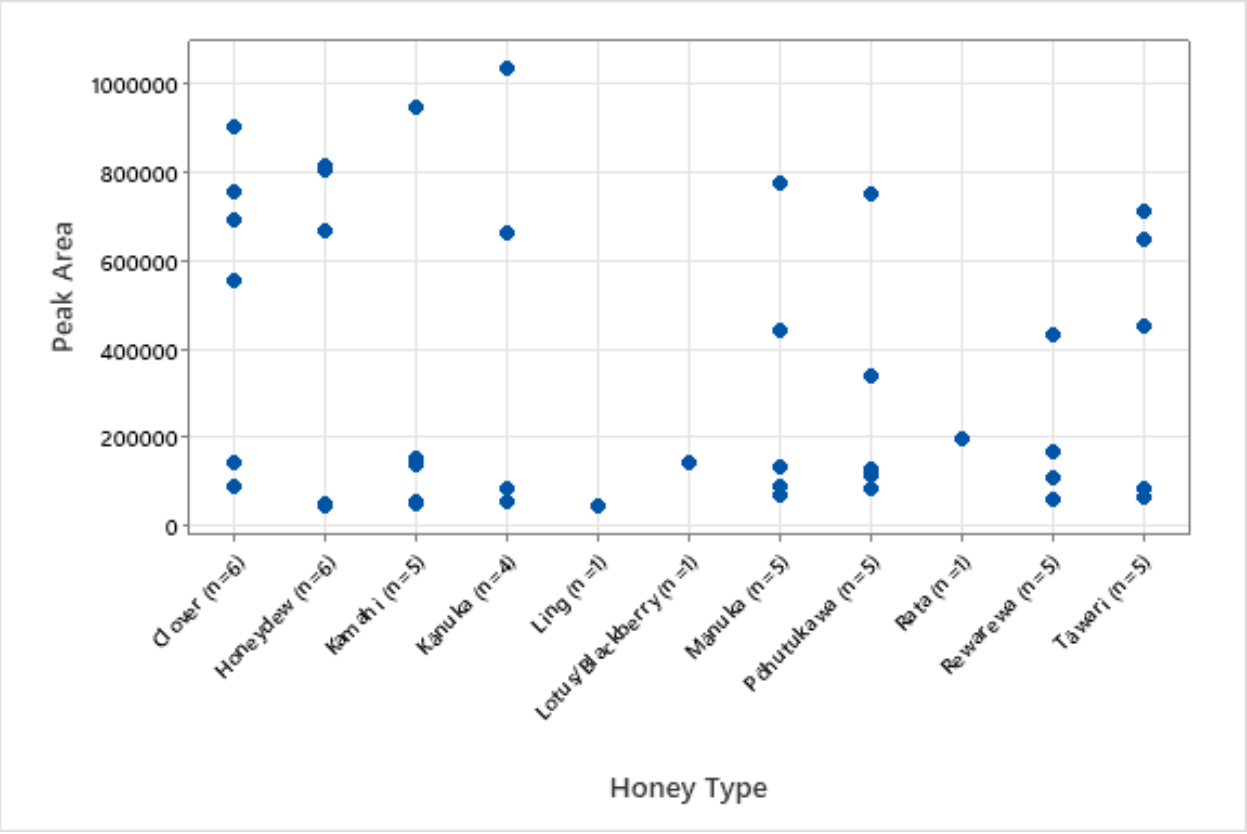

Figure 35. Individual value distribution plot of ᴅ-limonene peak area by honey type.

ᴅ-limonene is the principal compound responsible for the aroma of oranges and was first reported as a honey constituent in 2005. [183] Though it was identified in citrus honey, no sensory studies have yet confirmed that it is aroma-active in this honey. However, it has been reported as contributing to the aroma of buckwheat, cloudberry-bog, lingonberry, sweet clover, willowherb and astralagus honeys. [29,51] An extended literature search suggests that this

is the first reported occurrence of D-limonene in New Zealand honey, though it is present in mānuka leaf oil. D-limonene was detected in all honey types analysed, with clover honeys displaying the highest average peak area but one kānuka sample (NZ334) having the highest peak area overall at 1030000. The odour threshold of D-limonene is low at 60 μg kg^{-1}, [29] so it is likely that it has some contribution to the aroma of the honeys analysed.

5.3.2. Guaiacol

Clover honey also had the highest average peak area for guaiacol, however, this appears to be due to an outlier (Figure 36). In fact, one sample each of clover (NZ202) and pōhutukawa (NZ105) honeys displayed high peak areas for guaiacol. Reported concentrations for all compounds were above the range of linearity, but typically were estimated at around 4.8 mg kg^{-1} for most honeys. The calculated concentrations for NZ202 and NZ105 respectively were 9.64 mg kg^{-1} and 8.39 mg kg^{-1}. The two samples were sourced from nearby areas in the Bay of Plenty during the 2015 season. Heather honey has typically been characterised by guaiacol (Figure 30 (**i**)), as well as other phenolic compounds such as *p*-anisaldehyde, propylanisole and *p*-cresol. [24,49] One possibility is that the honeys contained some nectar contribution from heather, which is a pest plant throughout New Zealand, including in the Bay of Plenty region. Pollen analysis would be needed to confirm this.

Guaiacol possesses a medicinal aroma and has an odour threshold of 0.34 μg kg^{-1}. [9]It has been reported as aroma-active in cashew, [58] rape, [48] astralagus[53] and haze[50] honeys. Due to the exceptionally low odour threshold, guaiacol can be considered a likely aroma contributor in the samples NZ202 and NZ105, though no comment can be made on the other samples, which had low peak areas.

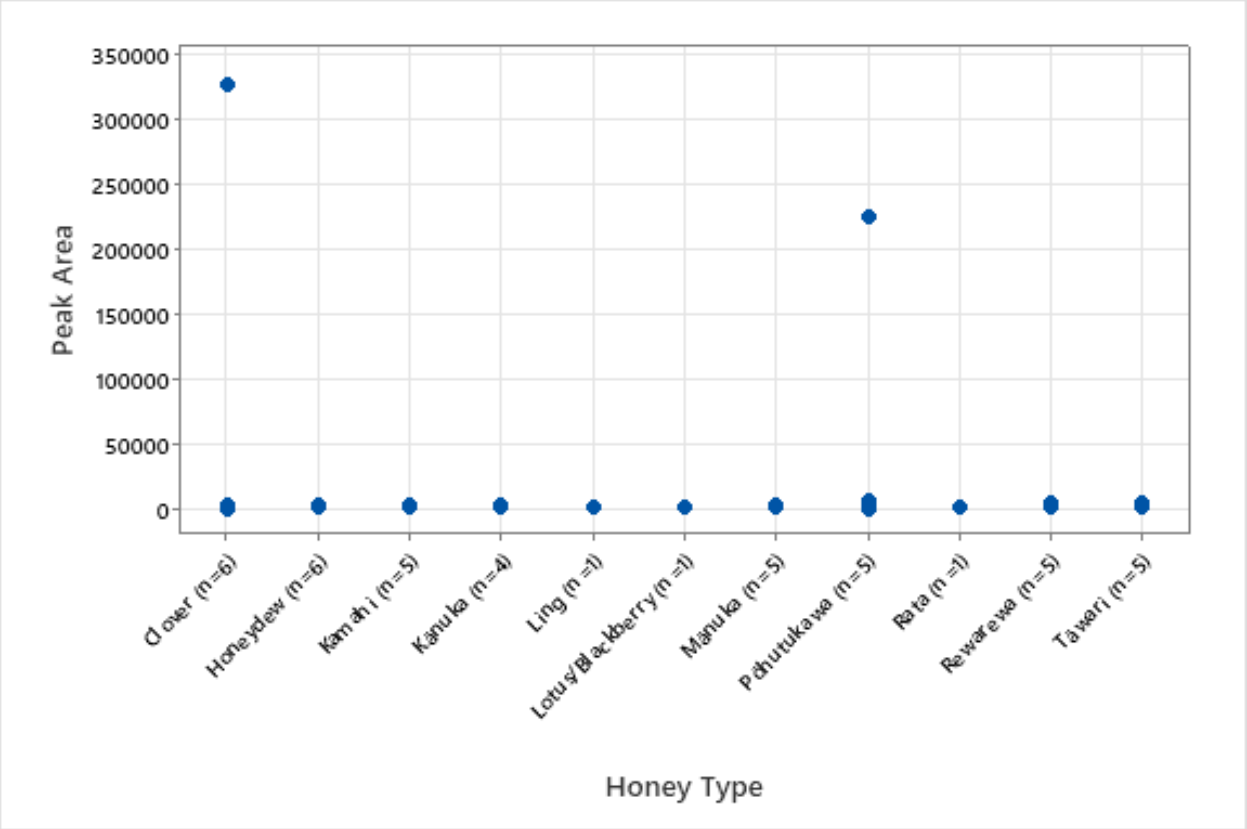

Figure 36. Individual value distribution plot of guaiacol peak area by honey type. One sample each of clover and pōhutukawa honeys had much higher peak areas than the rest of the samples.

5.4.Kāmahi honey

Kāmahi honey samples had comparable peak areas for most of the analysed compounds. The dominant compounds in kāmahi honey were 4-oxoisophorone, *o*-methoxyacetophenone, and D-limonene. They also had the highest average peak areas of all honey types for nonanal and 2-phenylethanol of all the honeys.

5.4.1. 4-oxoisophorone

The distinguishing feature was the high peak area of 4-oxoisophorone (Figure 30 (**m**)). The average peak area was 2370000 ± 2280000, which was one order of magnitude higher than kānuka and rātā honeys and two orders of magnitude higher than the other honey types (Figure 37). However, due to the high standard deviation of the peak areas reported, the 4-oxoisophorone concentration was not significantly different to that of any of the other honey types ($p>0.05$). All kāmahi honey samples contained high levels of 4-oxoisophorone except for NZ342. This was labelled as kāmahi but visual pollen analysis revealed that it was multifloral. Kāmahi was the dominant pollen type, however there were also significant contributions from rātā, lotus, and clover honeys. The peak area was comparable with that of

the rātā and clover honeys, so this explains why it had a lower level of 4-oxoisophorone than the other kāmahi honeys.

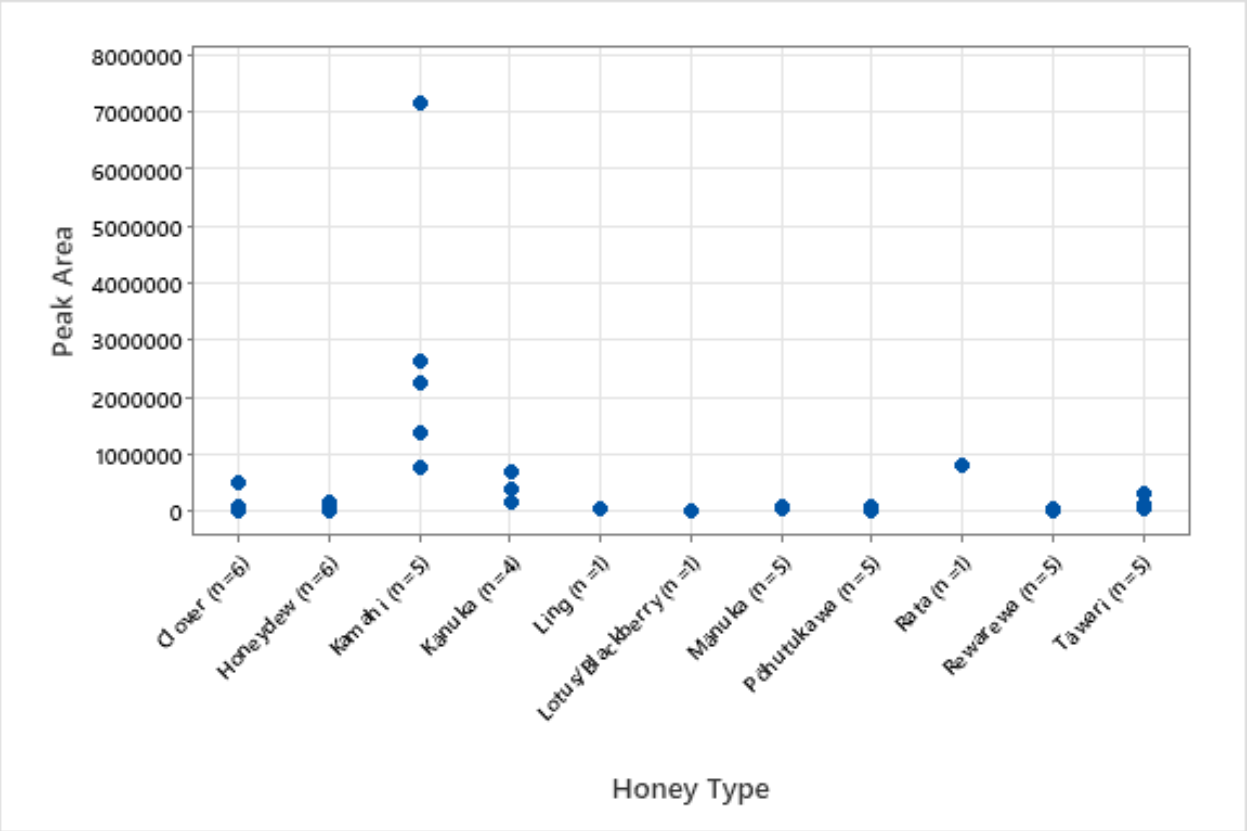

Figure 37. Individual value distribution plot of 4-oxoisophorone peak area by honey type.

4-oxoisophorone is a C_9 norisoprenoid. [110] These compounds are derived from carotenoids, the compounds responsible for the red, orange and yellow colours of many fruits and vegetables. For example, β-carotene is responsible for the orange colour of carrots. [184] 4-oxoisophorone has been identified in New Zealand heather honeys at an average concentration of 22 mg kg^{-1}, and in kāmahi honeys in lower levels (average concentration 4.9 mg kg^{-1}). [171] It has also been identified in Greek thyme honey with an average concentration of 236.7 µg kg^{-1}. [185] Spanish thyme honey has a lower concentration (29.7 µg kg^{-1}), whereas Spanish eucalyptus honey contained 178 µg kg^{-1}. [24] α-isophorone, β-isophorone and 4-oxoisophorone were identified in Sardinian strawberry tree honeys by dynamic headspace extraction and were proposed as marker compounds for this honey. [23] It is also found in heather, rosemary, citrus and eucalyptus honeys. [186] Karabagias (2018) suggested that total isophorone concentration (including α-isophorone, β-isophorone, 4-oxoisophorone and 2-hydroxyisophorone) in honey may be influenced by many factors, including botanical origin, extraction method, harvest year, moisture content, microbial conversion, or sunlight exposure

of the plants from which bees collect nectar or honeydew. [185] The honeys harvested during 2018 and 2019 displayed the highest peak areas compared to those from 2021.

The aroma of 4-oxoisophorone is “sweet, floral, honey, tea, woody and musty” and its odour threshold is 0.19 mg kg^{-1}. [180] The previously reported concentrations indicate that 4-oxoisophorone is not a common odour contributor in all honey types, but it may indeed be a contributor for heather, eucalyptus and kāmahi honeys previously reported in the literature. Based on the previously reported concentration in kāmahi honey by Senanayake (2006), and the large difference in peak area between kāmahi and the other honey types, it is likely that 4-oxoisophorone is a contributor to the aroma of the kāmahi honeys analysed.

The TIC chromatograms (Figure 38) of kāmahi honeys had large peaks for 4-oxoisophorone (24.7 min) and D-limonene (16.7 min), which reflected the high concentrations of these compounds. The average peak area for this compound in the SIM chromatogram was 293000 ± 343000 which was one of the highest for kāmahi honey. The RTs of the D-limonene peaks in both chromatograms match, and since the SIM method was created using RTs of the external standards, the identification of this peak is confirmed. Though *o*-methoxyacetophenone had the third highest peak area for kāmahi honey, a peak was not seen in the TIC chromatogram for this compound.

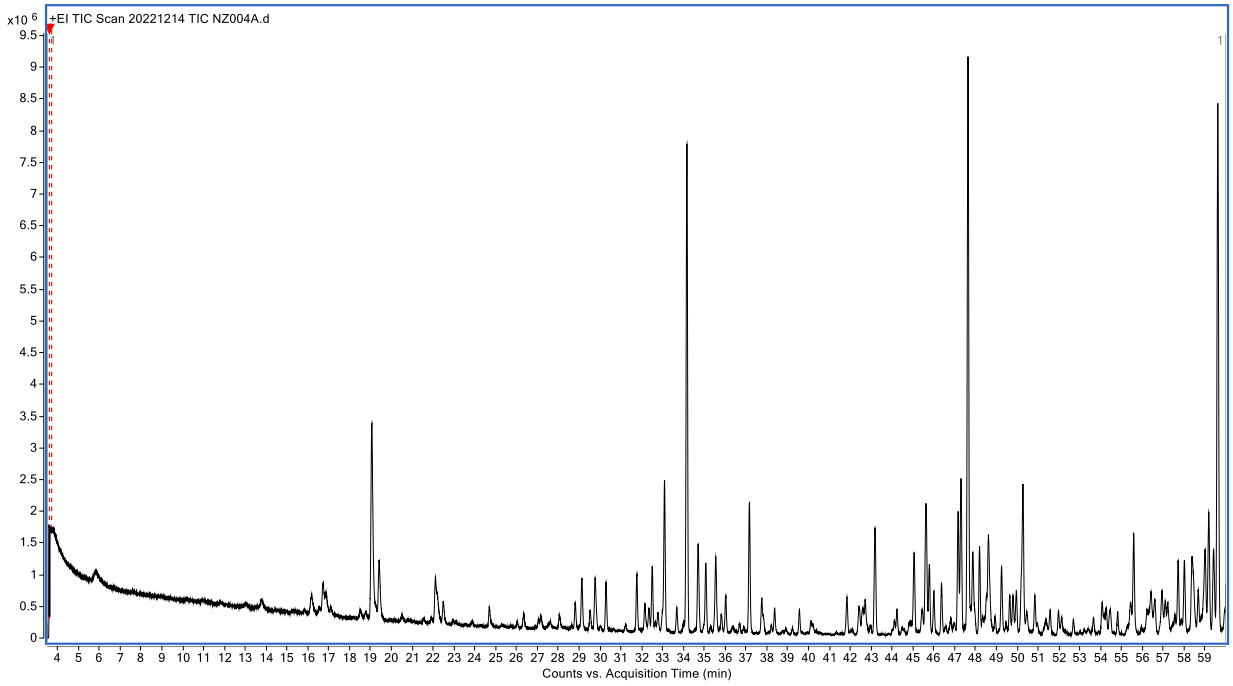

Figure 38. TIC chromatogram of a typical kāmahi honey (NZ004). Important peaks include D-limonene (16.7 min), 4-oxoisophorone (24.7 min), octanoic acid (27.6 min), hotrienol (28.1 min) and 3,5-bis(1,1-dimethylethyl)-phenol (48.5 min).

5.4.2. Nonanal

Kāmahi honey also had the highest average peak area for nonanal and 2-phenylethanol out of all the honey types. Linear aldehydes such as nonanal (Figure 30 (**k**)) are commonly reported in the volatile fraction of honey. Nonanal and decanal, for example, have been found in honeys of various floral sources and are reported as typical honey flavour compounds. [105] Nonanal has a low odour threshold at 0.17 μg kg^{-1} in air, and contributes a "fatty, floral" aroma owing to the long carbon chain. [74] Nonanal was reported as a strong aroma contributor in black mangrove honey, and was detected even at an FD of 1024. [53] It has also been identified as aroma-active in haze and astralagus honeys, with concentrations of 30-100 μg kg^{-1} typically reported [50,53] Here nonanal can also be reported as a strong contributor to the aroma of all the New Zealand honey types analysed, the average concentration for kāmahi honey being 15.8 mg kg^{-1}, much higher than those reported previously. The odour activity value can thus be calculated according to the following equation:

$$Odour\ activity\ value = \frac{concentration}{odour\ threshold}$$
$$= \frac{15.8\ mg\ kg^{-1}}{0.00017\ mg\ kg^{-1}}$$
$$= 92{,}941$$

Equation 4. Calculation of odour activity value (OAV).

This is an extremely high value – recall that an OAV > 1 signifies that a compound is aroma-active in a sample. Thus, nonanal has a very strong contribution to the aroma of kāmahi honeys. The fact that it was detected in all honey types was to be expected, as nonanal has previously been reported in beech honeydew, clover, kāmahi, mānuka, rātā, rewarewa, tāwari, thyme, viper's bugloss and pōhutukawa honeys. [79,91]

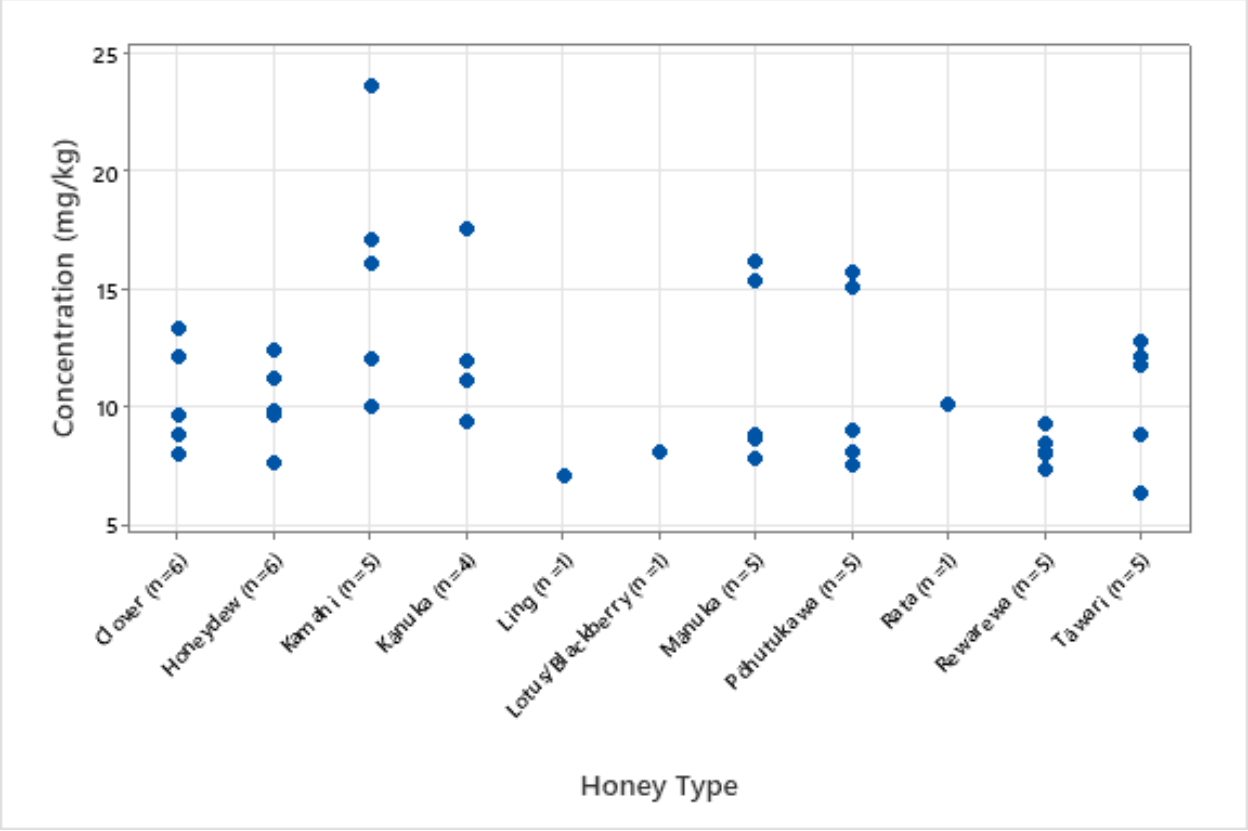

Figure 39. Individual value distribution plot of nonanal concentration by honey type.

5.4.3. 2-phenylethanol

Like benzaldehyde, 2-phenylethanol (Figure 30 (l)) is a benzene derivative that is widely reported in honey worldwide, regardless of botanical origin. The odour threshold for 2-phenylethanol varies between reports, with a value of 1 mg kg^{-1} being reported in water and

89 μg kg^{-1} reported in an aqueous fructose-glucose solution. [48,60] Castro-Vázquez *et al.* (2007) reported a mean concentration of 484 ppb in Spanish citrus honey, yet the compound was not considered aroma-active, lending the former value more credibility. However, FD factors of 16-64 have been reported in an extensive variety of honeys, indicating 2-phenylethanol contributes significantly to the aroma of these honeys with a floral and spicy aroma. [27,48-53,55,58] 2-phenylethanol has been identified in clover, honeydew, kāmahi, mānuka, pōhutukawa, southern rātā, rewarewa, tāwari, thyme, viper's bugloss, heather, nodding thistle, beech honeydew and kānuka honeys. [79,81,84,86,91,93] Concentrations of 5[187] to 5928[188] μg kg^{-1} have been reported internationally. In New Zealand honey, the range (59 to 900 μg kg^{-1}) is lower, so it is unknown if 2-phenylethanol is an aroma contributor. [81,91] Judging by the low peak areas displayed for 2-phenylethanol across all honey types, it is unlikely that the compound is aroma-active in the analysed honeys. Kāmahi honey did display a wider range of peak areas, however the maximum peak area was only 31000 in sample NZ011.

Overall, it is likely that the major aroma contributors for kāmahi honey were 4-oxoisophorone, ɒ-limonene and nonanal, considering the extremely high peak area of the former and the low odour thresholds of the latter two.

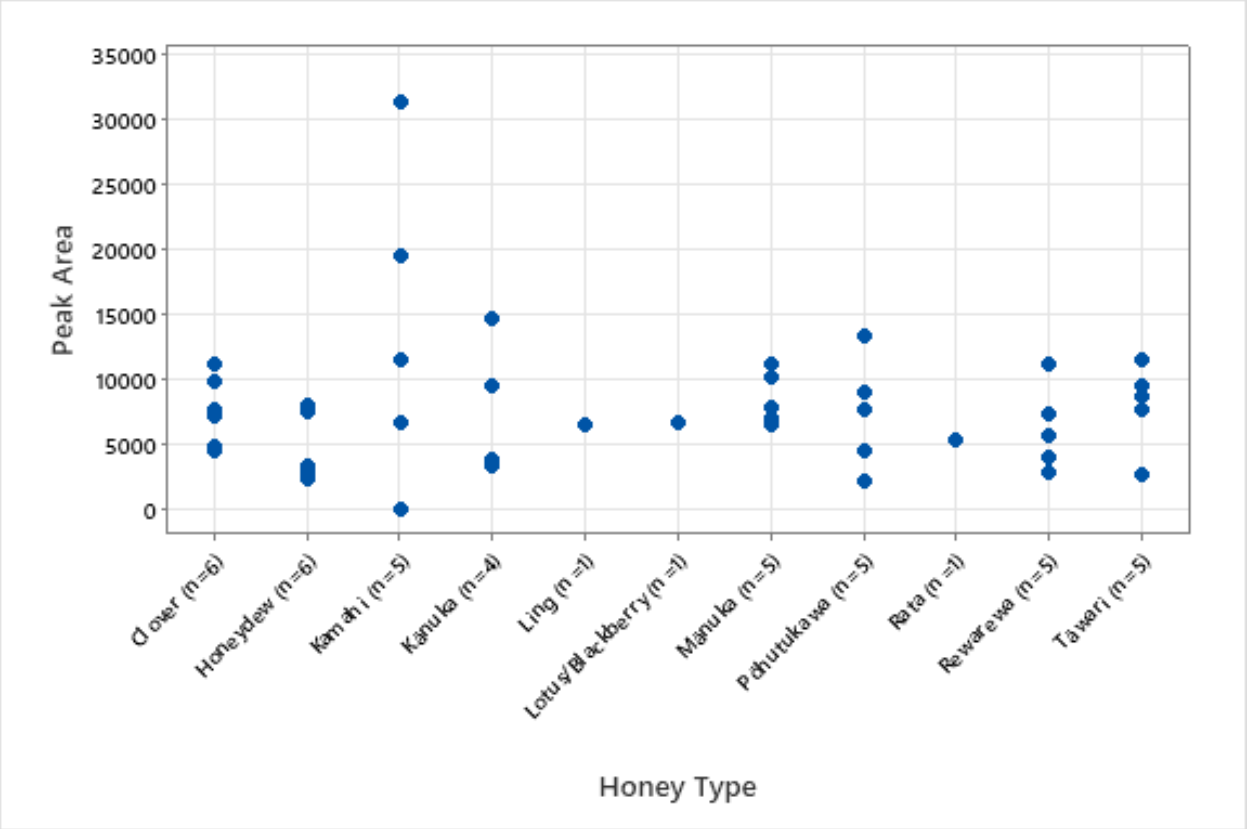

Figure 40. Individual value distribution plot of 2-phenylethanol peak area by honey type.

5.5. Kānuka honey

Although kānuka honey is similar to mānuka in many respects, and is difficult to distinguish by pollen analysis as discussed in section 5.2, the two parent plants are from different families, so it is not surprising that the volatile profiles of the samples analysed were quite different. While mānuka had higher average peak areas for benzaldehyde, β-pinene, *o*-methoxyacetophenone, eugenol, (*E*)-β-damascenone and β-ionone, kānuka honeys had around double the average peak area of mānuka for terpene derivatives D-limonene, cineole, linalool oxide and linalool, as well as the benzene derivative phenylacetaldehyde. The average peak area of 4-oxoisophorone was around 16 times higher than that of mānuka honeys, yet still much lower than kāmahi. The dominant peaks in the kānuka honey chromatograms were phenylacetaldehyde, 4-oxoisophorone, and D-limonene, while kānuka had the highest peak areas of all honey types for phenylacetaldehyde (Figure 41), linalool oxide and cineole.

5.5.1. Phenylacetaldehyde

The presence of phenylacetaldehyde (Figure 30 (g)) in honey has many contributing factors. It is partially a product of the botanical origin. [189] Phenylacetaldehyde is a dominant volatile

in cotton honey, and has also been identified in lavender honey. [109,127,155] It has been identified as aroma-active in a large range of honey types, including linden, acacia, heath, citrus, buckwheat, cloudberry-bog, lingonberry, sweet clover, willowherb, black mangrove, rape, raspberry, heather, alder buckthorn and astralagus, giving a honey-like, rosy, floral aroma. [11,27,39,48,49,51,53,55] This is largely due to its low odour threshold of 4 μg kg^{-1}. [190] This is the first instance that phenylacetaldehyde has been reported in kānuka honey, though it has been identified previously in beech honeydew, clover, kāmahi, mānuka, rātā, rewarewa, tāwari, thyme, viper's bugloss, heather and pōhutukawa honeys in New Zealand. [79,83,85,86,90,91]

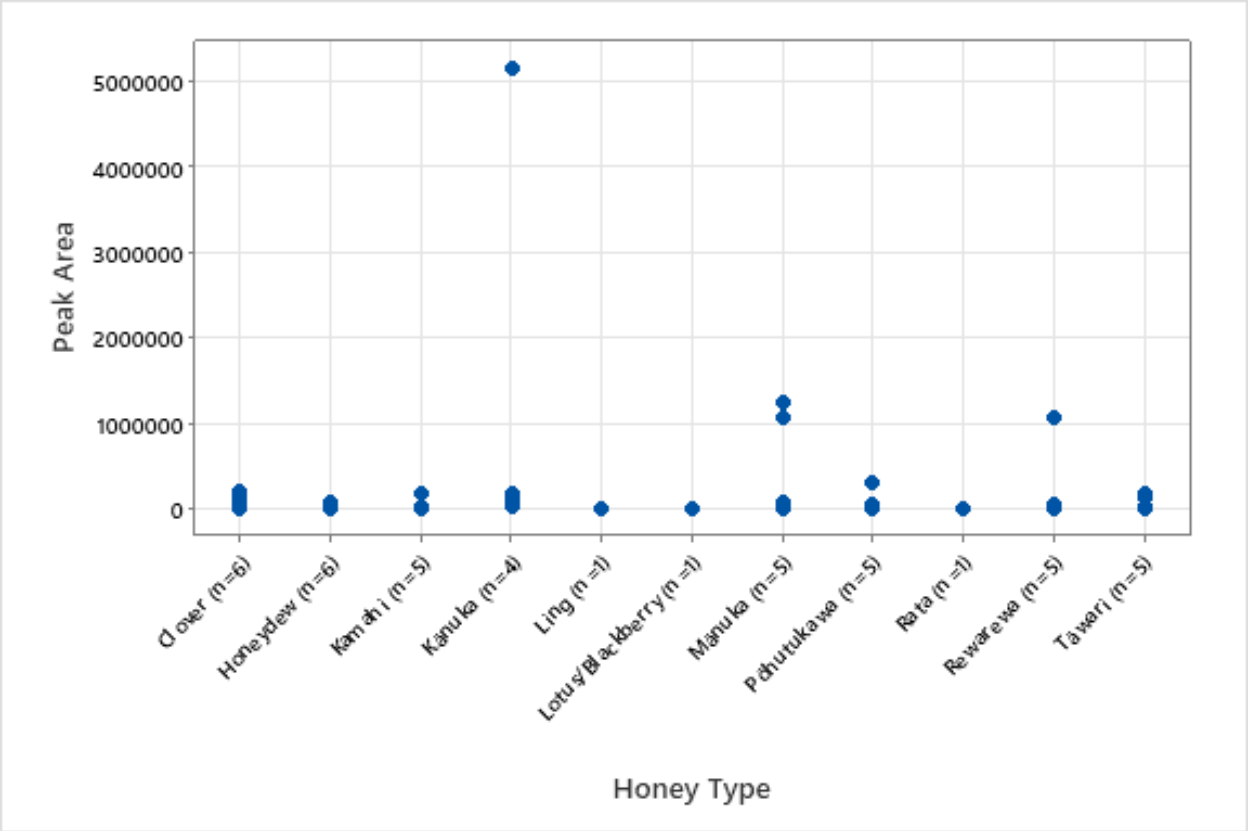

Figure 41. Individual value distribution plot of phenylacetaldehyde peak area by honey type.

Phenylacetaldehyde can also be produced in the honey from the amino acid phenylalanine, either by enzymes or Strecker degradation reaction (Figure 42).[121,191] Strecker degradation of amino acids to aromatic aldehydes occurs when the honey is subjected to increased temperatures or UV light. Beckmann *et al.* (2007) proposed that this reaction is likely to occur during heated headspace extraction of honey volatiles, emphasising the need for a room-temperature extraction. [191] Jerkovic *et al.* (2007) identified phenylacetaldehyde as a thermal artefact after extracting honey samples using hydrodistillation, supporting this idea. [121] However, this extraction was carried out at room temperature, and the samples were stored

in the fridge (4 °C) or freezer (−18 °C) when obtained and only removed for analysis, making Strecker degradation an unlikely cause of phenylacetaldehyde in the kānuka honey samples. Of course, the initial phenylalanine content would also affect the amount of phenylacetaldehyde in this way, so to be certain that Strecker degradation was not a contributing factor phenylalanine content would need to be analysed.

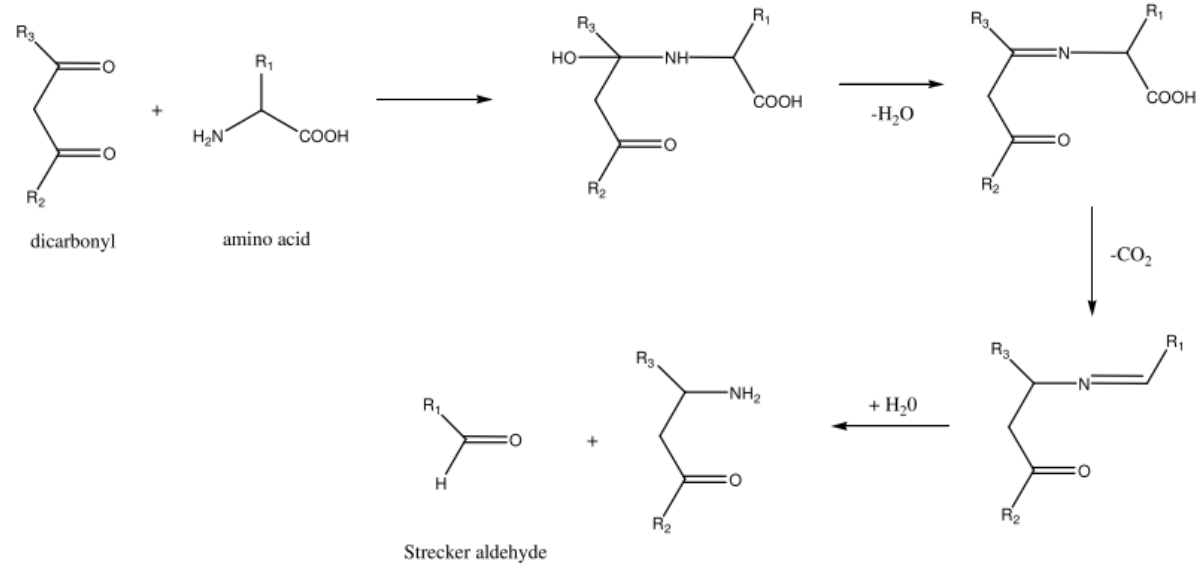

Figure 42. General scheme of Strecker degradation, one method by which phenylalanine is converted to phenylacetaldehyde in honey. ***Reproduced from Jerkovic et al. (2009)*** [137] ***(open access).***

Lastly, phenylacetaldehyde can be used as a bee repellent to aid in the harvesting of honey. [190,191] In this way, residues can end up in the honey, increasing the phenylacetaldehyde concentration. However, there is no evidence of the compound's use as a popular bee repellent in New Zealand, and if this were the cause, elevated peak areas would be expected in other honey types as well, which was not the case.

Although phenylacetaldehyde is present in honeys regardless of botanical origin, and there are many mechanisms in which it can become present in the honey, phenylalanine content does vary widely depending on the floral source. [192] This means that the floral source does have some impact on the phenylacetaldehyde content, but because the sources are often unclear, it should not be used as a marker compound.

5.5.2. Linalool oxide

Kānuka honeys also had the highest average peak concentration of linalool oxide (Figure 43). The linalool oxides (present in *cis*- and *trans*- isomers which could not be distinguished using GC-MS) are monoterpenes that are typically formed from linalool. A 2010 study by

Alissandrakis *et al.* found that the formation of linalool oxides was favoured upon addition of linalool to honey, catalysed by enzymes secreted by bees and possibly using 6,7-epoxylinalool as an intermediate. This supports the idea that linalool derivatives are eventually formed in honey from linalool. This reaction is affected by the acidity and heat in the hive, and also produces hotrienol, another monoterpene which was observed in the TIC chromatogram of kāmahi honey (Figure 38).

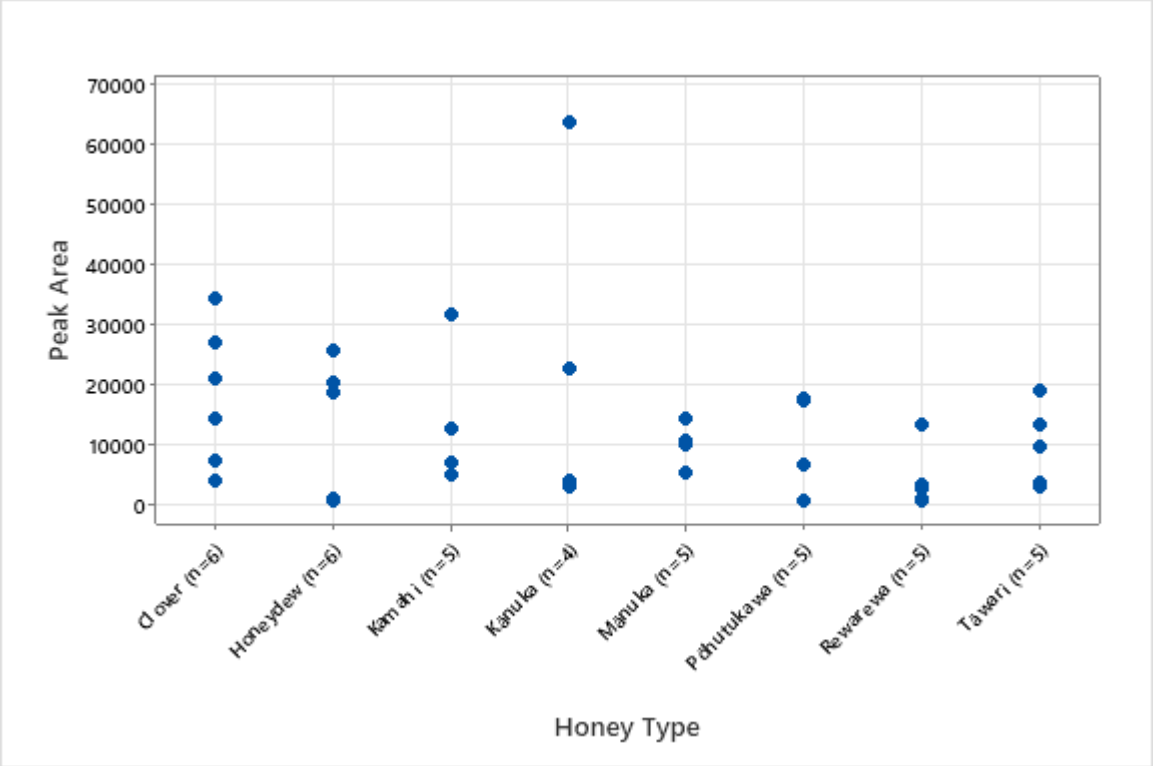

Figure 43. Individual value distribution plot of linalool oxide peak area by honey type.

As the kānuka honey had a high average peak area for linalool, it follows that the average peak area of linalool oxides would also be high. In fact, the kānuka samples with the highest linalool peak area (NZ334 and NZ376) had higher linalool oxide peak areas than the samples with lower linalool peak area (NZ277 and NZ283). A slight positive correlation (R^2 = 0.3810) was seen between the average peak areas for linalool and linalool oxide when all honey samples were considered (Figure 44). Of course, the fact that some linalool is used up in the conversion would affect this relationship; the longer the sample is stored, the further the reaction would progress so this figure should be approached critically.

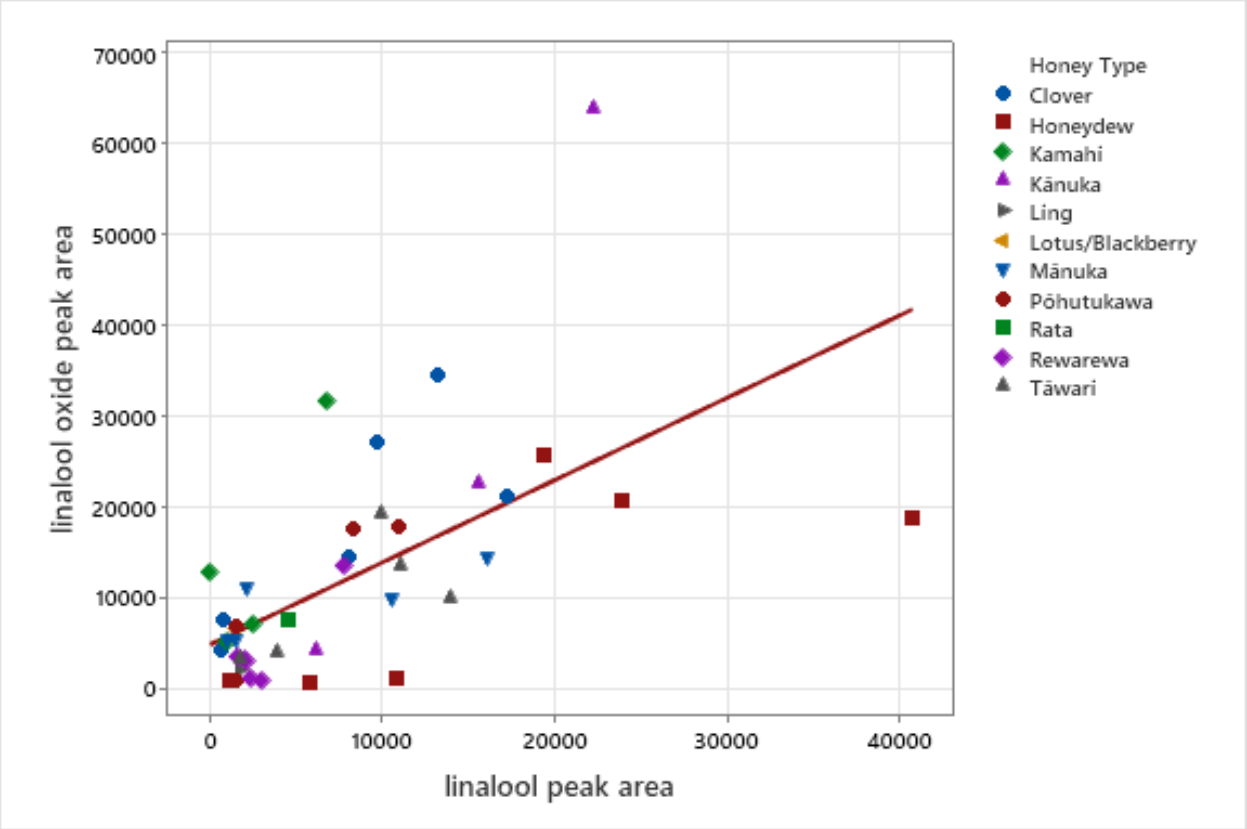

Figure 44. Scatter plot showing a slight positive correlation between the average peak areas for linalool and linalool oxide in all honey types. (R^2 = 0.3810, n=44).

Linalool oxides have a fresh, sweet and floral aroma and an odour threshold of 6 μg kg^{-1}. They have been identified as aroma-active in citrus, buckwheat, lingonberry, sweet clover, willowherb, [51] black mangrove, astralagus, cloudberry-bog, raspberry, rape, heather and alder buckthorn honeys. [11,29,39,49,51,53] In New Zealand, linalool oxides have already been identified in clover, kāmahi, mānuka, pōhutukawa, rātā, rewarewa, tāwari, thyme and viper's bugloss honeys. [79,90] Like phenylacetaldehyde, linalool oxides have not previously been reported in kānuka honey, so it is interesting that they have the highest average peak area of all the compounds monitored.

5.5.3. Cineole

Cineole (Figure 30 (**e**)) is another monoterpene compound with a very similar structure to D-limonene. Also known as eucalyptol, it is the main component of eucalyptus oil. As no studies have yet been carried out to determine the aroma-active compounds in eucalyptus honey, cineole has not been reported as aroma-active in any honeys to date. However, it has been identified as one of the dominant volatile compounds in lavender honey, [8,9] as well as being present in trace amounts in rape, robinia, fir tree, chestnut, linden, apple and orange honeys. [9,124] The odour threshold is 1.1 μg kg^{-1}. The average concentration of cineole in

kānuka honey is 1.47 mg kg^{-1} ± 1.33 mg kg^{-1}. Using Equation 4, the OAV is 1,336, indicating a strong contribution from cineole to the aroma of kānuka honey. OAVs were greater than 1 for clover, honeydew, and tāwari honeys as well. This is the first report of cineole in New Zealand honeys, and the first instance that cineole has been identified as aroma-active in honey.

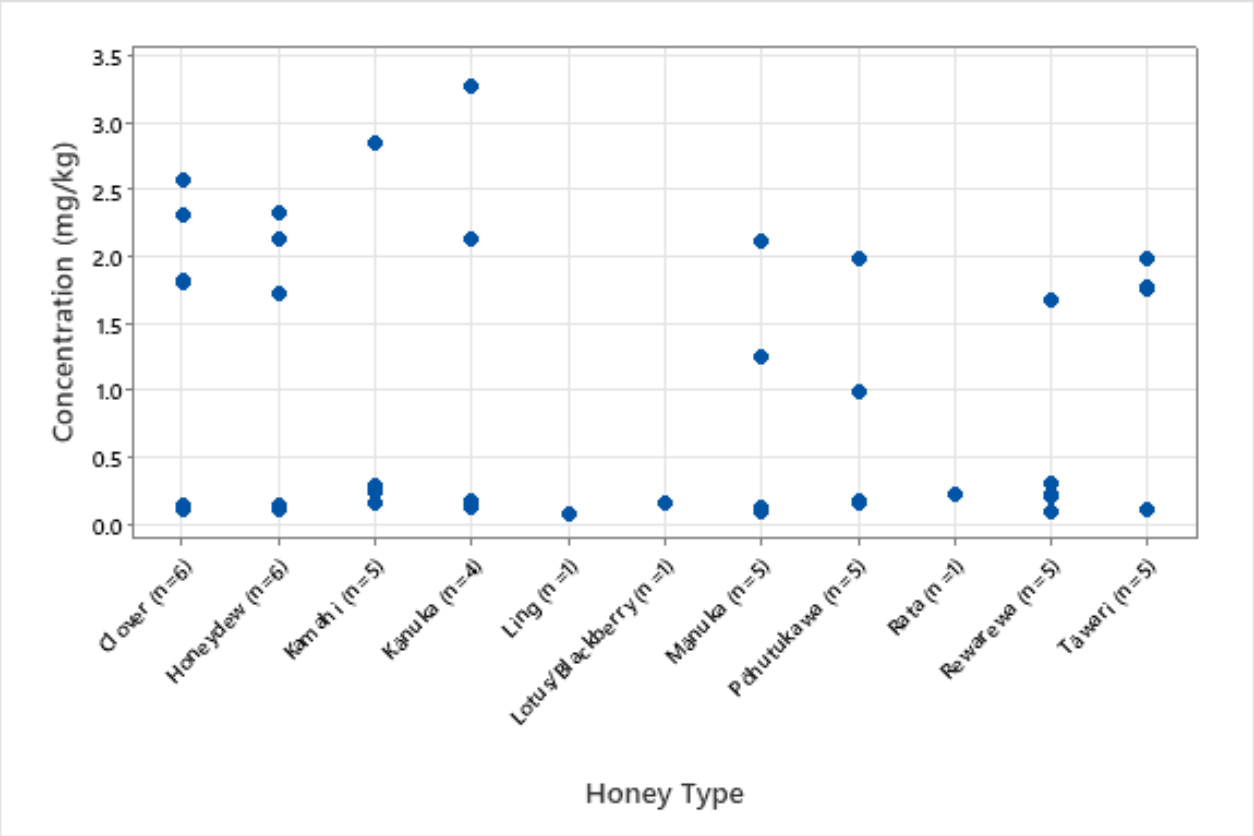

Figure 45. Individual value distribution plot of cineole concentration by honey type.

5.6. Pōhutukawa and rātā honeys

As pōhutukawa and rātā both belong to the *Metrosideros* genus, and usually show similarities in their volatile profiles (Chapter 1, section 1.4.3) so they are discussed together here. Five pōhutukawa samples were analysed, though only one rātā sample was included due to time constraints. Thus, conclusions cannot be drawn on the contributions of these compounds to all rātā honeys, but comments can be made.

The TIC chromatograms (Figure 46, Figure 47) of pōhutukawa and rātā honeys had similarities, but also showed marked differences in the peak areas of the SIM chromatograms. D-limonene was one of the dominant volatiles in both honey types, but while the pōhutukawa honeys had high average peak areas for *o*-methoxyacetophenone and phenylacetaldehyde, the

rātā honey sample had low peak areas for these compounds and was instead characterised by a high peak area for 4-oxoisophorone.

Though pōhutukawa honey did not display the highest average peak area for any of the volatiles, rātā honey displayed the highest peak areas for α-pinene, eugenol, (*E*)-β-damascenone and β-ionone of all honey types, while pōhutukawa honey did not have the highest peak area for any compounds. However, it is important to remember the sample size of one for rātā, so these results should be approached with caution. While individual samples from other honey types displayed higher peak areas than the rātā sample for each of these compounds, considerable spread in the results of those honey types reduced their averages. For consistency of reporting, these compounds will be discussed here, but the samples with higher peak areas for each compound are also mentioned.

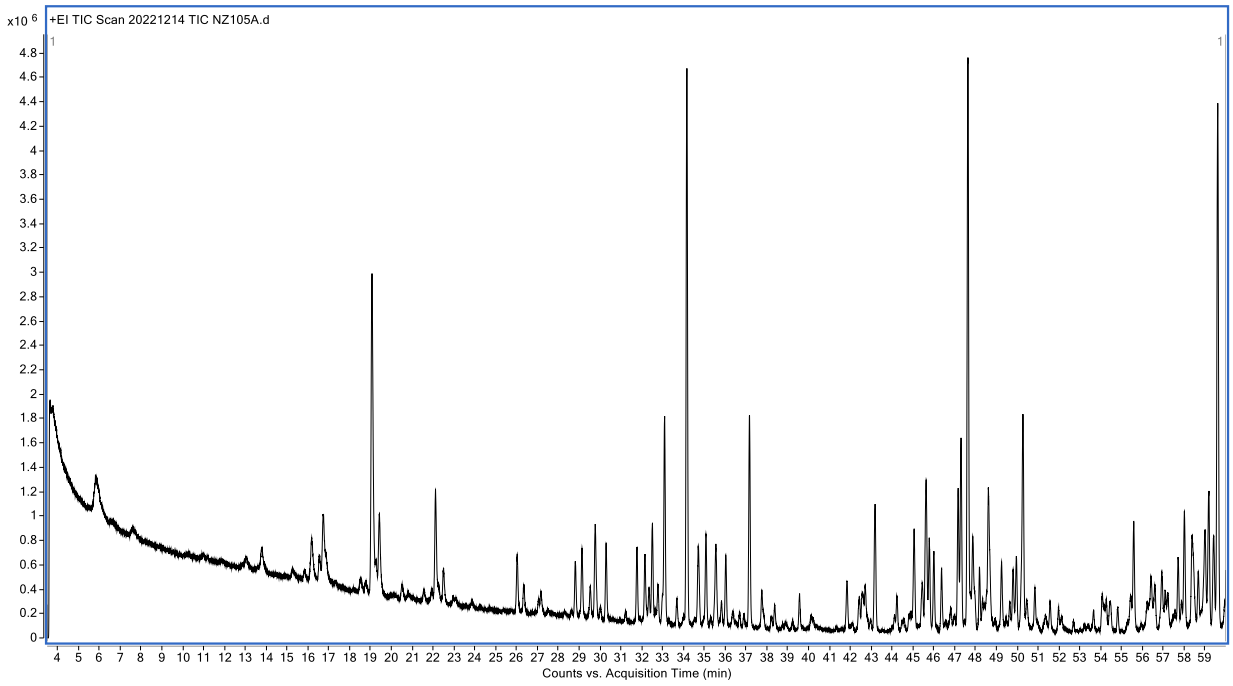

Figure 46. TIC chromatogram of a typical pōhutukawa honey (NZ105).

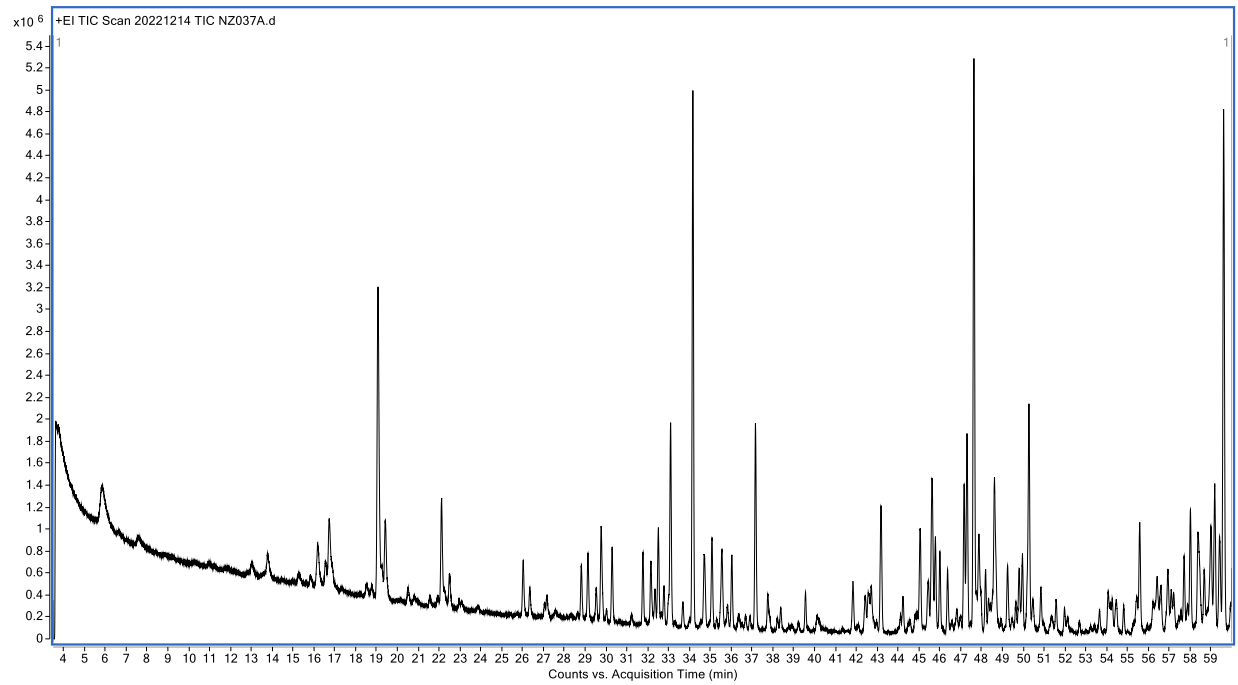

Figure 47. TIC chromatogram of rātā honey (NZ037).

5.6.1. Eugenol

Eugenol (Figure 30 (**o**)) is a derivative of guaiacol. It has been reported as a minor component of the volatile composition of international honeys, accounting for less than 1% of total peak area in most cases. [76,77,166] It is the major component of clove oil, and thus has a spicy, clove-like aroma. [27,48,49,51] In apple honeys, the peak area of eugenol was 2.5% of the total peak area, and was shown to be characteristic of the floral origin as it was found in apple flowers. [124] Clove trees are not typically grown in New Zealand and any contribution from apple nectar would be much lower, which may explain why the peak area of eugenol was so low in all the samples analysed. Clove oil may be present in some honeys since it is sometimes used by beekeepers to combat the *Varroa destructor* mite which threatens beehives. [9] Siegmund *et al.* (2018) reported likely contamination of eugenol from application to the hive in dandelion honey that was analysed in their study due to a much higher contribution compared other honey types, and the fact that eugenol had not previously been identified in dandelion honey. [9] It is possible that this contamination was a contributor to eugenol appearing in the New Zealand honeys, as the "high" amount reported by Siegmund *et al.* was still only 0.21% of total peak area.

Despite the low peak areas, eugenol is likely to have some contribution to the aroma of all the honeys analysed due to the low odour threshold of 1.1 μg kg^{-1}. [48] Moreira *et al.* (2010) detected eugenol in cashew honeys by GC-O even when it could not be detected by GC-MS,

indicating it contributes to honey aroma even when present in very low amounts. [58] NZ011 (kāmahi) had the highest peak area for eugenol, but NZ004 (kāmahi), NZ298 (mānuka), and NZ216 (rātā) also had similar amounts (Figure 48). A t-test was carried out at the 95% confidence level which determined the difference in eugenol peak area was not significant. Since the peak areas were low for all samples, any contribution from eugenol to the aroma of these honeys is probably similar.

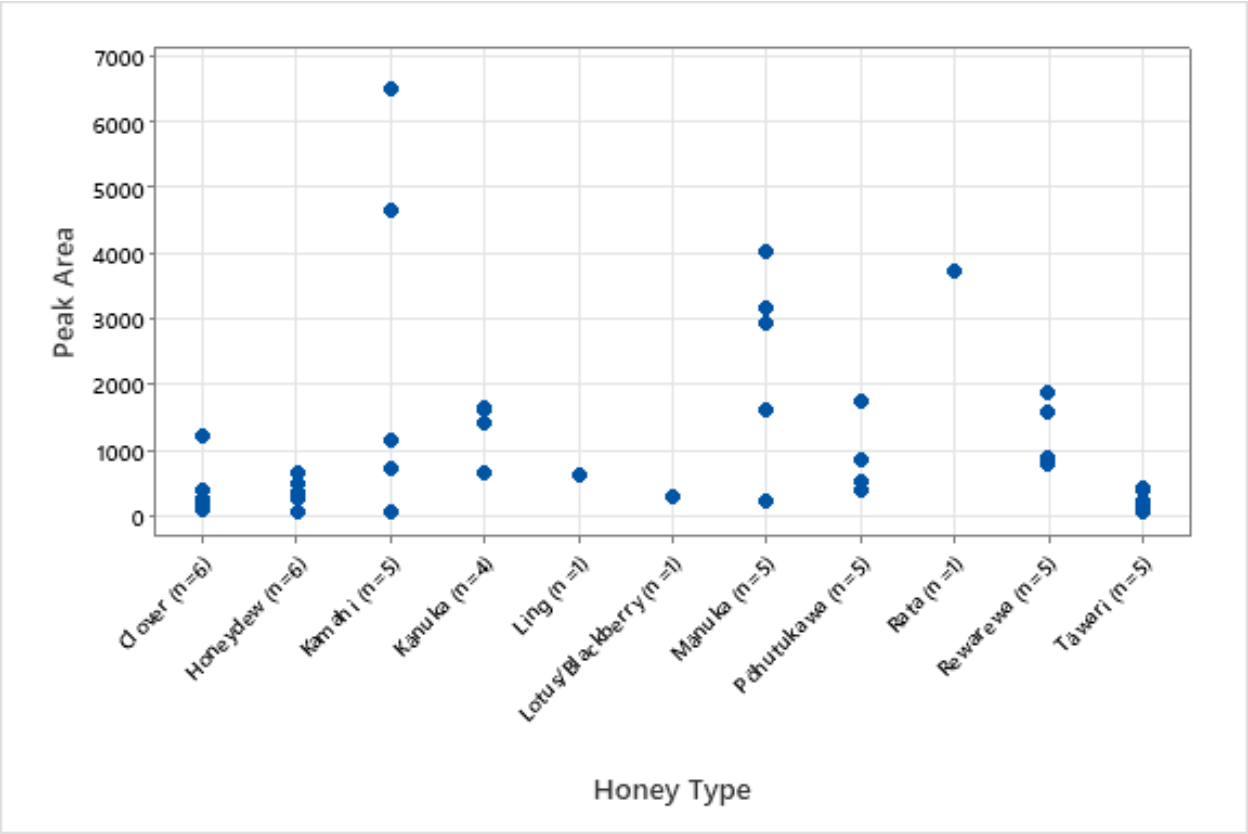

Figure 48. Individual value distribution plot of eugenol peak area by honey type.

5.6.2. Norisoprenoids: (E)-β-damascenone and β-ionone

(*E*)-β-damascenone (Figure 30 (**p**)) and β-ionone (Figure 30 (**q**)) are norisoprenoids, and are commonly known as rose ketones due to their significant contribution to the aroma of rose oil. [193] These compounds have the lowest odour thresholds of all the C_{13} norisoprenoids, at 2 ng kg^{-1} and 7 ng kg^{-1} for (*E*)-β-damascenone and β-ionone respectively. [54,104,194] (*E*)-β-damascenone has a fruity, sweet, apple-like aroma, which has also been described as honey-like. [27,39,51] Its extremely low odour threshold led to (*E*)-β-damascenone being identified as one of the key aroma contributors in most types of honey, with FDs of 1024 in linden and black mangrove honeys and 2048 in rape honey. [27,39,48] Its OAV in citrus honey has been reported at 575-1800, and in buckwheat honey as 2000-4808. [11,55]

Peak areas for (*E*)-β-damascenone were below 50,000 for most samples, other than the rātā honey and some samples from the clover, kāmahi and mānuka honeys (Figure 49). However, due to the low odour threshold of the compound, which is far below the detection limit of this method, it can be assumed that (*E*)-β-damascenone has significant contribution to the aroma of all honeys analysed.

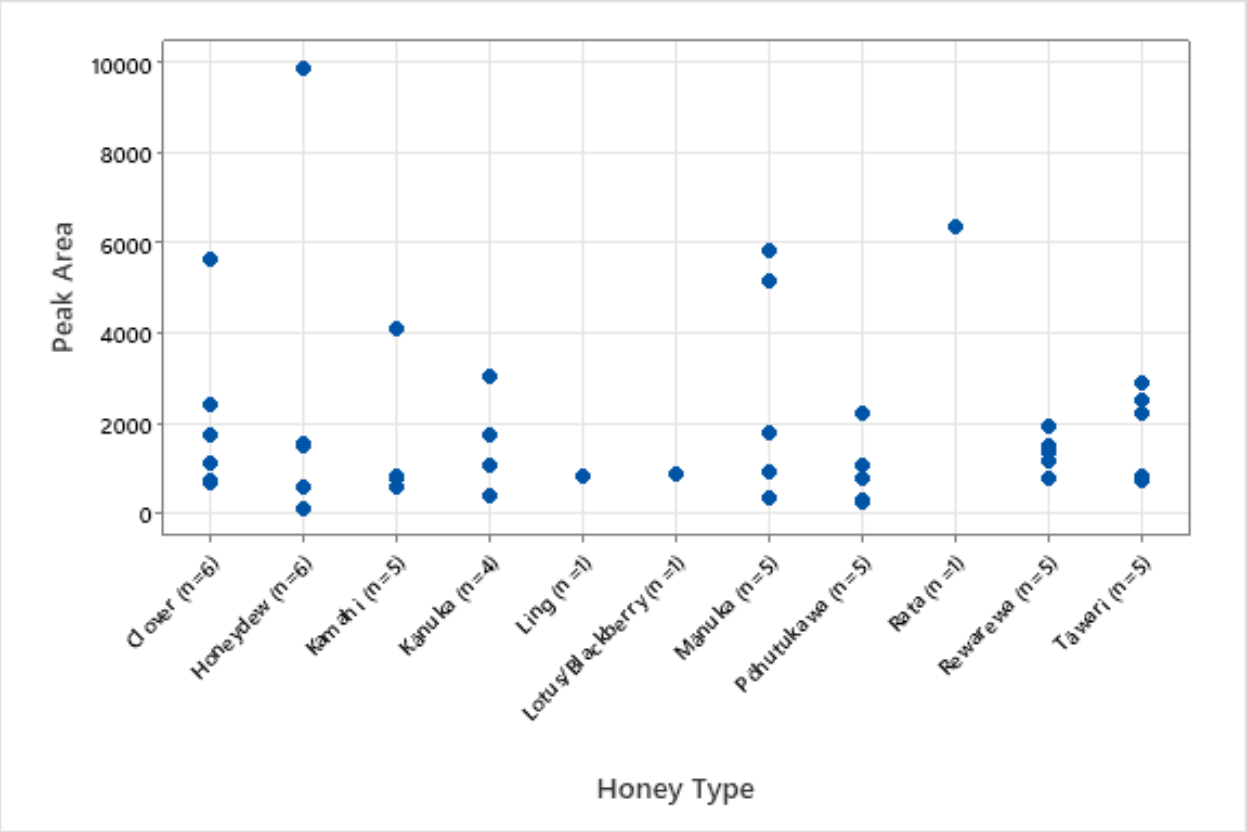

Figure 49. Individual value distribution plot of (*E*)-β-damascenone peak area by honey type.

Though β-ionone has a similarly low odour threshold to (*E*)-β-damascenone, it has not been reported as aroma-active in any honey type to date, because it has rarely been reported as a honey constituent at all. Mannaş and Altuğ (2007) reported β-ionone in thyme honey, making up 1% of peak area, and Yildiz *et al.* (2022) recently identified it in rhododendron, chestnut, astralagus, chaste tree, oak and pine honeys, though all were below 0.5% of total peak area.[26,105] This is the first report of β-ionone in New Zealand honey, and it was present in all honey types analysed (Figure 50). The peak area was very low for all samples, with a honeydew honey (NZ372) having the highest peak area. However, due to the low odour threshold, it is likely that β-ionone contributes its honey-like aroma to at least this sample, if not all the samples analysed.

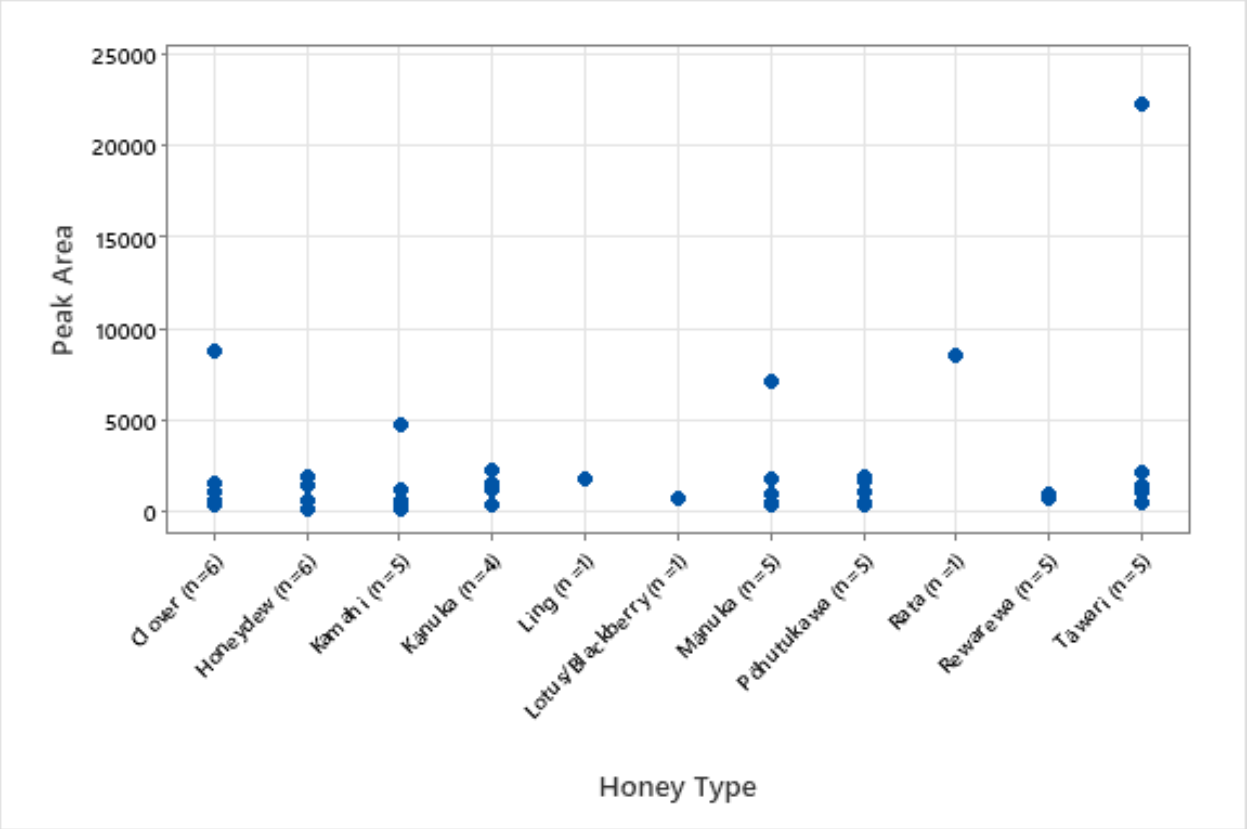

Figure 50. Individual value distribution plot of β-ionone peak area by honey type.

Of the pōhutukawa honeys, NZ248 and NZ276 had higher concentrations than the other samples for most compounds. Unfortunately, no data was available on the harvest year and location for NZ276, but NZ248 was the latest in a series of samples collected from the Bay of Plenty. NZ246, NZ247 and NZ248 were collected in 2013, 2014 and 2015 respectively. NZ246 and NZ247 had similar peak areas for all compounds, while the peak areas for NZ248 were vastly increased. Interestingly, the sample NZ105 was also harvested in the Bay of Plenty in 2015 and did not display the same elevated peak areas for any compounds except linalool oxide (isomer 1). Pollen counts would be useful to help understand the dramatic increase in total volatiles between 2014 and 2015, however it is unlikely that there would be much change in the nectar contributions of honeys harvested at the same site unless a large amount of planting occurred.

5.7. Tāwari and rewarewa honey

Tāwari and rewarewa honeys produced similar chromatograms, possibly on the basis that they are both forest honeys. Both floral types are under-represented in pollen count analysis and may contain significant contributions from other honey types. Two of the tāwari samples (NZ287 and NZ364) were sent for visual pollen analysis and confirmed as monofloral tāwari.

NZ287 showed the greatest pollen contribution from clover, mānuka/kānuka and lotus, while clover and rose pollen were found in NZ364. Tāwari honey produced unremarkable chromatograms in TIC (Figure 51) and SIM, and peak areas for all compounds were lower than other honey types. The SIM chromatogram was dominated by D-limonene, phenylacetaldehyde and 4-oxoisophorone, although it must be reiterated that the peak area of the compound does not necessarily reflect the concentration, which depends on the sensitivity of the detector to each compound. Goss (2009) also reported that tāwari honey is low in extractable organic compounds, and that organic acids such as 4-methoxybenzoic acid dominated the GC-MS profile. [195] These acids were not detected in this method as no derivatisation was used.

Tāwari honey is described as tasting of rosehip syrup, so it can be expected to have significant contributions from the rose ketones (*E*)-β-damascenone and β-ionone described above (section 5.6). One sample of tāwari honey (NZ323) displayed the highest peak area for β-ionone, suggesting a significant contribution from this compound to the aroma of the honey.

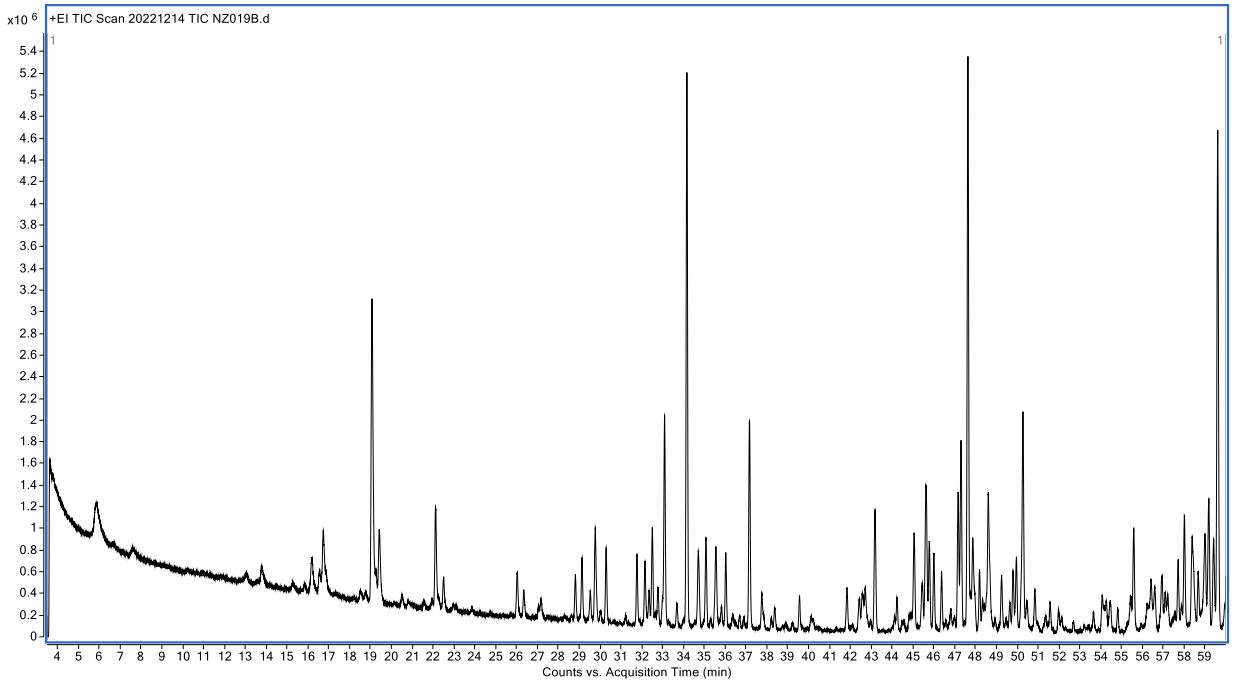

Figure 51. TIC chromatogram of a typical tāwari honey (NZ037).

5.7.1. Benzyl alcohol

Rewarewa honeys also displayed low total volatiles by peak area, and an unremarkable TIC chromatogram (Figure 52). Like tāwari, rewarewa honey chromatograms were dominated by D-limonene, phenylacetaldehyde and 4-oxoisophorone. Rewarewa honey had the highest

average peak area for benzyl alcohol (Figure 30 (**f**)), although this was low in all samples analysed. The slope of the calibration curve for benzyl alcohol was comparable to most other compounds, so the low peak areas reported suggest low concentrations for all honey types. Benzyl alcohol has been identified in all New Zealand honeys to date at high concentrations (100[82,84] to 300[84] μg kg^{-1}). Even in these cases, benzyl alcohol was unlikely to contribute to the honey aroma. Its odour threshold is 625 μg kg^{-1}, which has resulted in it only being reported as an aroma contributor for citrus, cashew and cambará honeys internationally.[48,52,53,58] In the case of the samples analysed here, the peak area was very low, with the highest peak area coming from the rewarewa sample NZ233. Combined with the high odour threshold, it is unlikely that benzyl alcohol contributed to the aroma of any of the honeys analysed.

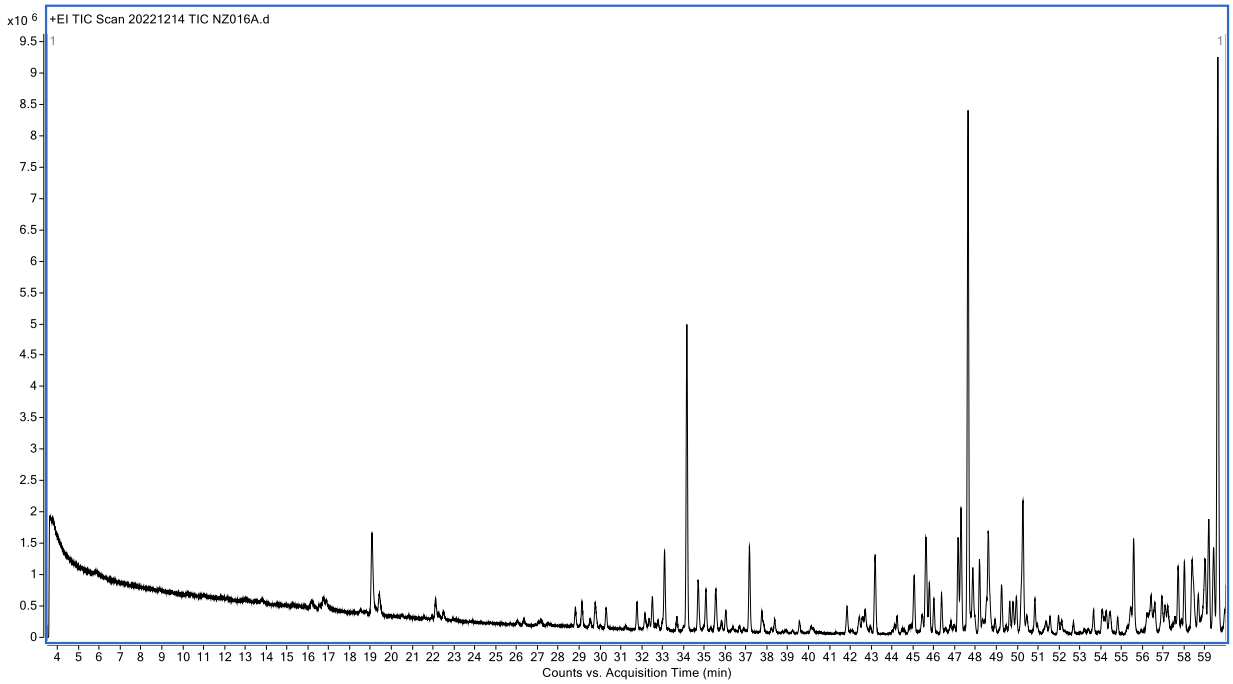

Figure 52. TIC chromatogram of a typical rewarewa honey (NZ016).

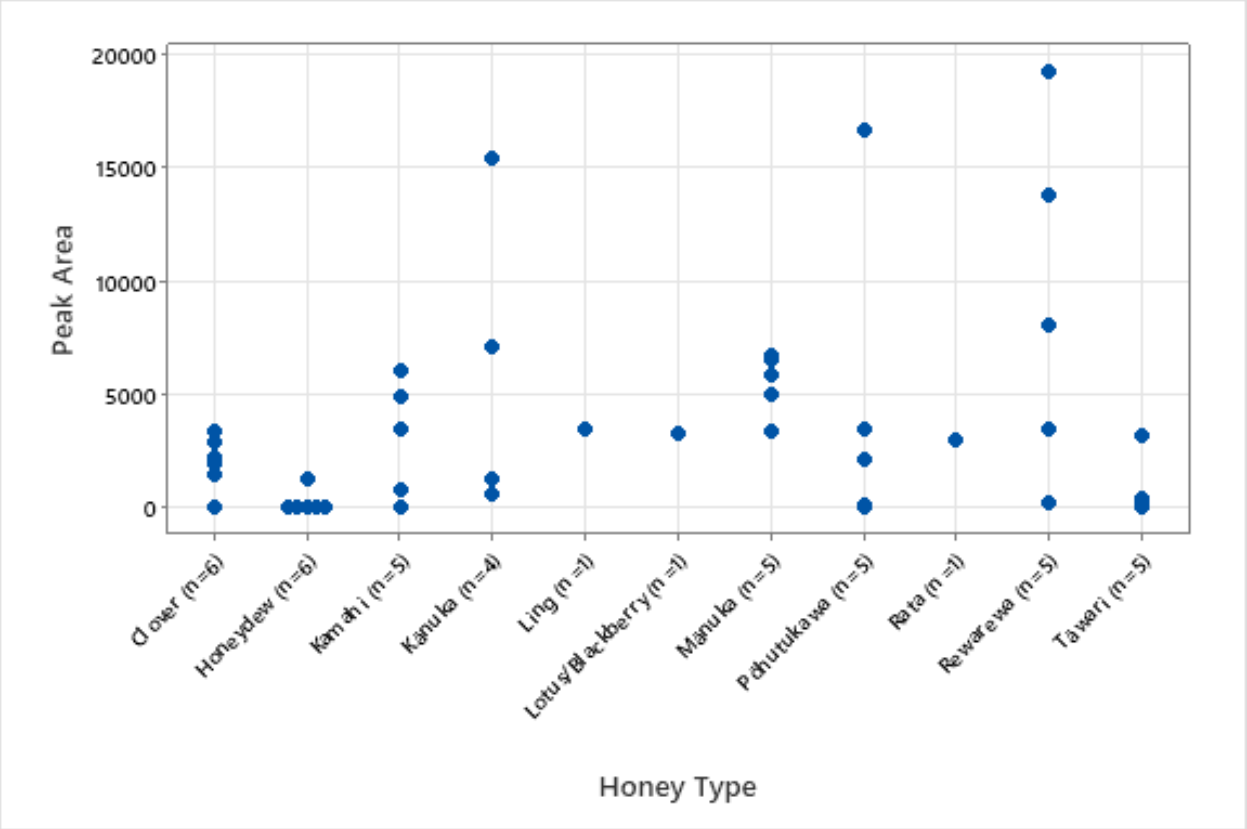

Figure 53. Individual value distribution plot of benzyl alcohol peak area by honey type.

5.8. Honeydew honey

Six honeydew honey samples were analysed, and though the TIC chromatogram was similar to the bush honeys with no distinctive peaks, the SIM chromatograms showed some interesting features. Honeydew honeys had the highest average peak area for linalool, with an average of 16900 ± 13300. The sample NZ360, harvested in North Canterbury in 2021, had the highest peak area (Figure 54).

5.8.1. Linalool

Linalool is a common monoterpene, derived from geranyl pyrophosphate. [104] It is present in over 200 species of plant, which means its presence in honey does not point towards a specific floral source. Indeed, linalool has been identified as an aroma-active component in linden, acacia, citrus, raspberry, rape, alder buckthorn, haze and astralagus honeys internationally, as well as clover, kāmahi, [79,91] mānuka, rātā, rewarewa, tāwari, thyme, viper's bugloss and pōhutukawa honeys in New Zealand. [11,27,29,49,50,53,79,91] It has a floral aroma, and an odour threshold of 6 µg kg^{-1}. [72] As discussed in section 5.5, linalool is converted into numerous derivatives during honey storage such as linalool oxides, hotrienol, lilac aldehydes, lilac alcohols, and 8-hydroxylinalool. If the peak areas for linalool oxide (section 5.5.2,

Figure 43) are considered for the honeydew honeys, and the relationship between linalool and linalool oxide peak areas (section 5.5.2, Figure 44), some suggestions can be made. Honeydew honeys typically fall beneath the regression line in Figure 44, indicating that the ratio of linalool oxide to linalool is lower than the average across all honey types. Additionally, in kānuka honey where linalool was likely being converted to the linalool oxides, peak area for both compounds was reasonably high. In the case of the honeydew samples, linalool peak areas were high, but linalool oxide peak areas were low, suggesting that the conversion occurring in kānuka honeys did not occur in the honeydew honeys to the same extent.

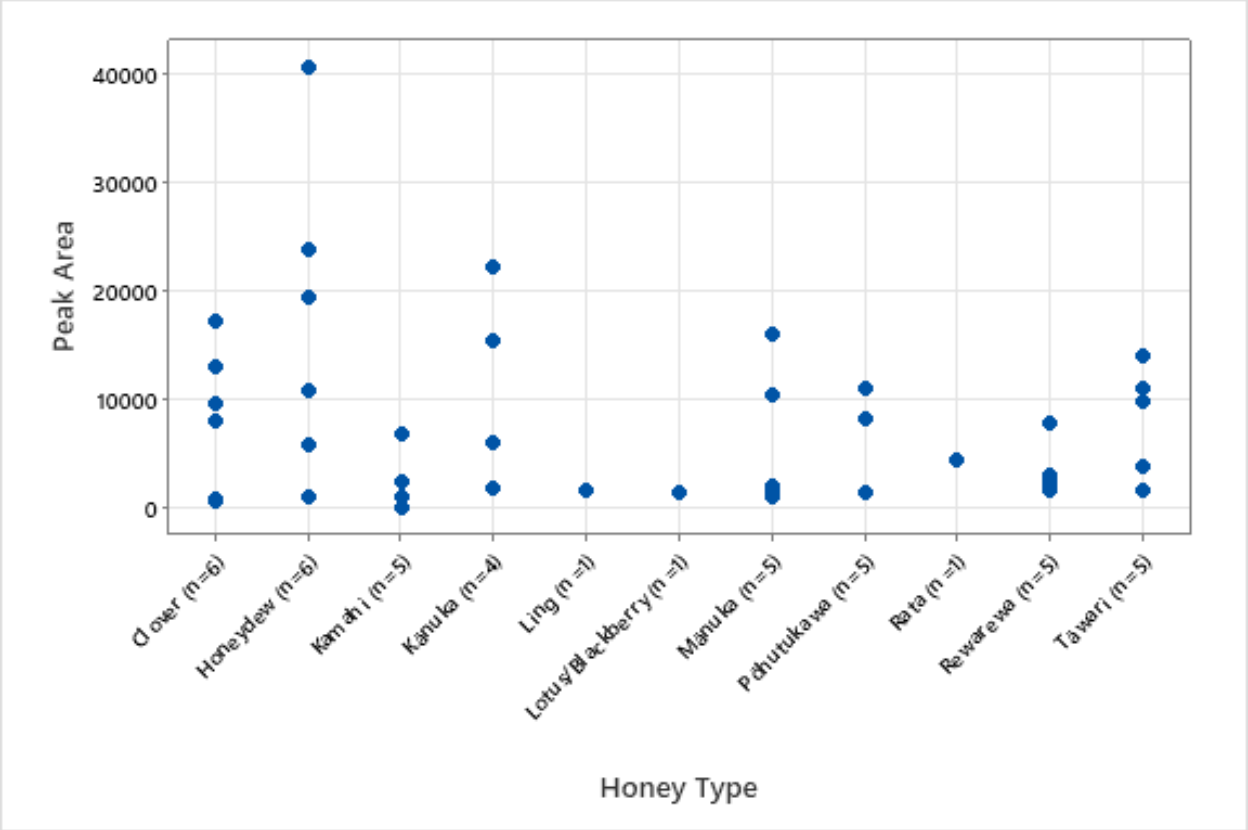

Figure 54. Individual value distribution plot for linalool peak area by honey type.

Revell (2014) also reported linalool in New Zealand honeydew honeys, whereas internationally, the derivatives were more common. Linalool oxides, hotrienol and epoxylinalool characterise Croatian honeydew honey; [196] Spanish; [197] and Polish honeydew honeys. [198] This difference may be due to the geographic origin or the botanical origin of the honeydew, and further research is required to understand why linalool does not appear to be converted to its derivatives in New Zealand beech honeydew honeys.

5.9. Other honeys

One sample each of a New Zealand ling (heather) honey and a lotus/blackberry blend were analysed. Both these honeys displayed low amounts of the volatile compounds analysed, with the lotus/blackberry blend having the lowest peak areas of all honey types for α-pinene, benzaldehyde, phenylacetaldehyde, 4-oxoisophorone, *o*-methoxyacetophenone, eugenol and β-ionone, and the ling honey having the lowest peak areas for D-limonene, cineole, linalool oxide, nonanal and (*E*)-β-damascenone. There were also no characteristic peaks present in the TIC chromatograms of these two samples (Figure 55, Figure 56).

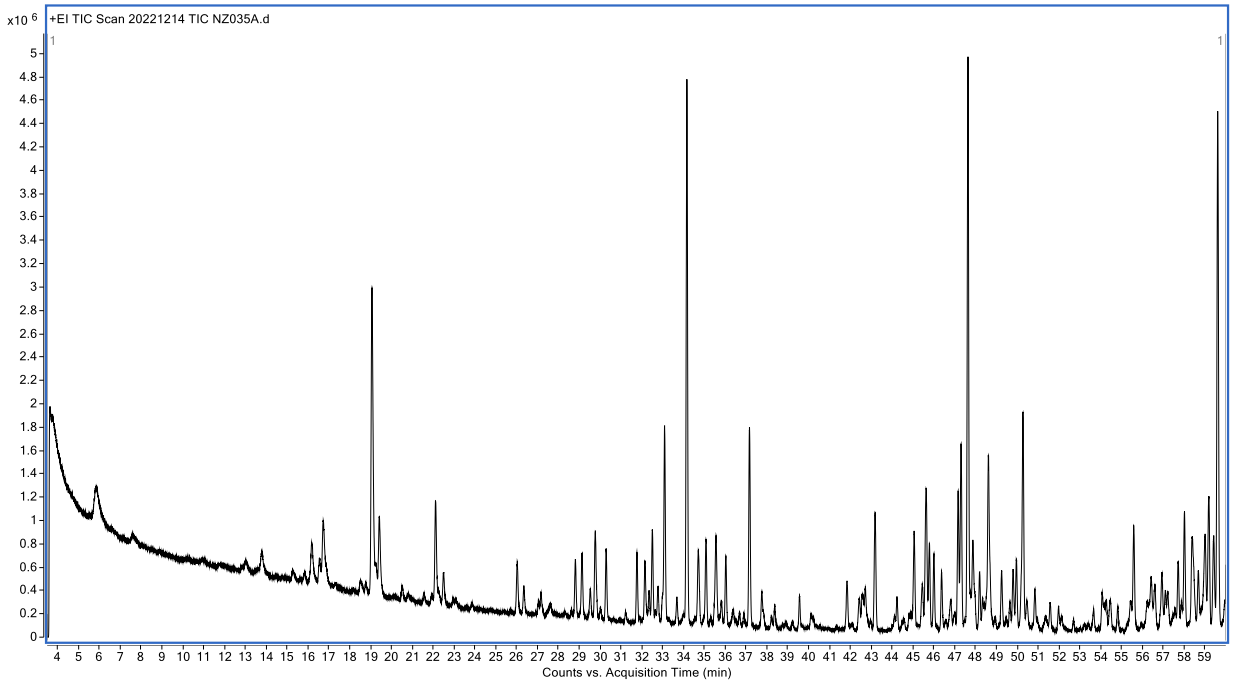

Figure 55. TIC chromatogram of the New Zealand ling honey sample (NZ035).

The dominant peaks in the ling honey SIM chromatogram were 4-oxoisophorone, D-limonene, and phenylacetaldehyde. The presence of 4-oxoisophorone is not surprising, given that New Zealand ling honey is typically characterised by the presence of norisoprenoids (see Chapter 1, section 1.4.10). The presence of β-ionone and (*E*)-β-damascenone in high amounts was not expected, even though these are also norisoprenoids, as β-ionone has not been reported in New Zealand honey before and (*E*)-β-damascenone has not been reported in New Zealand ling honey, though it has in international heather honeys. [109] The presence of phenylacetaldehyde is also expected, as it has been reported internationally in heather honey recently. [110]

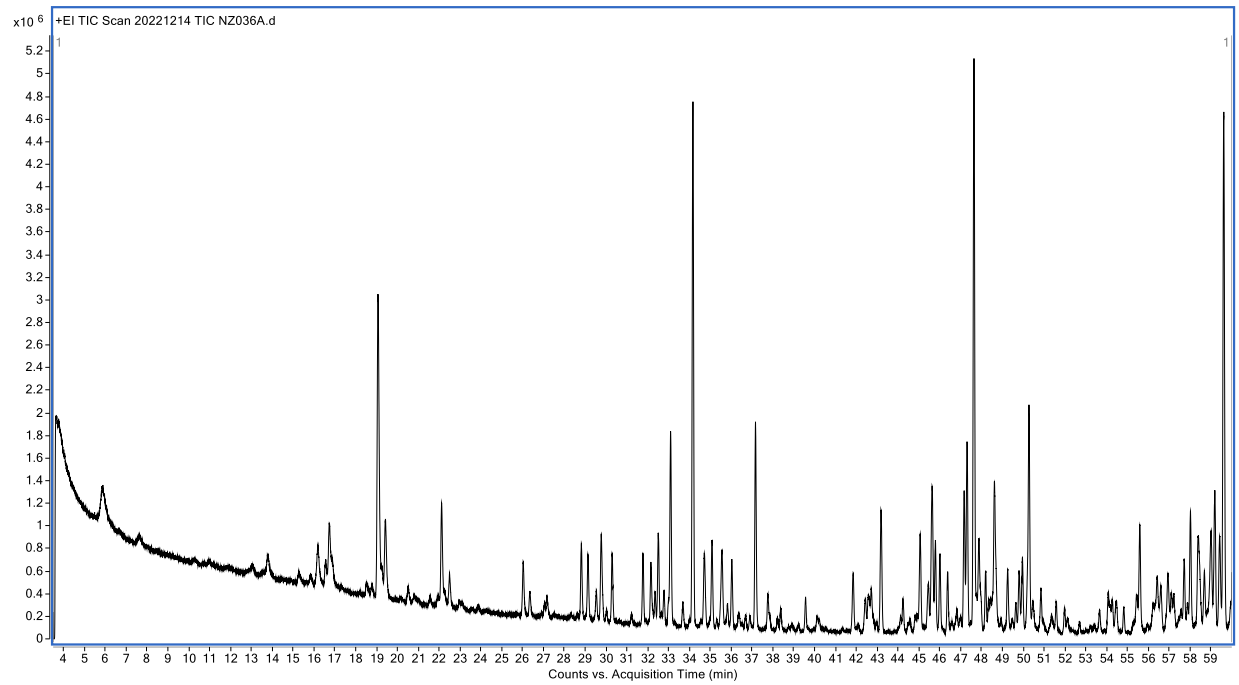

Figure 56. TIC chromatogram of the New Zealand lotus/blackberry honey sample (NZ036).

The SIM chromatogram of the lotus/blackberry honey sample was characterised by D-limonene, 4-oxoisophorone and nonanal, so it was quite similar to the ling sample. Unfortunately, no studies have been published on the volatile profiles of either lotus or blackberry honeys as they are not commonly produced. However, Choi (2017) reported the presence of α- and β-pinene, nonanal and isoeugenol in various parts of the lotus plant. [199] An earlier study on the volatile composition of blackberries also identified nonanal as one of the dominant compounds, as well as *n*-alcohols including 2-heptanol, 1-hexanol and 1-octanol. [200] Thus, it seems the presence of nonanal in this honey originated from the floral sources. D-limonene and 4-oxoisophorone were common among all the honeys analysed, and they do not appear to be characteristic of the floral sources in this case.

5.10. Summary of findings

Most of the honeys analysed had features in their volatile profiles that were characteristic of the floral source. Mānuka honey was defined by the high peak areas of *o*-methoxyacetophenone, and also had the highest peak areas of all honey types for benzaldehyde and β-ionone. It is likely that *o*-methoxyacetophenone and benzaldehyde contributed to the earthy, mineral aroma of mānuka honey. Meanwhile, kāmahi honey was characterised by high peak areas for 4-oxoisophorone, and the low odour threshold of this compound suggests that it contributes to the aroma threshold of the samples analysed. Nonanal was also determined as an aroma contributor to kamahi honey, with an OAV of

92,941. Though clover honeys typically had low peak areas for most compounds, D-limonene and guaiacol were suggested as aroma contributors for at least some of the clover samples. Kānuka honey had the highest peak areas for phenylacetaldehyde, and several suggestions were discussed towards the origin of this compound in the honey samples. Cineole was also confirmed as an aroma contributor for kānuka honey, with an OAV of 1,336. Rātā honey displayed the highest peak areas for α-pinene, eugenol, and the norisoprenoids (*E*)-β-damascenone and β-ionone.

Some honeys had less distinctive volatile profiles. Pōhutukawa, ling and lotus/blackberry samples did not have the highest peak areas for any compounds, and their chromatograms did not show any unique features. Tāwari and rewarewa honeys displayed similar chromatograms, likely owing to the nectar contributions from other floral sources. However, links between linalool and linalool oxide peak areas were explored with regards to kānuka and honeydew honeys, suggesting the possibility that linalool is not converted to linalool oxide in honeydew honeys to the same extent that it is in nectar honeys.

5.11. Chemometrics

Statistical analysis was carried out to investigate relationships between the volatile profiles and honey type. This combination of analytical chemistry and multivariate statistics is called chemometrics. [201]

5.11.1. Principal component analysis

Principal component analysis (PCA) is an unsupervised machine learning technique that uses pattern recognition to explain the variance of variables within a large dataset. [125] In this study, PCA was used to explain the variation between honey samples based on the peak areas of the 18 volatiles analysed. The grouping of the samples (honey type) is not used in the classification, making it an *unsupervised* technique. The output of PCA is a set of equations called principal components, which are combinations of the variables (volatile compounds). These components each account for different proportions of the variation, until eventually 100% of the variance is explained. Typically, two of the principal components are plotted against each other in a score plot, allowing the separation of the samples to be viewed. [15,201]

PCA was performed on the entire data set. The first two principal components explained 52.7% of the variation, with 17 components required to explain 100% of the variation. The first principal component was influenced by strong positive contributions from α-pinene, D-limonene, linalool oxide (isomer 2), cineole and β-pinene. Eugenol, 4-oxoisophorone,

benzyl alcohol and 2-phenylethanol made strong negative contributions to the second principal component, while linalool made a moderate positive correlation.

The score plot (Figure 57) of the first two principal components did not show much distinction between the honey types, with all groups showing some overlap. This is not surprising, given that for most compounds, variation in peak area between honey types was not significantly different from variation within a honey type. The low number of compounds used is likely to be another contributing factor here. Typically, PCA analyses that have been able to successfully classify samples by botanical origin use results from TIC chromatograms, meaning that many more compounds are available for the analysis. For example, Yildiz *et al.* (2022) used 103 volatile compounds and were able to explain 98.7% of the variation in their samples with the first two principal components, and achieved excellent separation between samples of different honey types. [105]

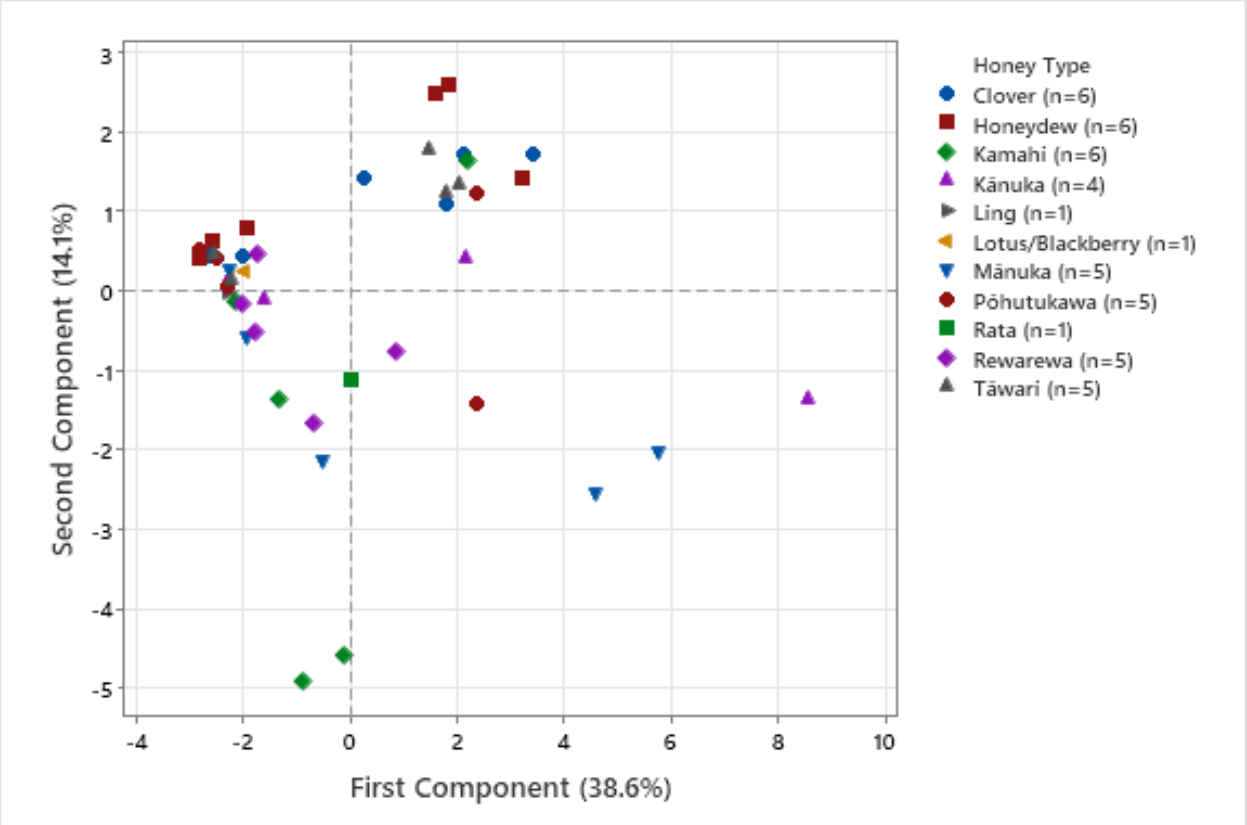

Figure 57. PCA score plot based on 18 volatile compounds, with 53.2% of variance explained by the first two principal components.

A cluster containing all honey types except rātā can be seen toward the left of the plot. The loading plot (Figure 58), which shows how each compound influences the two principal components used in the score plot, showed that this cluster was negatively associated with

guaiacol. Most kāmahi honeys clustered toward the bottom left of the score plot, which was associated with 4-oxoisophorone and eugenol. This makes sense, considering that the kāmahi honey samples were characterised by high peak areas for 4-oxoisophorone. Mānuka honeys typically sat around the x-axis, indicating they were influenced more by the compounds associated with the first component than those for the second component. The characterising feature of the mānuka honeys, *o*-methoxyacetophenone, did not contribute strongly to the first or second components, which may be why they did not cluster well in the score plot.

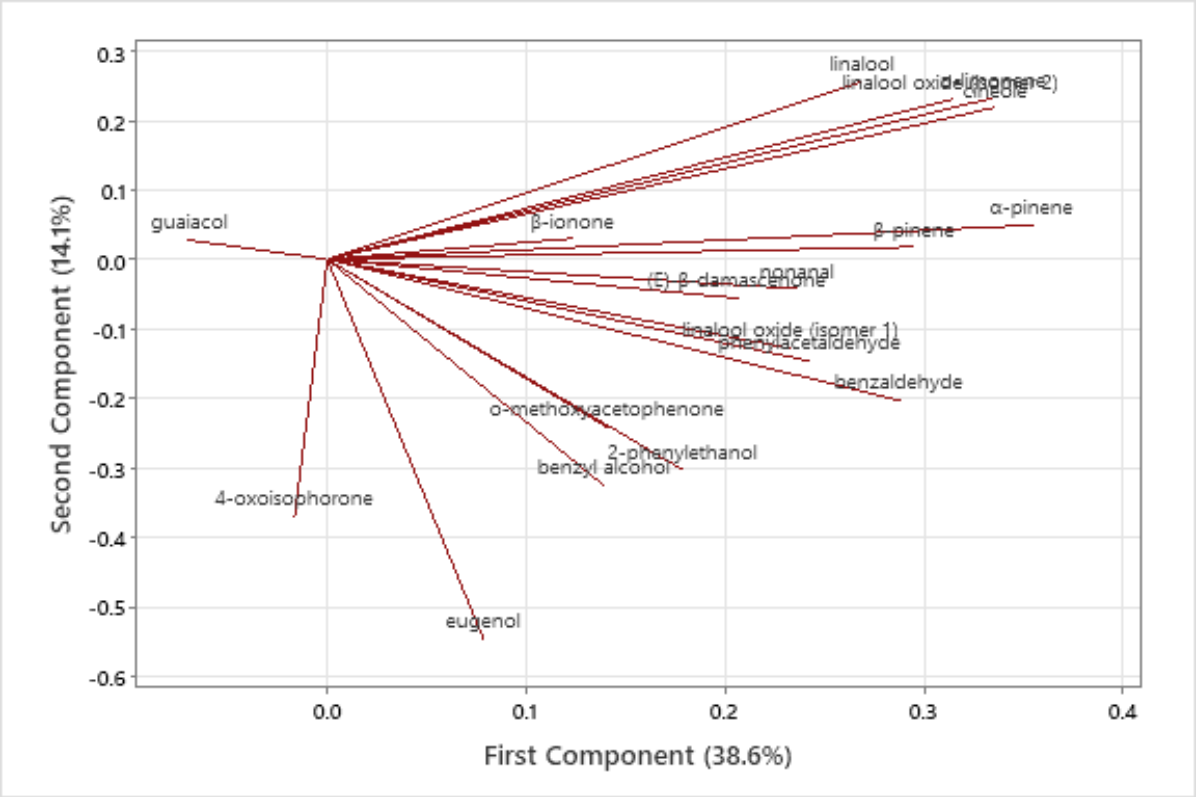

Figure 58. Loading plot showing the influence of each compound on the first two principal components for the PCA plot in Figure 57.

5.11.2. Classification and regression tree

The classification of samples into groups based on honey type was not possible using PCA, so it was necessary to use an alternative method. A classification and regression tree (CART) method was selected as it is suitable for classifying data into multiple categories based on any number of continuous predictor variables. As opposed to PCA, an unsupervised learning technique, the purpose of CART classification is to use the data available, in which the groups (honey types) are known, to attempt to classify future samples by honey type. CART classification was performed on the entire data set and a 9-node decision tree was produced

(Figure 59). The decision tree presents the process for determining which group (honey type) an unknown future sample belongs to based on its peak areas for the 18 analysed volatiles. At each split point in the tree, a condition is posed based on the peak area of the sample for a certain compound. If the sample's peak area for that compound is higher than the conditional amount, the path furthest on the right is taken; if not, the path furthest on the left is taken. These conditions continue down the tree, so that any future sample is classified into one of the nine groups.

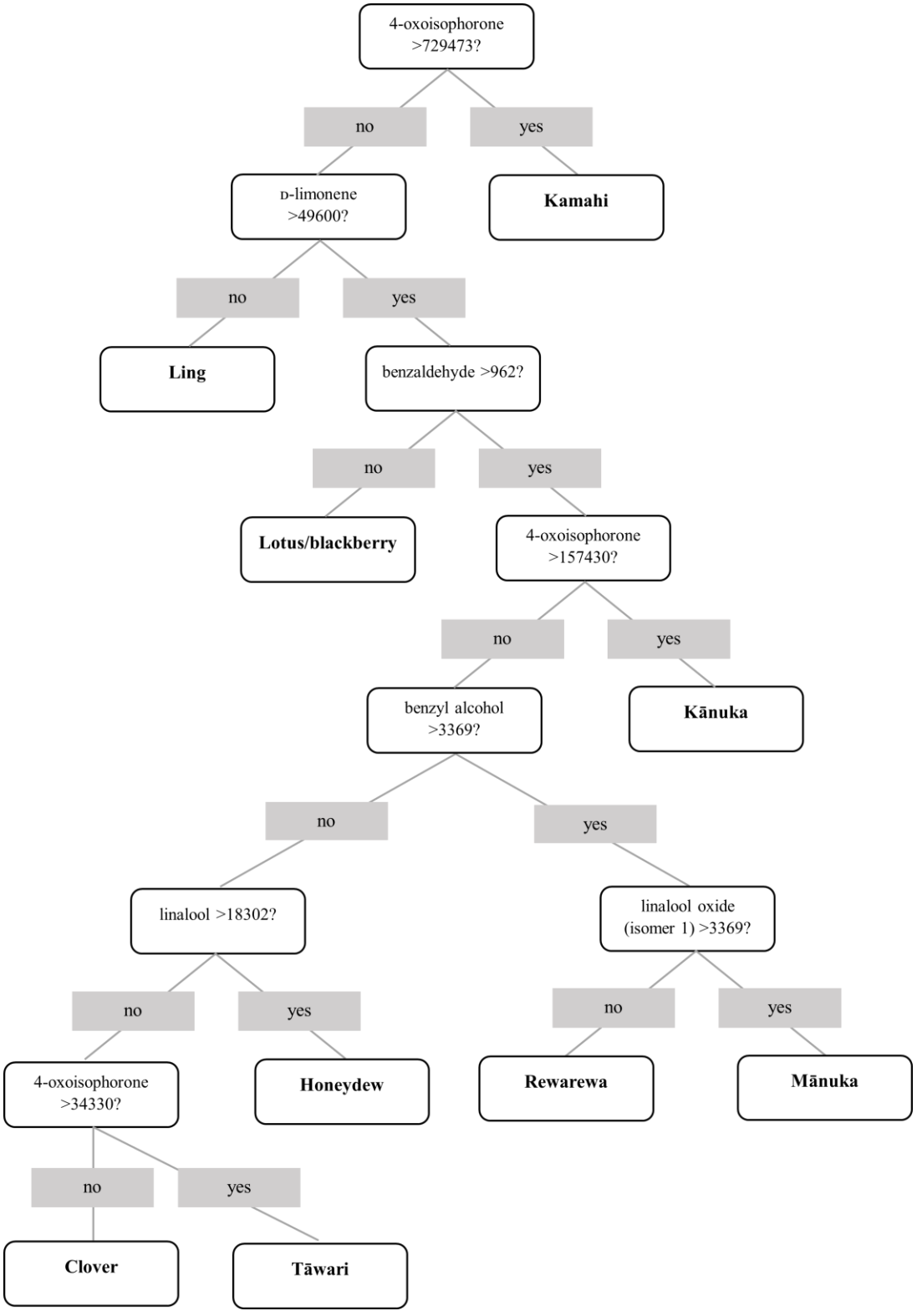

Figure 59. CART classification decision tree for classifying honey samples into nine terminal nodes based on peak areas for the analysed volatile compounds. All 18 compounds were used to build the model.

The decision tree is tested by running through the conditions with each of the existing samples used to build the tree, and calculating how many samples are mislabelled. The results

are shown in Table 34. The tree in Figure 59 had an average accuracy of 65.9%, meaning that this percentage of samples can be correctly classified using the tree. Kāmahi, kānuka, ling, lotus/blackberry and mānuka honeys were classified 100% correctly.

While eleven honey types were analysed, only nine terminal nodes are present on the decision tree – there is no terminal node for rātā or pōhutukawa honeys. This means that these honeys cannot be classified correctly using the decision tree. This is because CART classification builds the optimal decision tree as that with the least miscalculations. The rātā sample was classified as kāmahi due to its high peak area for 4-oxoisophorone, which was higher than three of the kāmahi samples. If the threshold for the 4-oxoisophorone condition at the first split in the decision tree were raised, then three kāmahi samples would have been mislabelled as opposed to just one rātā sample being mislabelled as the tree is constructed currently. Therefore, though the sample is mislabelled, this threshold for 4-oxoisphorone has minimised mislabelling of the two honey types. Pōhutukawa honeys, on the other hand, were mislabelled as clover, lotus/blackberry and mānuka. This reflects the large differences between the volatile profiles of the pōhutukawa honey samples, as these three categories all branch off at different splits in the decision tree, meaning more than one of the criteria separated each honey.

Honeydew samples were classified with 50% accuracy. The misclassified honeydew samples were determined to be ling and blackberry/lotus by the CART classification. These three honey types were characterised by low peak areas across most volatiles, and their groups in the CART classification were determined as having a lower peak area than other compounds for a certain compound. It makes sense that the groups would be misclassified as the volatile profiles of these three honey types were so similar. Indeed, in the PCA plot (Figure 57) three honeydew samples were closely clustered with the ling and lotus/blackberry samples.

Overall, the CART classification was able to correctly classify kāmahi, kānuka, ling, lotus/blackberry and mānuka honeys based on their volatile profiles. The classification of tāwari honey was acceptably accurate at 80%, however the misclassification rates of clover, honeydew, pōhutukawa, rātā and rewarewa were too great for this to be a successful method of predicting botanical origin of the honey samples.

5.11.3. Linear discriminant analysis

The final chemometrics method used to attempt to predict a honey sample's botanical origin based on its volatile profile was linear discriminant analysis (LDA). LDA functions similarly

to PCA, except that being a supervised method, separation between the groups is maximised as those groups are already known. The result of the LDA is a classification of the existing samples using the model, identical to that of the CART classification (Table 35). However, the method provided a more accurate classification than the CART classification, with an accuracy of 82.9%. It is important to note, before comparing the results of these two methods, that the dataset used in each classification was slightly different. While CART classification was carried out on the full data set, LDA did not include rātā, lotus/blackberry and ling samples. These honey types contained only one sample each, and since LDA aims to maximise the distance between the *means* of each compound per honey type, those with only one sample could not be included. However, for LDA and the classification tree, whether these samples were included or not did not change the results.

Interestingly, samples misclassified using this model were only classified as rewarewa or tāwari. Mānuka and pōhutukawa each had one sample misclassified as rewarewa, while clover, honeydew, rewarewa and mānuka samples were misclassified as tāwari. In contrast, kāmahi, kānuka and tāwari were classified 100% correctly. Even pōhutukawa honey, which was classified completely incorrectly in the CART classification, had an 80% accuracy using this method. Overall, an 82.9% accuracy rate of classification makes LDA the most suitable method for predicting botanical origin of New Zealand monofloral honey samples based on the volatile compounds analysed in this method. Using the three chemometrics-based approaches in this section, similarities between the volatile compositions of the eleven honey types were explored and different models of separating samples were investigated. The trends in volatile compositions of each honey type, discussed individually earlier in this chapter, were combined using these techniques to show a bigger picture. The unsupervised technique, PCA, did not show significant clustering of samples, but both supervised techniques were able to classify the samples into groups based on botanical origin with varying degrees of accuracy.

Table 34. Comparison of actual honey type with the honey type predicted by CART classification.

	Predicted Class											
Actual Class	**Clover**	**Honey-dew**	**Kāmahi**	**Kānuka**	**Ling**	**Lotus/ Black-berry**	**Mānuka**	**Pōhutu-kawa**	**Rātā**	**Rewa-rewa**	**Tāwari**	**% Correct**
Clover	3	0	0	1	0	0	0	0	0	0	2	**50**
Honeydew	0	3	0	0	2	1	0	0	0	0	0	**50**
Kāmahi	0	0	5	0	0	0	0	0	0	0	0	**100**
Kānuka	0	0	0	4	0	0	0	0	0	0	0	**100**
Ling	0	0	0	0	1	0	0	0	0	0	0	**100**
Lotus/ Blackberry	0	0	0	0	0	1	0	0	0	0	0	**100**
Mānuka	0	0	0	0	0	0	5	0	0	0	0	**100**
Pōhutukawa	2	0	0	0	0	1	2	0	0	0	0	**0**
Rātā	0	0	1	0	0	0	0	0	0	0	0	**0**
Rewarewa	1	0	0	0	0	0	1	0	0	3	0	**60**
Tāwari	0	0	0	1	0	0	0	0	0	0	4	**80**
All	6	3	6	6	3	3	8	0	0	3	6	**65.9**

Table 35. Comparison of actual honey type with the honey type predicted by linear discriminant analysis.

		Predicted Class								
Actual Class	**Count**	**Clover**	**Honeydew**	**Kāmahi**	**Kānuka**	**Mānuka**	**Pōhutukawa**	**Rewarewa**	**Tāwari**	**% Correct**
Clover	6	4	0	0	0	0	0	0	2	**67**
Honeydew	6	0	5	0	0	0	0	0	1	**83**
Kāmahi	5	0	0	5	0	0	0	0	0	**100**
Kānuka	4	0	0	0	4	0	0	0	0	**100**
Mānuka	5	0	0	0	0	3	0	1	1	**60**
Pōhutukawa	5	0	0	0	0	0	4	1	0	**80**
Rewarewa	5	0	0	0	0	0	0	4	1	**80**
Tāwari	5	0	0	0	0	0	0	0	5	**100**
All	44	4	5	5	4	3	4	6	10	**65.9**

6. Chapter Six: Conclusions and Future Recommendations

6.1. Conclusions

A wide variety of monofloral honeys are produced in New Zealand that have unique flavours and aromas, which are a product of their volatile composition. However, research linking the volatile profiles of New Zealand honeys with their sensory attributes has not been carried out before. Thus, the aims of this research were to develop, optimise and validate a method for the analysis of volatile compounds in honey and use this method to compare the volatile profiles of New Zealand monofloral honeys. While the final method unfortunately was not fit for quantification of compounds, the results gained from the analysis of New Zealand monofloral honeys allowed qualitative comparison between honey types and between the individual samples within them. The relative amounts of each compound were compared with sensory information to suggest possible aroma contributors in each honey type, and many of the compounds analysed were identified in their respective honey type for the first time. The application of chemometric techniques allowed honeys to be separated and classified according to their volatile compositions, and a classification technique with satisfactory accuracy was identified.

Internationally, a number of studies have combined analysis of volatile compounds in honey with quantitative sensory techniques such as aroma extract dilution analysis and odour activity values. In Chapter 1, a review of these publications was conducted and an extensive compilation of the known odour active compounds in honey was produced. Additionally, since this work has not been carried out in New Zealand, a compilation of the volatile compounds identified in New Zealand honeys was also created, to indicate the variety of compounds that may be identified in this work. It was found that while some compounds, such as 2-phenylethanol, (*E*)-β-damascenone and phenylacetaldehyde are aroma-active in most honey types throughout the world due to their low aroma thresholds, compounds that gave honeys their unique sensory properties were often a result of the botanical origin. The extraction method and geographic origin also influenced the volatile profiles.

GC-MS was used for the instrumental analysis as this is the most suitable method of separating volatile compounds and allowed confirmation of compound identification using comparison of the mass spectrometric data with databases, and comparison of retention times

with external standards. While almost all recent methods have used GC-MS, the methods of extracting volatiles from the complex honey matrix continue to evolve. Chapter 3 detailed the development of a suitable extraction method, in which solvent extraction displayed the best performance over static headspace and solid-phase microextraction. A literature review of these techniques, as well as solid-phase extraction, simultaneous distillation-extraction and ultrasound extraction, was carried out, followed by experiments on the three chosen procedures in order to optimise their sensitivity and recovery. Solvent extraction was chosen due to superior extraction efficiency and sensitivity over the headspace methods, though significant analytical challenges were also experienced with this method. Recoveries were improved by eliminating the solvent evaporation step, which was causing loss of analytes, and the resulting loss in sensitivity caused by increased sample dilution was compensated for by optimising the instrumental method. Selected ion monitoring was used to monitor characteristic ions of 18 volatile compounds in each sample, allowing for increased sensitivity and selectivity, but meaning the total volatile profile of honeys could not be determined.

Validation of the method was detailed in Chapter 4 and determined that the method was fit for the purpose of identification and semi-quantification of compounds, but was not suitable for full quantification. Weighting was applied to calibration curves to increase the accuracy at lower concentrations, which allowed a wide calibration range to be used while maintaining a linear response. Limits of detection and quantification were sufficiently low considering the endogenous levels of each monitored compound in honey, and both intra-day and inter-day precision were excellent, with 83% intra-day repeatability measurements being under 5% RSD. Unfortunately, spike recovery results did not fall into the ideal range of 80 – 120% for all compounds at all concentrations, due to a combination of spike concentration selection, SIM ion selection and a lack of baseline separation on some peaks. However, recoveries for nonanal were sufficient for complete quantification, and benzyl alcohol, cineole and guaiacol were able to be quantified within a reduced calibration range.

Once validation was complete, 44 honeys from 11 floral types were analysed and findings were summarised in Chapter 5. Each of the 18 compounds was identified in all honey types, though not in all samples. Of these, D-limonene, β-ionone and cineole were reported for the first time in any New Zealand honey, and phenylacetaldehyde and nonanal were reported for the first time in kānuka honey. Mānuka honey samples were characterised by *o*-methoxyacetophenone, which was not surprising given that this is the only volatile marker

compound for mānuka honeys used by MPI.[21] The peak areas varied widely between samples. Kāmahi honeys had much higher peak areas than other honey samples for 4-oxoisophorone, and due to the low odour threshold, this compound was determined as a likely contributor to the aroma of these honeys. As quantitative data could not be used for all compounds, OAVs could not be calculated to determine the relative contributions of each compound towards the aroma of the honeys. However, nonanal was determined to be aroma-active in all honeys analysed, and cineole in all but ling, lotus/blackberry and rātā. For the other compounds, odour thresholds were considered and compared with the relative peak areas to make suggestions about which compounds may be aroma-active in each honey type. Clover, pōhutukawa, tāwari, rewarewa, ling and lotus/blackberry honeys typically had low levels of volatiles compared to the other honeys; despite this, chemometric methods were able to elucidate differences between all the honey types. One unsupervised learning method and two supervised learning methods were used. Principal component analysis (PCA) was not able to separate any of the honey types clearly by their volatile profiles in the first two principal components, and CART classification was not able to classify samples into their floral origins with sufficient accuracy. Linear discriminant analysis proved successful at classifying the honeys based on the peak areas of the 18 volatile compounds. This method was a supervised modelling technique, which allowed the separation between honey types to be maximised. The classification of honeys according to the linear discriminant analysis into their floral origins was 82.9%, with the most accurate classification achieved for kāmahi and kānuka honeys.

6.2. Suggestions for future work

Throughout the development and validation of this method, several opportunities for improvement were identified. Incorporating these suggestions would improve the performance of the method for the intended purpose stated in the aims of this research, through increased quantification accuracy and better ability for comparison with literature.

Accurate quantification of the 18 compounds analysed would vastly increase the relevance of this work, giving a more complete picture of the volatile profiles of each honey type and allowing calculation of OAVs to determine the relative contributions of each compound to the honey's aroma. There were several factors that contributed to quantification not being possible for all compounds, but these can be improved upon:

- No internal standard was used, largely because a suitable compound could not be identified. Of the potential standards that were trialled, none were found that behaved similarly to all of the analytes while not being present in the sample. As an example, β-ionone is commonly used as an internal standard, yet it was identified in all the honey types analysed here. [10,202] An internal standard would improve the accuracy of the quantification by accounting for fluctuations in the instrumental analysis. Commonly used compounds that were not trialled due to availability include esters such as methyl anthanilate, [163] methyl undecanoate[140] and methyl heptadecanoate, [89] linear alcohols such as 4-nonanol[29,53] and 2-pentanol, [117] and long-chain linear alkanes such as *n*-hexadecane[94] and undecane. [80] Of these, the esters are the most promising as 1-hexanol, which was already trialled, was found to be present in all honeys, and long-chain alkanes were the dominating peaks in the TIC chromatograms of all honeys. Ideally, isotopically labelled internal standards for each compound would be used, as these behave identically to the compounds of interest yet can be distinguished by mass spectrometry, but are very expensive, so the esters should be trialled as possible internal standards.
- Additionally, an SMC should be used to account for any analyte loss during sample extraction. The requirements for an SMC are the same as for the internal standard. Inclusion of an SMC would improve the accuracy of recovery as the analyte and SMC are assumed to be lost at the same rate. Even without complete quantification, the use of an internal standard and SMC would improve the reliability of the data, allowing for better comparisons between honey types.
- Very wide calibration ranges, spanning up to 8.7 mg kg^{-1}, were used as the concentrations of volatiles in the honeys to be analysed was largely unknown. Though the response was linear over the calibration ranges, a reduction in this range may result in increased accuracy. It is recommended that the calibration ranges be reduced to reflect the concentrations found so far in the honeys analysed, which did not appear to vary widely for most compounds.
- The primary contribution to the poor recoveries reported in Chapter 4 was due to the choice of spike concentration. Spike concentration was decided using the average endogenous concentration of five honey samples of different floral origins, and where the spiked sample would fall on the calibration curve was not considered. In future, spike concentrations should be chosen as 50% and 100% of the endogenous

concentration of each honey type, and the calibration curve should encompass the range of concentrations expected upon 100% recovery of those spikes. To reach a compromise between this and the previous suggestion about calibration ranges, a control sample – a honey sample with no analyte present – could be used. Spiking with 50% and 100% into a control sample would allow recovery to be calculated without risking the spiked sample falling outside the calibration range. A control sample would be difficult to find due to the large number of analytes, so using an artificial honey matrix like that used in the static headspace experiments in Chapter 2 may be more suitable.

Improvements can be made to other aspects of the method to provide a more well-rounded knowledge of the volatile profiles of the analysed honeys:

- The iterative nature of the method development was such that optimisation of one aspect of the method affected the other aspects. Often, it was clear that the results of earlier experiments may have been different if the parameters decided upon in later experiments were used. For example, the comparison of extraction solvents was repeated after the SIM method was finalised to check whether the solvents performed differently under different instrumental conditions. With more time available, it would be advantageous to repeat all experiments using the SIM method to further optimise the extraction. It would also be worth repeating the trials of static headspace and SPME extraction methods using the SIM method, as the sensitivity of this method was much greater and may have been sufficient for these techniques.
- Peak identities were confirmed by comparing retention times with those of the external standards and by comparison of the mass spectra with the NIST mass spectral database. While these methods were sufficient to confirm peak identities, they do not allow for comparison with other literature because retention time is dependent on a number of factors including column dimensions, solvent, and oven programming in the gas chromatograph. To account for this, RTs for a peak are commonly reported alongside the Kováts retention index (RI). This system-independent value is calculated using the RT of the peak and of two standards, eluted before and after the peak, using Equation 5.[203] In gas chromatography, the standards used are typically a series of *n*-alkanes. Yildiz *et al.* (2022) used a mixture of C_8-C_{20} alkanes, whereas Kuś *et al.* (2021) used a C_9-C_{25} mixture, with many others using similar standards ranging between C_6-C_{40}.[77,89,105,155,157,160,166,185,188,204,205] It would be useful to analyse a

similar set of *n*-alkanes using this analysis method to calculate RIs for the analysed compounds. This would allow better comparison with other literature with regards to peak retention and identity.

$$RI = 100\left[\frac{log_{10}X_i - log_{10}X_z}{log_{10}X_{z+1} - log_{10}X_z} + z\right]$$

Equation 5. Calculation of the Kovats retention index of a peak.

Where *X* refers to retention time, *i* refers to the peak of interest, and *z* and *z+1* are the number of carbon atoms of the *n*-alkanes eluting before and after the peak of interest.

- It was not possible to distinguish between the two isomers of linalool oxide using mass spectrometry. Identifying the two peaks as either the *cis*- or *trans*- isomers would give a more complete knowledge of the volatile profiles of the honeys, particularly for the kānuka and honeydew honeys which had interesting profiles with regards to these peaks. However, the individual isomers cannot be purchased as external standards, so they could not be identified this way, and of course the mass spectra are identical. Nuclear magnetic resonance (NMR) spectroscopy would be a useful technique for distinguishing between the two isomers, as coupling constants for *cis*-oriented hydrogens are smaller than those for *trans*-oriented hydrogens. Another approach would be to use GC with an optically-active coating on the column, as employed by Wang *et al.* (1994).[206] This study reported *trans*-linalool oxide to elute earlier than this *cis*- isomer, but this should not be assumed for this research given that RIs were not calculated here.
- Another suggestion that would improve the quality of discussion around individual samples would be to carry out visual pollen analysis of all samples. Visual pollen analysis was only carried out on a selected set of the honey samples analysed here, and this provided valuable information, especially for NZ342 which was labelled as kāmahi honey but was revealed to be multifloral. The peak area of 4-oxoisophorone for this sample was comparable to rātā and clover honeys, which had significant nectar contributions. If this analysis were carried out on all honeys samples, better conclusions could be made about the variation between samples of the same honey type. Volatile compound analysis of the leaves of each plant would also be useful, as it would provide more information on which compounds are likely to be characteristic of the floral origin and which were more dependent on other factors.

Lastly, there is potential for the sensory aspect of this research to be expanded upon. As detailed in Chapter 1, there are many potential methods for analysing the aroma-active compounds in a honey sample, but a suggested path forward is detailed here.

- Firstly, samples of each honey type should be evaluated by a trained sensory panel to provide a database of terms used to describe the aroma and flavour of each honey type. Typically, the vocabulary used is determined by the panel, though the reader is directed to examples such as the UC Davis honey flavour wheel. [181] Ideally, all samples would be analysed this way in order to make links between variations in the volatile composition and the aroma, though the cost of this would be prohibitive.
- GC-O could be carried out for each sample alongside GC-MS, along with dilution experiments. Accurate quantification by GC-MS would enable the calculation of OAVs as detailed earlier, however, the practical meaning of these is better elucidated by AEDA (see Chapter 1, section 1.2.2). A series of dilutions of each honey sample would be analysed to determine the most dilute sample in which aroma compounds are still detected. This gives a quantitative value for the contribution of each compound to the honey aroma. It would be interesting to compare the dilution factors of the major aroma compounds such as (*E*)-β-damascenone and phenylacetaldehyde in New Zealand honeys with previously reported values for international honeys. It can be expected that the compounds would have stronger contributions to aroma due to the typically high volatile concentrations of New Zealand honeys compared with honeys from Europe and North and South America.
- Other experiments involving synthetic mixtures of aroma compounds similar to that by Buttery *et al.* (1987) could also be carried out to better understand the interactions between aroma compounds. [47] Once the various contributions of each analysed compound were determined, a synthetic mixture of these compounds can be analysed by a sensory panel to determine whether it is a good representation of the honey aroma. Additionally, omission of certain compounds from these mixtures can elucidate their importance.

References

1. Aparna, A. R.; Rajalakshmi, D. Honey—its characteristics, sensory aspects, and applications. *Food Reviews International* **1999**, *15*, 455-471.
2. Codex Alimentarius Commission Standards. *Standard for honey.* Rome, 1981.
3. Tian, H.; Shen, Y.; Yu, H.; Chen, C. Aroma features of honey measured by sensory evaluation, gas chromatography-mass spectrometry, and electronic nose. *International Journal of Food Properties* **2018**, *21*, 1755-1768.
4. Pontes, M.; Marques, J. C.; Câmara, J. S. Screening of volatile composition from Portuguese multifloral honeys using headspace solid-phase microextraction-gas chromatography–quadrupole mass spectrometry. *Talanta* **2007**, *74*, 91-103.
5. *Apiculture: Ministry for Primary Industries 2020 apiculture monitoring programme.* Ministry for Primary Industries: Wellington, New Zealand, 2020.
6. Grainger, M. N. C. Kinetics of conversion of dihydroxyacetone to methylglyoxal in honey. . Doctoral Thesis, University of Waikato, Hamilton, New Zealand, 2015.
7. Molan, P. C.; Russell, K. M. Non-peroxide antibacterial activity in some New Zealand honeys. *Journal of Apicultural Research* **1988**, *27*, 62-67.
8. Machado, A. M.; Miguel, M. G.; Vilas-Boas, M.; Figueiredo, A. C. Honey volatiles as a fingerprint for botanical origin - a review on their occurrence on monofloral honeys. *Molecules* **2020**, *25*
9. Siegmund, B.; Urdl, K.; Jurek, A.; Leitner, E. "More than honey": investigation on volatiles from monovarietal honeys using new analytical and sensory approaches. *Journal of Agricultural and Food Chemistry* **2018**, *66*, 2432-2442.
10. Alissandrakis, E.; Kibaris, A. C.; Tarantilis, P. A.; Harizanis, P. C.; Polissiou, M. Flavour compounds of Greek cotton honey. *Journal of the Science of Food and Agriculture* **2005**, *85*, 1444-1452.
11. Castro-Vázquez, L.; Díaz-Maroto, M. C.; Pérez-Coello, M. S. Aroma composition and new chemical markers of Spanish citrus honeys. *Food Chemistry* **2007**, *103*, 601-606.
12. Ministry for Primary Industries. *Apiculture monitoring report.* 2014.
13. Creasey, S. The great manuka honey swindle? In *The Grocer*, 2014.
14. Louveaux, J.; Maurizio, A.; Vorwohl, G. Methods of melissopalynology. *Bee World* **1978**, *59*, 139-157.
15. Petchell, L. E. Identification of the floral source of New Zealand honeys. Thesis, University of Waikato, Hamilton, New Zealand, 2009.
16. Holt, K. A.; Bebbington, M. S. Separating morphologically similar pollen types using basic shape features from digital images: a preliminary study. *Applications in Plant Sciences* **2014**, *2*
17. McIntyre, D. J. Pollen morphology of New Zealand species of *Myrtaceae*. *Transactions of the Royal Society of New Zealand* **1963**, *2*, 83-107.
18. Mildenhall, D. C.; R., T. *Pollen analysis of New Zealand honey*; Institute of Geological and Nuclear Sciences: Lower Hutt, N.Z., 2005.
19. da Silva, P. M.; Gauche, C.; Gonzaga, L. V.; Costa, A. C.; Fett, R. Honey: Chemical composition, stability and authenticity. *Food Chemistry* **2016**, *196*, 309-323.
20. Moar, N. T. Pollen analysis of New Zealand honey. *New Zealand Journal of Agricultural Research* **1985**, *28*, 39-70.
21. Ministry for Primary Industries. *Criteria for identifying mānuka honey.* 2017.
22. Zhu, M.; Sun, J.; Zhao, H.; Wu, F.; Xue, X.; Wu, L.; Cao, W. Volatile compounds of five types of unifloral honey in Northwest China: Correlation with aroma and floral origin based on HS-SPME/GC–MS combined with chemometrics. *Food Chemistry* **2022**, *384*, 132461.

23. Bianchi, F.; Careri, M.; Musci, M. Volatile norisoprenoids as markers of botanical origin of Sardinian strawberry-tree (*Arbutus unedo L.*) honey: Characterisation of aroma compounds by dynamic headspace extraction and gas chromatography–mass spectrometry. *Food Chemistry* **2005**, *89*, 527-532.
24. Castro-Vázquez, L.; Díaz-Maroto, M. C.; González-Viñas, M. A.; Pérez-Coello, M. S. Differentiation of monofloral citrus, rosemary, eucalyptus, lavender, thyme and heather honeys based on volatile composition and sensory descriptive analysis. *Food Chemistry* **2009**, *112*, 1022-1030.
25. Dairou, V.; Sieffermann, J.-M. A comparison of 14 jams characterized by conventional profile and a quick original method, the flash profile. *Journal of Food Science* **2002**, *67*, 826-834.
26. Mannaş, D.; Altuğ, T. SPME/GC/MS and sensory flavour profile analysis for estimation of authenticity of thyme honey. *International Journal of Food Science & Technology* **2007**, *42*, 133-138.
27. Blank, I.; Fischer, K.-H.; Grosch, W. Intensive neutral odorants of linden honey. Differences from honeys of other botanical origin. *Zeitschrift für Lebensmittel Untersuchung und Forschung* **1989**, *189*, 426-433.
28. Steeg, E.; Montag, A. Quantitative bestimmung aromatischer carbonsäuren in honig. *Zeitschrift für Lebensmittel-Untersuchung und Forschung* **1988**, *187*, 115-120.
29. Türk, G.; Şen, K. Changes of various quality characteristics and aroma compounds of astragalus honey obtained from different altitudes of Adana-Turkey. *Journal of Food Processing and Preservation* **2021**, *45*
30. Puratos. The sensory panel. https://www.puratos.com/blog/the-sensory-panel (accessed 31 October, 2021).
31. Leonardos, G.; Kendall, D.; Barnard, N. Odor threshold determinations of 53 odorant chemicals. *Journal of the Air Pollution Control Association* **1969**, *19*, 91-95.
32. Dörrscheidt, W.; Friedrich, K. Trennung von aromastoffen des honigs mit hilfe der gas-chromatographie. *Journal of Chromatography A* **1962**, *7*, 13-18.
33. Merz, J. H. The objective assessment of honey aroma by gas chromatography. *Journal of Apicultural Research* **1963**, *2*, 55-61.
34. ten Hoopen, H. J. G. Flüchtige carbonylverbindungen in honig. *Zeitschrift für Lebensmittel-Untersuchung und Forschung* **1963**, *119*, 478-482.
35. Cremer, E.; Riedmann, M. Notizen: Identifizierung von gaschromatographisch getrennten Aromastoffen in Honigen. *Zeitschrift Fur Naturforschung* **1964**, *19*
36. Grosch, W. Evaluation of the key odorants of foods by dilution experiments, aroma models and omission. *Chemical Senses* **2001**, *26*, 533-545.
37. Patton, S.; Josephson, D. V. A method for determining significance of volatile flavor compounds in food. *Journal of Food Science* **1957**, *22*, 316-318.
38. Reineccius, G.; Peterson, D. Principles of food flavor analysis. In *Instrumental Assessment of Food Sensory Quality*; D. Kilcast, Ed.; Woodhead Publishing, 2013; pp 53-102.
39. Pino, J. A. Analysis of odour-active compounds of black mangrove (*Avicennia germinans L.*) honey by solid-phase microextraction combined with gas chromatography-mass spectrometry and gas chromatography-olfactometry. *International Journal of Food Science & Technology* **2012**, *47*, 1688-1694.
40. Czerny, M.; Christlbauer, M.; Christlbauer, M.; Fischer, A.; Granvogl, M.; Hammer, M.; Hartl, C.; Hernandez, N. M.; Schieberle, P. Re-investigation on odour thresholds of key food aroma compounds and development of an aroma language based on odour qualities of defined aqueous odorant solutions. *European Food Research and Technology* **2008**, *228*, 265-273.

41. Blank, I. Flavor, fragrance and odor analysis. In *Gas chromatography-olfactometry in food aroma analysis*; R. Marsili, Ed.; Marcel Dekker: New York, NY, USA, 2002; pp 297-331.
42. Delahunty, C. M.; Eyres, G.; Dufour, J. P. Gas chromatography-olfactometry. *Journal of Separation Science* **2006**, *29*, 2107-2125.
43. Fuller, G. H.; Steltenkamp, R.; Tisserand, G. A. The gas chromatograph with human sensor: perfumer model. *Annals of the New York Academy of Sciences* **1964**, *116*, 711-724.
44. Odournet. GC-Sniffing – most powerful tool in odour assessment. https://www.odournet.com/product-testing/molecular-analysis/gc-sniffing/ (accessed December 8 2022).
45. Acree, T. E.; Barnard, J.; Cunningham, D. G. A procedure for the sensory analysis of gas chromatographic effluents. *Food Chemistry* **1984**, *14*, 273-286.
46. da Silva, M. A. A. P.; Lundahl, D. S.; McDaniel, M. R. In *Trends in Flavour Research (Developments in Food Science)*; H. Maarse and D. van der Heij, Eds.; Elsevier Science: Amsterdam, 1994; pp 191-209.
47. Buttery, R. G.; Teranishi, R.; Ling, L. C. Fresh tomato aroma volatiles: a quantitative study. *Journal of Agricultural and Food Chemistry* **1987**, *35*, 540-544.
48. Ruisinger, B.; Schieberle, P. Characterization of the key aroma compounds in rape honey by means of the molecular sensory science concept. *Journal of Agricultural and Food Chemistry* **2012**, *60*, 4186-4194.
49. Seisonen, S.; Kivima, E.; Vene, K. Characterisation of the aroma profiles of different honeys and corresponding flowers using solid-phase microextraction and gas chromatography-mass spectrometry/olfactometry. *Food Chemistry* **2015**, *169*, 34-40.
50. Shimoda, M.; Wu, Y.; Osajima, Y. Aroma compounds from aqueous solution of haze (*Rhus succedanea*) honey determined by adsorptive column chromatography. *Journal of Agricultural and Food Chemistry* **1996**, *44*, 3913-3918.
51. Kortesniemi, M.; Rosenvald, S.; Laaksonen, O.; Vanag, A.; Ollikka, T.; Vene, K.; Yang, B. Sensory and chemical profiles of Finnish honeys of different botanical origins and consumer preferences. *Food Chemistry* **2018**, *246*, 351-359.
52. Moreira, R. F. A.; De Maria, C. A. B. Investigation of the aroma compounds from headspace and aqueous solution from the cambará (*Gochnatia velutina*) honey. *Flavour and Fragrance Journal* **2005**, *20*, 13-17.
53. Uckun, O.; Selli, S. Characterization of key aroma compounds in a representative aromatic extracts from citrus and astragalus honeys based on aroma extract dilution analyses. *Journal of Food Measurement and Characterization* **2016**, *11*, 512-522.
54. Buttery, R. G.; Teranishi, R.; Ling, L. C.; Turnbaugh, J. G. Quantitative and sensory studies on tomato paste volatiles. *Journal of Agricultural and Food Chemistry* **1990**, *38*, 336-340.
55. Zhou, Q.; Wintersteen, C. L.; Cadwallader, K. R. Identification and quanitification of aroma-active components that contribute to the distinct malty flavour of buckwheat honey. *Journal of Agricultural and Food Chemistry* **2002**, *50*, 2016-2021.
56. Blank, I.; Sen, A.; Grosch, W. Aroma impact compounds of Arabica and Robusta coffee. Qualitative and quantitative data. Presented at Fourteenth International Conference on Coffee Science, San Francisco, CA; Place,ASIC 91.
57. Buttery, R. G.; Takeoka, G. R.; Krammer, G. E.; Ling, L. C. Furaneol: odor threshold and importance to tomato aroma. *Journal of Agricultural and Food Chemistry* **1995**, *43*, 1638-1640.

58. Moreira, R. F. A.; De Maria, C. A. B.; Pietroluongo, M.; Trugo, L. C. Chemical changes in the volatile fractions of Brazilian honeys during storage under tropical conditions. *Food Chemistry* **2010**, *121*, 697-704.
59. Amoore, J. E. F., L. J.; Pelosi, P. . Specific anosmia to isobutyraldehyde: the malty primary odor. *Chemical Senses and Flavor* **1976**, *1*, 17-25.
60. Buttery, R. G. Quantitative and sensory aspects of flavor of tomato and other vegetables and fruits. In *Flavor Science: Sensible Principles and Techniques in Flavor Science*; T. E. Acree and R. Teranishi, Eds.; American Chemical Society: Washington, DC, 1993.
61. Guadagni, D. G.; Buttery, R. G.; Turnbaugh, J. G. Odour thresholds and similarity ratings of some potato chip components. *Journal of the Science of Food and Agriculture* **1972**, *23*, 1435-1444.
62. Kobayashi, A. Sotolon: identification, formation, and effect on flavor. Presented at ACS Symposium Series 388, Washington, DC; Place,American Chemical Society.
63. Kim, Y.; Lee, Y.; Kim, K. Optimum roasting and extraction conditions and flavor characteristics of roasted malt extract. *Cereal Chemistry* **1998**, *75*, 282-288.
64. Larsen, M.; Poll, L. Odour thresholds of some important aroma compounds in raspberries. *Zeitschrift für Lebensmittel-Untersuchung und Forschung* **1990**, *191*, 129-131.
65. Devos, M.; Patte, F.; Rouault, J.; Laffort, P.; Van Gemert, L. J. *Standardized human olfactory thresholds in air*; IRL Press: Oxford University, UK, 1990.
66. Buttery, R. G.; Turnbaugh, J. G.; Ling, L. C. Contribution of volatiles to rice aroma. *Journal of Agricultural and Food Chemistry* **1988**, *36*, 1006-1009.
67. Pyysalo, T.; Suihko, M.; Honkanen, E. Odor thresholds of the major volatiles identified in cloudberry rubus chamaemorus and arctic bramble rubus arcticus. *Lebensmittel Wissenschaft and Technologie* **1977**, *10*, 36-39.
68. Masanetz, C.; Grosch, W. Hay-like off-flavour of dry parsley. *Zeitschrift für Lebensmitteluntersuchung und -Forschung A* **1998**, *206*, 114-120.
69. Buttery, R. G.; Guadagni, D. G.; Ling, L. C.; Seifert, R. M.; Lipton, W. Additional volatile components of cabbage, broccoli, and cauliflower. *Journal of Agricultural and Food Chemistry* **1976**, *24*, 829-832.
70. Takeoka, G.; Buttery, R. G.; Flath, R. A.; R., T.; Wheeler, E. L.; Wieczorek, R. L.; Guentert, M. Volatile constituents of pineapple (*Ananas comosus [L.] Merr*.). In Flavor chemistry trends and developments. Presented at ACS Symposium Series 388, Washington, DC; Place,American Chemical Society.
71. Schnabel, K.-O.; Belitz, H.-D.; von Ranson, C. Untersuchungen zur struktur-aktivitäts-beziehung bei geruchsstoffen 1. mitteilung: wahrnehmungsschwellenwerte und geruchsqualitäten von gesättigten aliphatischen und alicyclischen verbindungen mit sauerstoff-funktion. *Zeitschrift für Lebensmittel-Untersuchung und Forschung* **1988**, *187*, 215-223.
72. Takeoka, G. R.; Buttery, R. G.; Flath, R. A. Volatile constituents of Asian pear (*Pyrus serotina*). *Journal of Agricultural and Food Chemistry* **1992**, *40*, 1925-1929.
73. Pittet, A. O.; Rittersbacher, P.; Muralidhara, R. Flavor properties of compounds related to maltol and isomaltol. *Journal of Agricultural and Food Chemistry* **1970**, *18*, 929-930.
74. Cometto-Muñiz, J. E.; Abraham, M. H. Odor detection by humans of lineal aliphatic aldehydes and helional as gauged by dose-response functions. *Chem Senses* **2010**, *35*, 289-299.

75. Semmelroch, P.; Laskawy, G.; Blank, I.; Grosch, W. Determination of potent odourants in roasted coffee by stable isotope dilution assays. *Flavour and Fragrance Journal* **1995**, *10*, 1-7.
76. Panseri, S.; Manzo, A.; Chiesa, L. M.; Giorgi, A. Melissopalynological and volatile compounds analysis of buckwheat honey from different geographical origins and their role in botanical determination. *Journal of Chemistry* **2013**, *2013*, 904202.
77. Pasini, F.; Gardini, S.; Marcazzan, G. L.; Caboni, M. F. Buckwheat honeys: screening of composition and properties. *Food Chemistry* **2013**, *141*, 2802-2811.
78. Radovic, B. S.; Careri, M.; Mangia, A.; Musci, M.; Gerboles, M.; Anklam, E. Contribution of dynamic headspace GC–MS analysis of aroma compounds to authenticity testing of honey. *Food Chemistry* **2001**, *72*, 511-520.
79. Revell, L. E.; Morris, B.; Manley-Harris, M. Analysis of volatile compounds in New Zealand unifloral honeys by SPME–GC–MS and chemometric-based classification of floral source. *Journal of Food Measurement and Characterization* **2013**, *8*, 81-91.
80. Tan, S. T.; Holland, P. T.; Wilkins, A. L.; Molan, P. C. Extractives from New Zealand honeys. 1. white clover, mānuka and kanuka unifloral honeys. *Journal of Agricultural and Food Chemistry* **1988**, *36*, 453-460.
81. Tan, S. T.; Wilkins, A. L.; Holland, P. T.; McGhie, T. K. Extractives from New Zealand unifloral honeys. 2. degraded carotenoids and other substances from heather honey. *Journal of Agricultural and Food Chemistry* **1989**, *37*, 1217-1221.
82. Tan, S. T.; Wilkins, A. L.; Holland, P. T.; McGhie, T. K. Extractives from New Zealand honeys. 3. unifloral thyme and willow honey constituents. *Journal of Agricultural and Food Chemistry* **1990**, *38*, 1833-1838.
83. Wilkins, A. L.; Lu, Y.; Molan, P. C. Extractable organic substances from New Zealand unifloral manuka (*Leptospermum scoparium*) honeys. *Journal of Apicultural Research* **1993**, *32*, 3-9.
84. Wilkins, A. L.; Lu, Y.; Tan, S. T. Extractives from New Zealand honeys. 4. linalool derivatives and other components from nodding thistle (*Carduus nutans*) honey. *Journal of Agricultural and Food Chemistry* **1993**, *41*, 873-878.
85. Wilkins, A. L.; Tan, S.-T.; Molan, P. C. Extractable organic substances from New Zealand unifloral vipers bugloss (*Echium vulgare*) honey. *Journal of Apicultural Research* **1995**, *34*, 73-78.
86. Tan, S. T. A chemical investigation of some New Zealand honeys. Thesis, University of Waikato, Hamilton, New Zealand, 1989.
87. Bouseta, A.; Collin, S.; Dufour, J.-P. Characteristic aroma profiles of unifloral honeys obtained with a dynamic headspace GC-MS system. *Journal of Apicultural Research* **1992**, *31*, 96-109.
88. Overton, S.; Manura, J. J. Flavor and aroma in commercial bee honey: A purge-and-trap thermal desorption technique for the identification and quantification of volatiles and semivolatiles in honey. *American Laboratory* **1994**, *26*, 45,47.
89. D'Arcy, B. R.; Rintoul, G. B.; Rowland, C. Y.; Blackman, A. J. Composition of Australian honey extractives. 1. Norisoprenoids, monoterpenes, and other natural volatiles from blue gum (*Eucalyptus leucoxylon*) and yellow box (*Eucalyptus melliodora*) honeys. *Journal of Agricultural and Food Chemistry* **1997**, *45*, 1834-1843.
90. Visser, F. R.; Allen, J. M.; Shaw, G. J. The effect of heat on the volatile flavour fraction from a unifloral honey. *Journal of Apicultural Research* **1988**, *27*, 175-181.
91. Langford, V.; Gray, J.; Foulkes, B.; Bray, P.; McEwan, M. J. Application of selected ion flow tube-mass spectrometry to the characterization of monofloral New Zealand honeys. *Journal of Agricultural and Food Chemistry* **2012**, *60*, 6806-6815.

92. Wilkins, A. L.; Lu, Y. R.; Tan, S. T. Extractives from New Zealand honeys .5. aliphatic dicarboxylic acids in New Zealand rewarewa (*Knightia excelsa*) honey. *Journal of Agricultural and Food Chemistry* **1995**, *43*, 3021-3025.
93. Beitlich, N.; Koelling-Speer, I.; Oelschlaegel, S.; Speer, K. Differentiation of manuka honey from kanuka honey and from jelly bush honey using HS-SPME-GC/MS and UHPLC-PDA-MS/MS. *Journal of Agricultural and Food Chemistry* **2014**, *62*, 6435-6444.
94. Meloncelli, D. M.; Windsor, S. A. M.; Brooks, P. Chemical profiles of honeys originating from different floral sources and geographic locations examined by a combination of three extraction and analysis techniques. *Journal of Fundamental and Applied Sciences* **2015**, *7*, 169-184.
95. New Zealand honeys. https://apinz.org.nz/nz-honey/ (accessed November 9, 2021.
96. Mono-floral honeys. http://www.honeyland.co.nz/our-products/mono-floral-honeys/ (accessed November 9, 2021.
97. Oelschlaegel, S.; Gruner, M.; Wang, P. N.; Boettcher, A.; Koelling-Speer, I.; Speer, K. Classification and characterization of manuka honeys based on phenolic compounds and methylglyoxal. *Journal of Agricultural and Food Chemistry* **2012**, *60*, 7229-7237.
98. Novak, K. M. *Drug facts and comparisons*, 56 ed.; Wolters Klumer Health: St Louis, Missouri, 2002.
99. Acree, T. E.; Arn, H. Flavornet and human odor space. 2004.
100. Broom, S. J.; Wilkins, A. L.; Ede, R. M.; Lu, Y. Isolation and structural characterisation of Kamahine C: An unusual spiroketal found in a native New Zealand honey. *Tetrahedron Letters* **1992**, *33*, 6201-6204.
101. Tananaki, C.; Liolios, V.; Kanelis, D.; Rodopoulou, M. A. Investigation of volatile compounds in combination with multivariate analysis for the characterization of monofloral honeys. *Applied Sciences* **2021**, *12*
102. Broom, S. J.; Ede, R. M.; Wilkins, A. L. Synthesis of (±)- E-(1,2,4-trihydroxy-2,6,6-tirmethylcyclohexyl)-but-3-en-2-one: A novel degraded carotenoid isolated from New Zealand thyme (*Thymus vulgaris*) honey. *Tetrahedron Letters* **1992**, *33*, 3197-3200.
103. Airborne Honey. Consumer information: Vipers bugloss. https://www.airborne.co.nz/pages/vipers-bugloss (accessed October 12 2022).
104. Jerković, I.; Kuś, P. M. Terpenes in honey: occurrence, origin and their role as chemical biomarkers. *RSC Advances* **2014**, *4*, 31710-31728.
105. Yildiz, O.; Gurkan, H.; Sahingil, D.; Degirmenci, A.; Er Kemal, M.; Kolayli, S.; Hayaloglu, A. A. Floral authentication of some monofloral honeys based on volatile composition and physicochemical parameters. *European Food Research and Technology* **2022**
106. Serra Bonvehí, J.; Ventura Coll, F. Flavour index and aroma profiles of fresh and processed honeys. *Journal of the Science of Food and Agriculture* **2003**, *83*, 275-282.
107. Mendes-Pinto, M. M. Carotenoid breakdown products the-norisoprenoids-in wine aroma. *Archives of Biochemistry and Biophysics* **2009**, *483*, 236-245.
108. Panten, J.; Surburg, H. *Common fragrance and flavor materials: preparation, properties and uses*; John Wiley & Sons, Incorporated: Berlin, 2016.
109. Soria, A. C.; Martínez-Castro, I.; Sanz, J. Analysis of volatile composition of honey by solid phase microextraction and gas chromatography-mass spectrometry. *Journal of Separation Science* **2003**, *26*, 793-801.
110. Rodriguez-Flores, M. S.; Falcao, S. I.; Escuredo, O.; Seijo, M. C.; Vilas-Boas, M. Description of the volatile fraction of Erica honey from the northwest of the Iberian Peninsula. *Food Chemistry* **2021**, *336*, 127758.

111. Walsh, R. S. *Nectar and pollen sources of New Zealand: together with a list of suitable source trees for planting in various districts and a chapter on the function of the flower*, Fully rev. ed.; Wellington, N.Z. : National Beekeepers Association of New Zealand: Wellington, N.Z., 1978.
112. Anklam, E. A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chemistry* **1998**, *63*, 549-562.
113. Kaškonienė, V.; Venskutonis, P. R. Floral markers in honey of various botanical and geographic origins: a review. *Comprehensive Reviews in Food Science and Food Safety* **2010**, *9*, 620-634.
114. Food and Drug Administration. *Bioanalytical method validation: guidance for industry*. Food and Drug Administration: Maryland, USA, 2018.
115. Bicchi, C.; Belliardo, F.; Frattini, C. Identification of the volatile components of some Piedmontese honeys. *Journal of Apicultural Research* **1983**, *22*, 130-136.
116. Alissandrakis, E.; Tarantilis, P. A.; Harizanis, P. C.; Polissiou, M. Evaluation of four isolation techniques for honey aroma compounds. *Journal of the Science of Food and Agriculture* **2005**, *85*, 91-97.
117. Castro-Vázquez, L.; Perez-Coello, M. S.; Cabezudo, M. D. Analysis of volatile compounds of rosemary honey. Comparison of different extraction techniques. *Chromatographia* **2003**, *57*, 227-233.
118. Pino, J. A.; Fajardo, M. Volatile composition and key flavour compounds of spirits from unifloral honeys. *International Journal of Food Science & Technology* **2011**, *46*, 994-1000.
119. Kus, P. M.; Jerkovic, I. New sample preparation method for honey volatiles fingerprinting based on dehydration homogeneous liquid-liquid extraction (DHLLE). *Molecules* **2018**, *23*
120. Heischler. Ultrasonic extraction and its working principle. https://www.hielscher.com/ultrasonic-extraction-and-its-working-principle.htm#:~:text=Ultrasonic%20extraction%20is%20the%20preferred%20technique%20to%20isolate%20bioactive%20compounds%20from%20botanicals.&text=Being%20such%20an%20efficient%20extraction,for%20food%2C%20supplements%20and%20pharmaceuticals. (accessed February 21 2022).
121. Jerkovic, I.; Mastelic, J.; Marijanovic, Z.; Klein, Z.; Jelic, M. Comparison of hydrodistillation and ultrasonic solvent extraction for the isolation of volatile compounds from two unifloral honeys of *Robinia pseudoacacia L.* and *Castanea sativa L. Ultrason Sonochem* **2007**, *14*, 750-756.
122. Jerkovic, I.; Hegic, G.; Marijanovic, Z.; Bubalo, D. Organic extractives from Mentha spp. honey and the bee-stomach: methyl syringate, vomifoliol, terpenediol I, hotrienol and other compounds. *Molecules* **2010**, *15*, 2911-2924.
123. Jerković, I.; Radonić, A.; Kranjac, M.; Zekić, M.; Marijanović, Z.; Gudićc, S.; Kliškić, M. Red clover (*Trifolium pratense L.*) honey: volatiles chemical-profiling and unlocking antioxidant and anticorrosion capacity. *Chemical Papers* **2016**, *70*, 726-736.
124. Kuś, P. M.; Jerković, I.; Tuberoso, C. I. G.; Šarolić, M. The volatile profiles of a rare apple (*Malus domestica Borkh.*) honey: shikimic acid-pathway derivatives, terpenes, and others. *Chemistry & Biodiversity* **2013**, *10*, 1638-1652.
125. Sharin, S. N.; Sani, M. S. A.; Jaafar, M. A.; Yuswan, M. H.; Kassim, N. K.; Manaf, Y. N.; Wasoh, H.; Zaki, N. N. M.; Hashim, A. M. Discrimination of Malaysian stingless bee honey from different entomological origins based on physicochemical properties and volatile compound profiles using chemometrics and machine learning. *Food Chemistry* **2021**, *346*, 128654.

126. Nickerson, G. B.; Likens, S. T. Gas chromatographic evidence for the occurrence of hop oil components in beer. *Journal of Chromatography A* **1966**, *21*, 1-5.
127. Senyuva, H. Z.; Gilbert, J.; Silici, S.; Charlton, A.; Dal, C.; Gürel, N.; Cimen, D. Profiling Turkish honeys to determine authenticity using physical and chemical characteristics. *Journal of Agricultural and Food Chemistry* **2009**, *57*, 3911-3919.
128. Godefroot, M.; Sandra, P.; Verzele, M. New method for quantitative essential oil analysis. *Journal of Chromatography A* **1981**, *203*, 325-335.
129. Maignial, L.; Pibarot, P.; Bonetti, G.; Chaintreau, A.; Marion, J. P. Simultaneous distillation—extraction under static vacuum: isolation of volatile compounds at room temperature. *Journal of Chromatography A* **1992**, *606*, 87-94.
130. Bouseta, A.; Collin, S. Optimized Likens-Nickerson methodology for quantifying honey flavors. *Journal of Agricultural and Food Chemistry* **1995**, *43*, 1890-1897.
131. Zwir-Ferenc, A.; Biziuk, M. Solid phase extraction technique - Trends, opportunities and applications. *Polish Journal of Environmental Studies* **2006**, *15*, 677-690.
132. Jerković, I.; Marijanović, Z.; Kranjac, M.; Radonić, A. Comparison of different methodologies for detailed screening of *Taraxacum officinale* honey volatiles. *Natural Product Communications* **2015**, *10*, 1934578X1501000238.
133. Naef, R.; Jaquier, A.; Velluz, A.; Bachofen, B. From the linden flower to linden honey - Volatile constituents of linden nectar, the extract of bee-stomach and ripe honey. *Chemistry & Biodiversity* **2004**, *1*, 1870-1879.
134. Castro-Vázquez, L.; Díaz-Maroto, M. C.; de Torres, C.; Pérez-Coello, M. S. Effect of geographical origin on the chemical and sensory characteristics of chestnut honeys. *Food Research International* **2010**, *43*, 2335-2340.
135. Castro-Vázquez, L.; Díaz-Maroto, M. C.; Guchu, E.; Pérez-Coello, M. S. Analysis of volatile compounds of eucalyptus honey by solid phase extraction followed by gas chromatography coupled to mass spectrometry. *European Food Research and Technology* **2006**, *224*, 27-31.
136. Oroian, M.; Amariei, S.; Rosu, A.; Gutt, G. Classification of unifloral honeys using multivariate analysis. *Journal of Essential Oil Research* **2015**, *27*, 533-544.
137. Jerkovic, I.; Marijanovic, Z. A short review of headspace extraction and ultrasonic solvent extraction for honey volatiles fingerprinting. *Croatian Journal of Food Science and Technology* **2009**, *1*, 28-34.
138. Rowland, C. Y.; Blackman, A. J.; D'Arcy, B. R.; Rintoul, G. B. Comparison of organic extractives found in leatherwood (*Eucryphia lucida*) honey and leatherwood flowers and leaves. *Journal of Agricultural and Food Chemistry* **1995**, *43*, 753-763.
139. Tipler, A. *An introduction to headspace sampling in gas chromatography: fundamentals and theory.* PerkinElmer: Waltham, USA, 2014.
140. Radovic, B. S.; Careri, M.; Mangia, A.; Musci, M.; Gerboles, M.; Anklam, E. Contribution of dynamic headspace GC±MS analysis of aroma compounds to authenticity testing of honey. *Food Chemistry* **2001**, *72*, 511-520.
141. Zhu, Z.; Zhang, Y.; Wang, J.; Li, X.; Wang, W.; Huang, Z. Sugaring-out assisted liquid-liquid extraction coupled with high performance liquid chromatography-electrochemical detection for the determination of 17 phenolic compounds in honey. *Journal of Chromatography A* **2019**, *1601*, 104-114.
142. Merck. Carbopack™ adsorbent. https://www.sigmaaldrich.com/NZ/en/product/supelco/20273 (accessed December 12 2021.
143. Merck. Porapak™ porous polymer adsorbent. https://www.sigmaaldrich.com/NZ/en/product/SUPELCO/20332 (accessed December 6, 2021.

144. Arthur, C. L.; Pawliszyn, J. Solid phase microextraction with thermal desorption using fused silica optical fibers. *Analytical Chemistry* **1990**, *62*, 2145-2148.
145. Górecki, T.; Yu, X.; Pawliszyn, J. Theory of analyte extraction by selected porous polymer SPME fibres. *Analyst* **1999**, *124*
146. Merck. SPME fiber assembly divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS). https://www.sigmaaldrich.com/NZ/en/product/supelco/57328u (accessed August 23, 2022.
147. Zhang, Z.; Pawliszyn, J. Headspace solid-phase microextraction. *Analytical Chemistry* **1993**, *65*, 1843-1852.
148. Câmara, J. S.; Alves, M. A.; Marques, J. C. Development of headspace solid-phase microextraction-gas chromatography–mass spectrometry methodology for analysis of terpenoids in Madeira wines. *Analytica Chimica Acta* **2006**, *555*, 191-200.
149. Piasenzotto, L.; Gracco, L.; Conte, L. Solid phase microextraction (SPME) applied to honey quality control. *Journal of the Science of Food and Agriculture* **2003**, *83*, 1037-1044.
150. Daher, S.; Gulacar, F. O. Identification of new aromatic compounds in the New Zealand manuka honey by gas chromatography-mass spectrometry. *E-Journal of Chemistry* **2010**, *7*, S7-S14.
151. Perez, R. A.; Sanchez-Brunete, C.; Calvo, R. M.; Tadeo, J. L. Analysis of volatiles from Spanish honeys by solid-phase microextraction and gas chromatography-mass spectrometry. *Journal of Agricultural and Food Chemistry* **2002**, *50*, 2633-2637.
152. Baroni, M. V.; Nores, M. L.; Díaz, M. D. P.; Chiabrando, G. A.; Fassano, J. P.; Costa, C.; Wunderlin, D. A. Determination of volatile organic compound patterns characteristic of five unifloral honey by solid-phase microextraction−gas chromatography−mass spectrometry coupled to chemometrics. *Journal of Agricultural and Food Chemistry* **2006**, *54*, 7235-7241.
153. Dekebo, A.; Kwon, S.-Y.; Kim, D.-H.; Jung, C. Volatiles analysis of honey by gas chromatography-mass spectrometry (GC-MS): comparison of SPME volatiles extraction methods. *Journal of Apiculture* **2018**, *33*, 117-128.
154. Karabagias, I. K.; Badeka, A.; Kontakos, S.; Karabournioti, S.; Kontominas, M. G. Characterisation and classification of Greek pine honeys according to their geographical origin based on volatiles, physicochemical parameters and chemometrics. *Food Chemistry* **2014**, *146*, 548-557.
155. Karabagias, I. K.; Nikolaou, C.; Karabagias, V. K. Volatile fingerprints of common and rare honeys produced in Greece: in search of PHVMs with implementation of the honey code. *European Food Research and Technology* **2019**, *245*, 23-39.
156. Pattamayutanon, P.; Angeli, S.; Thakeow, P.; Abraham, J.; Disayathanoowat, T.; Chantawannakul, P. Volatile organic compounds of Thai honeys produced from several floral sources by different honey bee species. *PloS One* **2017**, *12*, e0172099-e0172099.
157. Starowicz, M.; Hanus, P.; Lamparski, G.; Sawicki, T. Characterizing the volatile and sensory profiles, and sugar content of beeswax, beebread, bee pollen, and honey. *Molecules* **2021**, *26*
158. Heinonen, J.; Sainio, T. Chapter five - chromatographic fractionation of lignocellulosic hydrolysates. In *Advances in Chemical Engineering*; D. Y. Murzin, Ed.; Academic Press, 2013; pp 261-349.
159. Sukola, K.; Koziel, J.; Augusto, F.; Pawliszyn, J. Diffusion-based calibration for SPME analysis of aqueous samples. *Analytical Chemistry* **2001**, *73*, 13-18.

160. de Lima, M.; da Silva, P.; de Lima, L. S.; Caetano, Í. K.; Torres, Y. R. Comparative analysis of the volatile composition of honeys from Brazilian stingless bees by static headspace GC–MS. *Food Research International* **2017**, *102*, 536-543.
161. Rowland, C. Y.; Blackman, A. J.; D'Arcy, B. R.; Rintoul, G. B. Comparison of organic extractives found in leatherwood (Eucryphia lucida) honey and leatherwood flowers and leaves. *Journal of Agricultural and Food Chemistry* **1995**, *43*, 753-763.
162. Chen, H.; Jin, L.; Fan, C. *Chemometric methods for botanical classification of Chinese honey based on the volatile compound profile.* Agilent Application Note 5991-8967: Beijing, China, 2018.
163. Graddon, A. D.; Morrison, J. D.; Smith, J. F. Volatile constituents of some unifloral Australian honeys. *Journal of Agricultural and Food Chemistry* **1979**, *27*, 832-837.
164. Turner, D. C.; Schäfer, M.; Lancaster, S.; Janmohamed, I.; Gachanja, A.; Creasey, J. *Gas chromatography–mass spectrometry: how do I get the best results?*; Royal Society of Chemistry, 2019.
165. Reineccius, G. *Flavor chemistry and technology*, 2nd ed.; CRC Press: Florida, 2005.
166. Kus, P. M.; Jerkovic, I. Application of the dehydration homogeneous liquid-liquid extraction (DHLLE) sample preparation method for fingerprinting of honey volatiles. *Molecules* **2021**, *26*
167. Afirm Group. Butylated hydroxytoluene (BHT). Chemical Information Sheet, 2021.
168. Hossaini, R.; Chipperfield, M. P.; Montzka, S. A.; Rap, A.; Dhomse, S.; Feng, W. Efficiency of short-lived halogens at influencing climate through depletion of stratospheric ozone. *Nature Geoscience* **2015**, *8*, 186-190.
169. Bonaga, G.; Giumanini, A. G.; Gliozzi, G. Chemical composition of chestnut honey: Analysis of the hydrocarbon fraction. *Journal of Agricultural and Food Chemistry* **1986**, *34*, 319-326.
170. Taylor, T. Pragmatic rules for GC column selection. *LCGC North America* **2015**, *33*, 150.
171. Senanayake, M. J. A chemical investigation of New Zealand unifloral honeys. Thesis, The University of Waikato, Hamilton, New Zealand, 2006.
172. PerkinElmer. What is peak fronting? https://www.perkinelmer.com/library/what-is-peak-fronting.html (accessed November 9 2022).
173. Swartz, M. E.; Krull, I. S. *Handbook of analytical validation*; CRC Press: Baton Rouge, 2012.
174. Crawford Scientific Correct weighting for regression analysis in analytical calibration. Element (formerly Crawford Scientific) Blog, 2018.
175. Almeida, A. M.; Castel-Branco, M. M.; Falcão, A. C. Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods. *Journal of Chromatography B* **2002**, *774*, 215-222.
176. Alankar, S.; Vipin, B. G. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of Young Scientists* **2011**, *2*, 21-25.
177. Burns, D. T.; Dillon, A.; Warren, J.; Walker, M. J. A critical review of the factors available for the identification and determination of mānuka honey. *Food Analytical Methods* **2018**, *11*, 1561-1567.
178. Bell, A. Personal Communication, 2022.
179. Adams, C. J.; Boult, C. H.; Deadman, B. J.; Farr, J. M.; Grainger, M. N. C.; Manley-Harris, M.; Snow, M. J. Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (*Leptospermum scoparium*) honey. *Carbohydrate Research* **2008**, *343*, 651-659.

180. The Good Scents Company information system. http://www.thegoodscentscompany.com/index.html (accessed February 12 2023).
181. Garvey, K. K. A taste of honey - can you describe it? https://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum=14862 (accessed 6 September 2021).
182. Aldred, E. M.; Buck, C.; Vall, K. Terpenes. In *Pharmacology*; E. M. Aldred; C. Buck and K. Vall, Eds.; Churchill Livingstone: Edinburgh, 2009; pp 167-174.
183. Alissandrakis, E.; Tarantilis, P. A.; Harizanis, P. C.; Polissiou, M. Aroma investigation of unifloral Greek citrus honey using solid-phase microextraction coupled to gas chromatographic–mass spectrometric analysis. *Food Chemistry* **2007**, *100*, 396-404.
184. Higdon, J.; Drake, V. J.; Delage, B.; Johnson, E. J. Carotenoids. https://lpi.oregonstate.edu/mic/dietary-factors/phytochemicals/carotenoids (accessed 29 October 2021.
185. Karabagias, I. K. A targeted metabolomic procedure for the identification of isophorone related compounds in honey. *Journal of Plant Biochemistry & Physiology* **2018**, *6*, 1-6.
186. de la Fuente, E.; Martínez-Castro, I.; Sanz, J. Characterization of Spanish unifloral honeys by solid phase microextraction and gas chromatography-mass spectrometry. *Journal of Separation Science* **2005**, *28*, 1093-1100.
187. Guyot, C.; Schierman, V.; Collin, S. Floral origin markers of heather honeys: *Calluna vulgaris* and *Erica arborea*. *Food Chemistry* **1999**, *64*, 3-11.
188. Guyot, C.; Collin, S.; Bouseta, A.; Schierman, V. Floral origin markers of chestnut and lime tree honeys. *Journal of Agricultural and Food Chemistry* **1998**, *46*, 625-633.
189. Jerković, I.; Kuś, P. M.; Carbonell-Barrachina, Á. A. Volatile organic compounds as artefacts derived from natural phytochemicals sourced form plants and honey. *Phytochemistry Reviews* **2019**, *18*, 871-891.
190. Deifel, A. Die chemie des honigs. *Chemie in unserer Zeit* **1989**, *23*, 24-33.
191. Beckmann, K.; Beckh, G.; Lüllmann, C.; Speer, K. Phenylacetaldehyde in honey—residue or natural compound. *Deutsche Lebensmittel Rundschau* **2007**, *103*, 154-158.
192. Jerković, I.; Tuberoso, C. I. G.; Kasum, A.; Marijanović, Z. Volatile compounds of *Asphodelus microcarpus Salzm. et Viv*. honey obtained by HS-SPME and USE analyzed by GC/MS. *Chemistry & Biodiversity* **2011**, *8*, 587-598.
193. Ohloff, G.; Pickenhagen, W.; Lawrence, B. M. *Scent and fragrances: the fascination of odors and their chemical perspectives*; Springer Berlin Heidelberg, 1994.
194. Wang, J.; Zhang, N.; Zhao, M.; Jing, T.; Jin, J.; Wu, B.; Wan, X.; Schwab, W.; Song, C. Carotenoid cleavage dioxygenase 4 catalyzes the formation of carotenoid-derived volatile β-ionone during tea (*Camellia sinensis*) withering. *Journal of Agricultural and Food Chemistry* **2020**, *68*, 1684-1690.
195. Goss, C. H. A. Indicators of bioactivity and floral origin of New Zealand honeys. Doctoral Thesis, University of Waikato, Hamilton, New Zealand, 2009.
196. Jerković, I.; Marijanović, Z. Oak (*Quercus frainetto Ten.*) honeydew honey—approach to screening of volatile organic composition and antioxidant capacity (DPPH and FRAP assay). *Molecules* **2010**, *15*, 3744-3756.
197. Castro-Vázquez, L.; Díaz-Maroto, M. C.; Pérez-Coello, M. S. Volatile composition and contribution to the aroma of Spanish honeydew honeys. Identification of a new chemical marker. *Journal of Agricultural and Food Chemistry* **2006**, *54*, 4809-4813.
198. Kuś, P. M.; Jerković, I.; Marijanović, Z.; Tuberoso, C. I. G. Screening of Polish fir honeydew honey using GC/MS, HPLC-DAD, and physical-chemical parameters: benzene derivatives and terpenes as chemical markers. *Chemistry & Biodiversity* **2017**, *14*, e1700179.

199. Choi, H.-S. Volatile flavour composition of white-flowered lotus by solid-phase microextraction. *The Korean Journal of Food and Nutrition* **2017**, *30*, 363-370.
200. Georgilopoulos, D. N.; Gallois, A. N. Über die aromastoffzusammensetzung von brombeeren (*Rubus laciniata L.*). *Zeitschrift für Lebensmittel-Untersuchung und Forschung* **1987**, *184*, 374-380.
201. Beebe, K. R.; Pell, R. J.; Seasholtz, M. B. *Chemometrics: a practical guide*; John Wiley & Sons, Inc.: New York, 1998.
202. Alissandrakis, E.; Mantziaras, E.; Tarantilis, P.; Harizanis, P.; Polissiou, M. Generation of linalool derivatives in an artificial honey produced from bees fed with linalool-enriched sugar syrup. *European Food Research and Technology* **2010**, *231*, 21-25.
203. IUPAC. *Compendium of chemical terminology (the "Gold Book").* 2nd ed.; Blackwell Scientific Publications: Oxford, UK, 1997.
204. Guyot-Declerck, C.; Renson, S.; Bouseta, A.; Collin, S. Floral quality and discrimination of *Lavandula stoechas, Lavandula angustifolia,* and *Lavandula angustifolia x latifolia* honeys. *Food Chemistry* **2002**, *79*, 453-459.
205. Quintanilla-López, J. E.; Lebrón-Aguilar, R.; Soria, A. C. Volatile fingerprinting by solid-phase microextraction mass spectrometry for rapid classification of honey botanical source. *Lebensmittel-Wissenschaft & Technologie* **2022**, *169*, 114017.
206. Wang, D.; Ando, K.; Morita, K.; Kubota, K.; Kobayashi, A. Optical isomers of linalool and linalool oxides in tea aroma. *Bioscience, Biotechnology, and Biochemistry* **1994**, *58*, 2050-2053.