An investigation of low diastase activity in mānuka honey

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Abstract

Mānuka honey with high non-peroxide antibacterial (NPA) activity is a valuable export product in the multi-million-dollar New Zealand honey export industry, with monofloral mānuka honey accounting for 68% (NZ$290 million) of the total export value (NZ$425 million) for the 2019/20 season. Exported honey must meet strict export regulations. Diastase enzyme (alpha-amylase) activity is a quality control indicator of detrimental storage conditions (excessive heat treatment or prolonged storage), decreasing over time. International export requires honey to have high diastase activity (Diastase Number, DN > 8). Medium to high NPA mānuka honeys often fail the diastase test, despite careful storage. It is important to determine the cause of low diastase activity in mānuka honey to prevent revenue loss for New Zealand honey exports. Bioactive compounds in the honey were hypothesised to inhibit alpha-amylase, therefore reducing diastase activity.

A database of fresh mānuka and non-mānuka honeys were analysed for various chemical parameters (including moisture content, mānuka chemical marker compounds, dihydroxyacetone (DHA), methylglyoxal (MGO), 5-hydroxymethylfurfural (HMF) and diastase activity) to determine if any correlations were present with diastase activity. Parameters between and within floral honey types varied largely, however no correlations were observed to diastase activity.

Storage experiments (20 °C, 161 days; 27° C, 198 days) of selected database honeys showed the mānuka marker compounds were stable. However, the rates of change observed for DHA, MGO, HMF and diastase activity were reliant on intrinsic honey characteristics (not identified). While time and temperature had significant impact on loss of diastase activity in honey, correlation was observed between changes in diastase activity and DHA, MGO and mānuka marker 3-phenyllactic acid (3-PLA) concentrations.

Additionally, a clover honey matrix was perturbed with single bioactive compounds (DHA (MGO-precursor), MGO, mānuka markers compounds, methyl syringate and tannic acid) to observe the effect on diastase activity over time (185 days) and at 20, 27 and 34 °C. MGO and 3-PLA caused diastase activity to decrease at a significantly faster rate than other compounds. The half-life of diastase at 27 °C for the control sample was 590 days compared to the much shorter half-life of diastase in the MGO (440 days) and

3-PLA (380 days) perturbed samples. The mechanism by which these two compounds decrease diastase activity is unknown, however MGO is a highly reactive molecule and has been reported to inhibit other enzymes. Time and temperature were also significant drivers of diastase activity loss; diastase half-life at 20 °C was more than eight times greater than it was at 34 °C (1340 vs. 160 days).

The experiments indicate that diastase activity is highly variable between fresh honeys and the rate at which it decreases may be dependent not only on temperature and time honey is stored at, but on intrinsic honey compounds and physico-chemical parameters suggesting that diastase activity regulations may not be suitable for mānuka honey.

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List of Abbreviations

°C	Degrees Celsius
2'MAP	2'-Methoxyacetophenone
2-MBA	2-Methoxybenzoic acid
3-PLA	3-Phenyllactic acid
4-HPLA	4-Hydroxyphenyllactic acid
ACN	Acetonitrile
AGEs	Advanced glycation end products
AH	Artificial honey
AOAC	Association of Official Analytical Chemists
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
A_w	Water activity
CE	Capillary electrophoresis
DAD	Diode array detection
DB	Database
DBSH	Database snapshot
DBT	Database trials
DHA	Dihydroxyacetone
DLLME	Dispersive liquid-liquid microextraction
DN	Diastase number
DP	Degrees of polymerisation
ECD	Electrochemical detection
ESI	Electron spray ionisation
FLD	Fluorescence detection
GAE	Gallic acid equivalents
GC	Gas chromatography
Glu	Glutamic acid
HA	Hydroxyacetone
His	Histidine
h-LLE	Homogenous liquid-liquid extraction
HMF	5-Hydroxymethylfurfural
HPLC	High performance liquid chromatography
IC50	Half maximal inhibitory concentration
IDLLME	Inverse liquid-liquid microextraction
IHC	International Honey Commission
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LSR	Least squares regression analysis

MeOH	Methanol
MGO	Methylglyoxal
MPI	Ministry for Primary Industries
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MStd	Mixed standard
MSY	Methyl syringate
NMR	nuclear magnetic resonance spectroscopy
NPA	Non-peroxide antibacterial activity
OPD	*O*-phenylenediamin*e*
PDA	Photodiode array detection
PFBHA	*O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine
PPA	Porcine pancreatic alpha-amylase
QC	Quality control
Q-TOF	Quadrupole-time of flight
QuEChERS	Quick, easy, cheap, effective, rugged, and safe method
RCF	Relative centrifugal force
Ref.	Reference
RP	Reversed phase
RSD	Relative standard deviation
RT	Retention time
SALLE	Salting-out assisted liquid-liquid extraction
SPE	Solid phase extraction
Std	Standard
SULLE	Sugaring-out assisted liquid-liquid extraction
TA	Tannic acid
TFC	Total flavonoid content
TPC	Total phenolic content
UHPLC	Ultra-high performance liquid chromatography
UMF™	Unique Mānuka Factor
UMFHA	Unique Mānuka Factor Honey Association
UV	Ultraviolet detection
vs.	Versus
WHO	World Health Organisation

1 Introduction and Literature Review

1.1 Introduction

Mānuka honey with high non-peroxide antibacterial (NPA) activity is a valuable export product for New Zealand, worth millions of dollars each year,[1] as such, this research is carried out for industry. A critical component for exportation of honey is passing the export regulations applied by both New Zealand and the destination country. One important criterion for the quality of exported honey is that honey must not be excessively heated nor stored for long periods of time. Poor quality can be indicated by low diastase enzyme activity and high 5-hydroxymethylfurfural (HMF) concentration. Some high grade mānuka honeys fail to pass the diastase activity (a measure of alpha-amylase activity) requirements even with acceptable HMF levels, therefore cannot be exported. Bioactive compounds (phenolic compounds, methylglyoxal (MGO) and MGO precursor dihydroxyacetone (DHA)) have been implicated in several studies to inhibit the activity of diastase,[2-6] thus will be investigated in this research.

1.2 Honey

1.2.1 Honey production

Honey is the high-energy, natural food source that the honey bee (*Apis mellifera*) produces from the nectar or excretions of plants.[7] Bees produce honey for storage in their hives to ensure they have a high energy feedstock to keep the hive fed during times where there are very few resources available in the environment.[8-11] It is crucial for honey to be stable and resistant to degradation over its storage period in the comb, hence nectar undergoes a significant transformation through processes catalysed by bees, altering nectar composition to form the substance known as honey.[10] The standardised definition of honey, as stated in the international food standard Codex Alimentarius[12], is as follows:

"Honey is the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature."[12]

Honey can be defined as floral honey (also blossom or nectar honey) when produced from plant nectar, or as honeydew honey when produced from the insect excretions and non-nectar plant secretions. Bees collect nectar through their proboscis, storing it in their honey stomach (up to 60 mg of nectar), for transportation back to the hive.[13] Two important processes happen to ripen the nectar to honey: enzymatic loading induces physicochemical transformation of sucrose into monosaccharides and simultaneous dehydration reduces moisture content to concentrate the sugar.[11] Storage of nectar within the honey stomach initiates the conversion of nectar to honey through an influx of enzymes into the nectar from the stomach and hypopharyngeal glands.[14]

In the hive, nectar is transferred to house bees,[15] who digest it further to increase the nectar's enzyme load and hasten ripening. Nectar is deposited into wax cells where passive and active processes are used to dehydrate it; hive temperature (approx. 35 °C),[16] airflow production from wing fanning and the transfer of unripened honey between cells by workers.[17] Once the honey has been dehydrated to a moisture content of 18 to 25%, full cells will be capped with wax and left to continue ripening.[10] Low moisture content, and therefore water activity (due to the hygroscopic nature of honey), prevents honey susceptibility to fermentation and spoilage by bacteria, yeast or mould, making honey one of the few natural food products that will not spoil if stored correctly.[18]

1.2.2 Beekeeping

Humans have taken advantage of honey as a food source since ancient times (earliest beekeeping records 2500 BC, Ancient Egypt), evolving into today's market where bees are used worldwide to produce a wide variety of floral honey types (mono- and multi-). European settlement in New Zealand saw the arrival of the first honeybees in 1839, with the first of many recorded bulk shipments of bees arriving in 1842. The New Zealand beekeeping industry has thrived ever since.[19]

In the past six years the number of registered beekeepers in New Zealand has almost doubled, from 4,814 in 2014 to 9,585 in 2020, a 99% increase. The number of registered beehives observed a smaller increase with a 71% rise over six years, from 507,247 in 2014 to 869,056 in 2020.[1]

Commercial honey operations extract and process honey, removing comb debris, so that it is suitable for consumers. During processing, honey requires heating between 30 and 40 °C due to its viscosity, before being strained through progressively smaller filters to remove debris, then packaged for retail. Alternatively, comb honey is also retailed for consumption, preventing the need for heating and straining.[19]

Honey demand has changed quite extensively over the years: initial preference was dependant on taste (light and smooth vs. dark, strong flavours), however improvements in honey analysis, particularly on honey medicinal properties has vastly increased demand for other honey types, especially mānuka honey (Section 1.4).

1.2.3 Composition and physico-chemical properties of honey

The major components in honey (glucose, fructose and water) are relatively consistent between floral types, however, minor components depend on a number of factors (e.g. floral origin, species variation, season, geographical location) and can vary significantly.[20] The minor components include nitrogenous substances (amino acids and protein), minerals, phenolic compounds and vitamins.[21]

A review by Solayman et al. (2016)[20] compiled results of approximately 1000 honey samples from around the world to determine the average values as well as the variation range of the main honey components and properties. There is some discrepancy in these values when compared to other studies, such as White (1962)[22] who examined 200 American honeys, showing how variable honey can be (Table 1.1).

Table 1.1. Comparison of honey composition and physicochemical properties from two different sources.

	Solayman et al. (2016)[20]		White et al. (1962)[22]	
Constituent	Average value (%, n = 1000)	Variation range (%)	Average value (%, n = 200)	Variation range (%)
Moisture	17.9	13.2-26.5	17.2	13.4-22.9
Fructose	39.4	37.1-42.7	38.2	27.3-44.3
Glucose	28.2	18.2-32.1	31.3	22.0-40.8
Sucrose	3.2	0.4-16.6	1.3	0.3-7.6
Other sugars	8.5	0.1-16.0	8.8	2.8-24.5
Nitrogenous compounds	1.13	0.22-2.93	0.04	0.00-0.13
Minerals (ash)	0.36	0.11-0.72	0.17	0.02-1.03
pH Value	3.9	3.4-4.7	3.9	3.4-6.1
Diastase value	14.27	0.40-22.08	20.8	2.1-61.2

1.2.3.1 Sugar

Honey is made up of 80-83% carbohydrates on average.[23] Sucrose, which is the main sugar in nectar,[24] is hydrolysed by enzymes into fructose and glucose as honey is ripened, resulting in an average of 1.3-3.2% in mature honey.[20, 22] Fructose and glucose are the primary sugars in honey, although maltose concentration can also be high (7.3%).[18] Literature shows a range of minor sugars have been found in honey (with disparity due to nectar variation), including maltulose, kojibiose, nigerose, isomaltose and maltotriose.[25-26] Oligosaccharides are also found in honey and can indicate nectar or honeydew origin due to transglucosylation enzymes in scale insects stomachs making the honeydew oligosaccharides more complex and larger than those that occur in nectar.[26]

1.2.3.2 Water content

Water content of honey is generally less than 20%, with an average of 17.2-17.9%,[20, 22] therefore it is stable over long periods of storage when correctly stored since the low water activity prevents bacterial growth and yeast fermentation. Honey pasteurisation kill yeasts, however unpasteurised honey usually does not ferment if water content is less than 19%.[27] Water content affects other honey properties including colour, crystallisation, density and viscosity,[18] and also reactions in honey as low water content limits available H^+ which is known to help facilitate reactions.[28]

1.2.3.3 Nitrogenous compounds

Amino acids

Honey nitrogenous compounds comprise of free amino acids, proteins and enzymes. Free amino acids make up approximately 1% of honey, with proline being the most abundant (50-85% of total amino acids), mainly occurring from bee secretion rather than botanical origin.[29] Proline varies greatly in honey, however a minimum of 180 mg/kg is required by the International Honey Commission (IHC) as lower levels suggest either unripe honey or honey adulteration; proline becomes more concentrated as the honey ripens. Other amino acids (alanine, leucine, histidine etc.) which are integrated from the nectar or pollen could be used to determine floral type due to variation in nectar amino acid profiles.[29] Other nitrogenous compounds include nucleosides and their derivatives.[30]

Proteins and enzymes

Proteins make up approximately 40-80% nitrogenous compounds in honey, primarily from the salivary and hypopharyngeal glands of bees, although some are of plant origin (nectar and pollen).[23, 31] Enzymes are the dominant protein group, with non-enzymatic proteins mainly consisting of the royal jelly proteins.[31-32] The main enzymes of interest found in honey are glucose oxidase, invertase, and diastase (alpha- and beta- amylase), while other less important enzymes include catalase (which decomposes H_2O_2)[33] and acid phosphatase.[23, 34] Glucose oxidase is responsible for the conversion of glucose to gluconic acid, producing hydrogen peroxide (H_2O_2) as a by-product, both the acidity and H_2O_2 preventing bacterial contamination in the ripening honey. Invertase is responsible for hydrolysing sucrose during ripening, resulting in a supersaturated sugar solution (honey); a rich energy source that is unlikely to spoil.[18] Diastase catalyses hydrolysis of starch into dextrins and mono-, di-, and oligo-saccharides, and is one of the properties that is an indicator of honey quality (refer to Chapter 4 for an in-depth review on diastase).[23]

1.2.3.4 Organic acids and pH

Due to the presence of glucose oxidase, the predominant organic acid in honey is gluconic acid from the oxidation of glucose as the honey ripens (see Section 1.3.2 for more details). More than 30 other acids can also be found in honey, including formic, citric, malic, succinic, acetic, butyric, lactic and pyroglutamic acids.[35] The presence of these acids make honey acidic, with pH ranging from 3.4 to 4.7.[20]

1.2.3.5 Minerals and trace elements

Minerals found in honey (0.04 – 0.2%)[36] are a result of expression of elements in the nectar due to uptake by the plant from the soil or from anthropogenic sources. Elemental profiles are heavily dependent on floral origin but also on geographical location, with research showing that elemental fingerprints could be a useful tool for authentication of honey botanical origin and geographical traceability.[36-38] The most abundant elements found in honey are potassium, phosphorus and calcium, with elevated potassium levels indicative of a honeydew.[39] Other minerals found in honey include but are not limited to sodium, magnesium, copper, iron, manganese, zinc and cadmium.[23, 38-39]

1.2.3.6 Maillard reaction products

The Maillard reaction is a non-enzymatic browning reaction which occurs in honey, a process that is sped up when honey is heated.[40] Interactions between a reducing sugars carbonyl groups and amino acid, or protein amino groups, result in a wide variety of brown products and by-products. The Maillard reaction is an extremely complex network of reactions that can be divided into three stages; the initial (reversible) stage produces colourless products, the intermediate stage produces colourless or yellow products, and the final stage producing highly coloured brown products. Honey that is stored or heated will progressively become darker in colour due to accumulation of the final stage products.[40]

1.2.3.7 Other compounds

Other compounds in honey can be divided into three groups: vitamins, aroma compounds and phenolic compounds. Ascorbic acid is the primary vitamin found in almost all honey types, however B complex vitamins (thiamine, niacin, pantothenic acid and pyridoxine) can also be present.[23, 41] The aroma compounds (over 600 identified in honey) cover a wide range of chemical families including acids, hydrocarbons, ketones, terpenoids, esters, benzene derivatives and cyclic compounds.[42] Phenolic compounds (predominantly phenolic acids and flavonoids) are an important class of compounds in honey, as a major phytonutrient, they have been shown to influence honey medicinal properties.[43-44] Phenolic compounds (as secondary plant metabolites) are characteristic or indicative of botanical origin, and as a key part of this research are reviewed in-depth in Section 1.7.

1.2.3.8 Colour

Honey colour can vary from almost white in colour (pale cream) to a dark, almost black shade. The colour of the honey is primarily due to the presence of plant pigments, several studies have found correlation between polyphenol content and honey colour, while others correlate it to mineral content.[45-47]

1.2.3.9 Viscosity and thixotropy

Honey is a viscous liquid, greatly hindering honey processing. Viscosity can be influenced by factors such as water content and nectar source, as well as temperature. For processing, honey is generally heated to approximately 40 °C to make pumping, straining and filtering of bulk honey easier.[27] Thixotropic honeys, such as mānuka and

heather honey (*Calluna vulgaris*),[48-49] are also an issue; colloidal protein in the honey causes a gel-like state that becomes more fluid only once physically agitated. Thixotropic honeys are hard to extract from the comb and thus may require specialised agitator equipment to maximise yields. The temperature of processing has the potential to affect other honey components.

1.3 Medicinal properties of honey

Honey is a unique substance that has been utilised for nutritional and medicinal purposes since ancient times, and continues to be used currently, due to its broad-spectrum antimicrobial properties in the fight against antibiotic resistant bacteria.[21, 50-51]

As a wound dressing, honey creates a physical barrier (keeping the wound moist), stimulates immune response, is anti-inflammatory, reduces scarring, assists wound debridement activity, and helps prevent infection (antibiotic).[21, 52] Various studies have found honey to be anti-bacterial, anti-inflammatory, antioxidant, antitumor, antimutagenic, antiviral and a prebiotic.[7, 53-56]

Several main properties contribute to the medicinal activity of honey, including osmotic effect, pH and hydrogen peroxide.[57] Phenolic compounds have also been found to contribute to the medicinal properties of honey, and will be discussed further in Section 1.7.2. Due to the synergistic medicinal action of honey, it is unlikely that bacteria will develop resistance to its action.

1.3.1 Osmotic effect

Due to its supersaturated sugar state, honey exerts high osmotic pressure; free water molecules interact strongly with the hygroscopic monosaccharides, limiting the free water[*] in the honey and inhibiting bacterial growth and activity.[58] Free water content can be measured by water activity (A_w), which is a ratio between 0 (no free water) and 1 (pure water). Most bacteria grow freely with A_w of 0.98 or greater, although certain bacterial strains are viable in lower water activity (Table 1.2).[59] Water activity in honey generally ranges between 0.50-0.65,[60] well below the threshold required for the growth of most microorganisms that lead to spoilage or infections; below a water activity of 0.6,

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[*] Non-bound water molecules, available for chemical and biological reactions.

no microbial growth will occur.[18] Dilution causes osmotic effect to be neutralised, such as honey application to wounds and subsequent wound seepage. While bacteria require high water activity to thrive, yeasts can survive in low water activity, with certain species capable of fermenting if honey water content is higher than 19%.[18]

Table 1.2 Water activity limit for growth of microorganisms. Reproduced from Majumdar *et al.* (2018).[59]

Water activity (A_W)	Microorganism	Example
0.91	Most bacteria	*E. coli*
0.88	Most yeasts	*Candida spp.*
0.80	Most moulds	*Aspergillus flavus*
0.75	Halophilic bacteria	*Wallemia sebi*
0.65	Xerophilic mould	*Aspergillus echinulatas*
0.60	Osmophilic yeast	*Saccharomyces bisporus*

1.3.2 pH

Honey is acidic (approximately pH 3.9)[20, 22] due to the formation of gluconic acid and gluconolactone during honey ripening. Glucose oxidase catalyses the formation of gluconic acid via gluconolactone from glucose (Figure 1.1).[61] This either partially or fully inhibits many pathogenic bacteria whose optimum growth pH is approximately neutral (pH 7).[62] Gluconic acid production is restricted in non-dilute honey due to the inhibitory effects of low water activity, low viscosity, high glucose concentration, low oxygen concentration and hydrogen peroxide competition.[61] When honey is applied to a wound, the dilution from wound seepage renders the pH neutral and the enzyme is reactivated, allowing for the continued production of gluconic acid and more importantly, hydrogen peroxide.[63]

α-D-Glucose   β-D-Glucose   D-Glucono-1,5-lactone   Gluconic acid
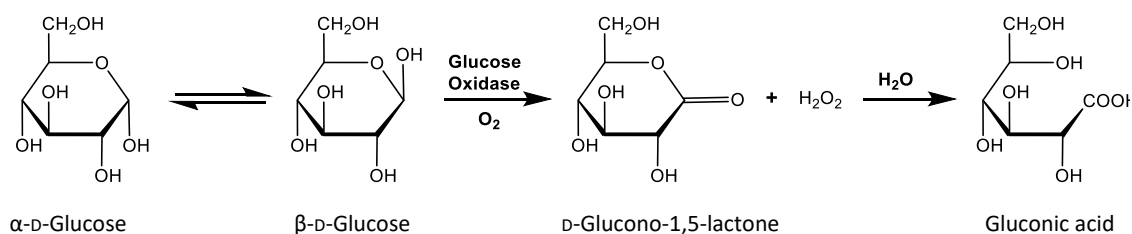
Figure 1.1. Scheme of glucose oxidase catalysing the reaction of glucose to gluconic acid via gluconolactone, with the formation of hydrogen peroxide (adopted from Brudzynski, 2020)[61]

1.3.3 Hydrogen peroxide

The activity of glucose oxidase also produces H_2O_2 (Figure 1.1).[61] Hydrogen peroxide is cytotoxic in high concentrations as it easily forms radicals that can damage cellular components and DNA.[64] In honey, hydrogen peroxide is dilute and slowly released, reducing risk of tissue damage when applied to wounds and instead stimulating blood vessel growth and repairing tissue damage, while also exhibiting high antibiotic activity.[65] It is the primary contributor to the overall antibacterial property in honey.[50]

In non-dilute honey, the effects of hydrogen peroxide are minimal due to the inhibition of glucose oxidase.[61] Honey dilution increases glucose oxidase activity, rapidly producing more hydrogen peroxide, however the dilution factor for maximum hydrogen peroxide level production is dependent on the honey type and generally ranges between 30-50% (*v/v*) dilution.[66] Further dilution past the maximum production results in decreasing levels, an inverted U-shaped relationship exists between H_2O_2 production and dilution.[61]

Catalase, an enzyme that occurs naturally in some floral honey types, neutralises hydrogen peroxide. Glucose oxidase is inactivated by heat and light, thus incorrect storage of honey can also impact peroxide levels.[61] Due to these factors, peroxide activity can be lost during honey ripening and can contribute to the inconsistency of activity across honey types.

1.3.4 Other bioactive compounds

There is ongoing research on other bioactive compounds found in honey that have potential health benefits, such as phenolic compounds which have been shown to exert strong anti-inflammatory action.[18, 21, 52]

1.4 Mānuka honey

Mānuka honey is produced from the nectar of the native New Zealand shrub *Leptospermum scoparium*, commonly known as mānuka, which is found widely across NZ. The leptospermum genus belongs to the Myrtaceae family, a family of dicotyledon plants containing 83 species distributed across NZ, AUS, New Guinea, and South-East Asia.[67] Only one leptospermum species (*L. scoparium*) is found NZ.[67]

Mānuka is generally a shrub that can grow up to 4 m but has been found to grow up to 15 m depending on its location. It is an evergreen, with small, sharp tipped foliage

(12 mm long and 4 mm wide) ranging from grey-green to dark bronze-green in colour. It carries woody seed capsules year-round, with seeds from burst capsules distributed by the wind. Wild mānuka flowers are generally white with a red receptacle, however blush pink and red flower varieties can also be found (mainly ornamental cultivars). The flowering period (September-March) often begins in the north, progressively moving south later in the season, with the occasional irregular flowering bursts occurring year-round.[19] Flowering of other plants within vicinity of the hive at the same time as mānuka honey results in multifloral honey, therefore careful hive management (placement and removal) is important for achieving mono-floral mānuka honey.

Mānuka honey is dark in colour which has been linked to the high polyphenol and mineral content. It has a strong, distinctive flavour, described as being earthy, woody and herbaceous in flavour. Due to its thixotropic nature, mānuka honey requires agitation and heating for extraction from the comb and processing to overcome the gel-like nature.

Mānuka honey has high non-peroxide antibacterial activity (NPA) which is not found in most other floral honey types, resulting in greater antioxidant, antibacterial, antifungal, and antidiabetic activity. Some of the other species in the Leptospermum genus (*Leptospermum polygalifolium*, jellybush) also have high NPA honey. The NPA has been attributed to the compound methylglyoxal (MGO) formed during the period of honey maturation and storage,[68] from dihydroxyacetone (DHA) found in mānuka nectar.[69]

1.4.1 Dihydroxyacetone (DHA)

DHA is a simple ketose (Figure 1.2) found in mānuka nectar (up to 5100 mg/kg) that is the precursor to MGO.[70] The origin of DHA in the mānuka nectar has been hypothesised as microbial origin[69] and nectary chloroplast origin[71]. Another uncertainty is the cause of DHA variation found between mānuka individuals, geographic locations and across seasons; it is not consistent. However, studies have found environment (light and temperature), plant genotype (cultivar type), flower sex and age, to have some correlation with DHA expression in mānuka nectar.[69, 71-72] Once collected by the honey bee, the nectar is converted to honey and during honey storage the DHA is converted to MGO via non-enzymatic dehydration (see Section 1.4.2 for further details).
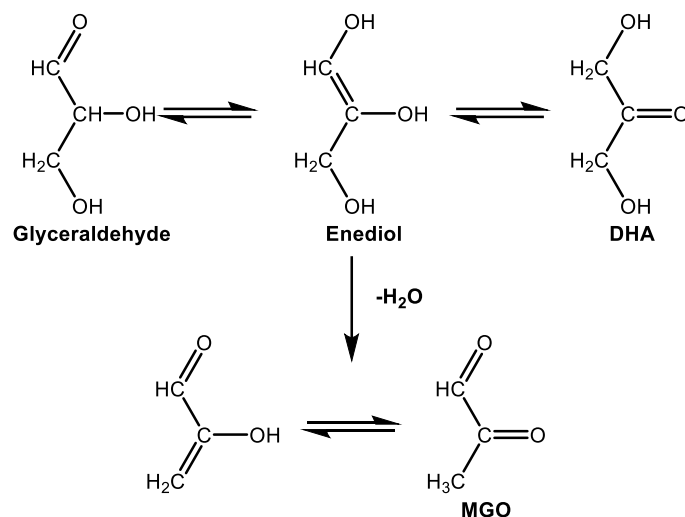
Figure 1.2. Conversion of DHA to MGO via an enediol dehydration. Reproduced from Lookhart and Feather (1978).[73]

1.4.2   Methylglyoxal (MGO) and non-peroxide antibacterial activity (NPA)

The NPA of mānuka honey was first discovered in 1982 but was initially termed the Unique Mānuka Factor (UMF™) as the compound responsible was unknown; both terms are still used interchangeably today. Historically, the NPA rating of mānuka honey was measured indirectly via well diffusion assays; honey samples with peroxide removed (using catalase) were compared against phenol standards in wells on *Staphylococcus aureus* inoculated agar plates. After incubation overnight, the inhibition zones (area where no *S. aureus* had grown) of the honey samples were compared to those of the standards. A sample with NPA 10 would have the same inhibition zone diameter as the 10% phenol solution, with samples being rated up to NPA 30.

Research isolated the fractions of mānuka honey that contained the NPA and determined that this activity came from MGO.[68, 74] Since the identification of MGO, its concentration can be directly measured using high performance liquid chromatography (HPLC), but also gas chromatography (GC) and nuclear magnetic resonance (NMR).[75] The unique mānuka factor honey association (UMFHA) use a correlation curve formed from data from independent laboratories to convert between NPA and MGO, shown in Table 1.3 in mg/kg, which is similar to the conversion used by Adams *et al*. (2008).[68]

Table 1.3. NPA to MGO conversions.

NPA	MGO (mg/kg)
5	134
10	263
15	478
20	779
25	1167
30	1640

The maximum MGO levels for honey depend on the initial DHA concentration in the mānuka nectar and thus are highly variable (Section 1.4.1). A study by Grainger *et al.* (2016)[28] demonstrated that the reaction of DHA to MGO is first-order using mānuka and DHA-spiked clover matrices. They reported that the MGO concentration reaches a maximum before declining over extended time. As the ratio of DHA:MGO nears 2:1, the rate of MGO formation decreases below the rate at which MGO is consumed in other reactions, hence the net concentration is a loss of MGO. The time taken to maximise the MGO concentration is dependent on the initial concentration of DHA and the temperature of honey processing and storage. While higher temperatures lead to faster conversion of DHA to MGO, this can adversely affect the honey due to production of 5-hydroxymethylfurfural (HMF) at higher temperature (Section 1.5.1) and also reduces the achievable maximum MGO concentration due to greater loss of both DHA and MGO in side reactions.

High NPA mānuka honey gives premium returns (Section 1.5.2), thus to hasten MGO formation, batches of honey are generally stored for a period of time before being sold. Predictive modelling, such as by Grainger *et al*. (2016)[76] and commercial laboratories,[77-78] gives indication of ideal storage times and temperatures depending on various constituents. If utilised effectively, accurate predictions for the optimal storage length could reduce costs related to storing samples and increase returns by selling mānuka honey near its peak MGO concentration. Once at this point, the honey should be stored at low temperatures to retain the MGO levels.

MGO levels are generally reported between 0-800 mg/kg, however levels up to ~1500 mg/kg have been found in more mature honeys.[68, 74, 79] The variation is attributed to DHA concentrations in the nectar (Section 1.4.1), variety of mānuka and also dilution by incorporation of nectar from other floral types by the honey bees.

Mānuka honey has been shown to be effective against several drug-resistant microorganisms, and therefore effective at wound healing, with no reports of resistance development to honey. Mānuka honey is effective against bacteria (*Helicobacter pylori*, *Staphylococcus aureus*, antibiotic resistant *Enterococci*, *Clostridium difficile* and *Pseudomonas aeruginosa)* as well other microorganisms including yeasts and filamentous fungi.[74]

While mānuka honey has the constituents for general honey medicinal properties, the reactivity of MGO leads to the inhibition of some of these constituents. MGO is a highly reactive compound due to the aldehyde and ketone functional groups, readily reacting with other compounds because of its highly hydrophilic and electrophilic nature. Majtan *et al.* (2014)[80] has demonstrated that mānuka honey with high MGO levels exhibit low or no peroxide activity due to MGO inhibiting glucose oxidase, while Li and Parikh (2018)[81] found H_2O_2 production increased after MGO was neutralised. Majtan *et al*. (2012)[82] found high MGO levels also inhibited defensin1, a protein found in honey that exhibits antibacterial properties.

Although MGO has antimicrobial activities when applied to wounds, multiple studies have demonstrated the toxicity of high MGO concentrations in organisms.[83-85] It causes irreversible, non-specific damage to cellular DNA and proteins, resulting in major cellular issues and eventual arrest of cell division. MGO is known to readily interact with lysine and arginine residues in proteins, as well as lipids and nucleic acids, forming advanced glycation end products (AGEs) that are implicated in the onset of diabetes and other age-related disorders.[86]

1.4.3 Mānuka honey definition

In February 2014, a 3-year project to develop a definition for mānuka honey was undertaken by Ministry for Primary Industries (MPI), with the finalised definition for mānuka honey and new export rules released in December 2017.[87] The official definition of mānuka honey is used to help to ensure the integrity and authentication of mānuka honey, assisting the continued growth of New Zealand honey exports and helping maintain premium positioning in international markets. The definition combines four chemical attributes of mānuka nectar origin (3-phenyllactic acid, 3-PLA; 2'-methoxyacetophenone, 2'-MAP; 2-methoxybenzoic acid, 2-MBA;

4-hydroxyphenyllactic acid, 4-HPLA) and mānuka pollen DNA levels less than a Cq of 36 (~3 fg/μL) DNA from mānuka pollen to identify mānuka from other floral types and specify it as monofloral or multifloral honey (Table 1.4). The four chemical markers (Figure 1.3a) were chosen as the most suitable markers due to their presence in mānuka and absence, or low concentrations in other honeys, as well as their stability over time.[87] Of these, only 2'-MAP of the chosen markers is considered unique to mānuka.[88] Other markers such as leptosperin, methyl syringate (MSY)[89] and lepteridine[90] (Figure 1.3b) have since been suggested as alternatives. A study by Kato *et al.* (2012)[91] found that varying leptosperin levels are also found in honey of other botanical origin in both Australia (*L. polygalifolium*) and New Zealand (honeydew, *Knightia excelsa* - rewarewa, *Ixerba brexioides* - tawari), however they suggested that leptosperin occurrence in the non-mānuka New Zealand honeys was due to the cross-over in flowering periods. The nectar of these species would need to be analysed for to confirm this. The study and findings by MPI determined that kānuka (*Kunzea ericoides*) and kāmahi (*Weinmannia racemose)* produce leptosperin, which resulted in exclusion of leptosperin as a mānuka marker.[88] MSY is unsuitable due to it instability, resulting in decreasing concentrations over time.[89] Lepteridine was proposed, but with only one study carried out on it,[90] there was not enough evidence to include it in the MPI definition.[88]

Table 1.4. The four chemical compounds and DNA limits required for the MPI mānuka honey definition.[88]

Attribute	Monofloral mānuka	Multifloral mānuka
3-phenyllactic acid	≥ 400 mg/kg	≥ 20 mg/kg to <400 mg/kg
2'-methoxyacetophenone	≥ 5 mg/kg	≥ 1 mg/kg
2-methoxybenzoic acid	≥ 1 mg/kg	≥ 1 mg/kg
4-hydroxyphenyllactic acid	≥ 1 mg/kg	≥ 1 mg/kg
Mānuka pollen DNA	<Cq 36 (~3 fg/μL)	<Cq 36 (~3 fg/μL)
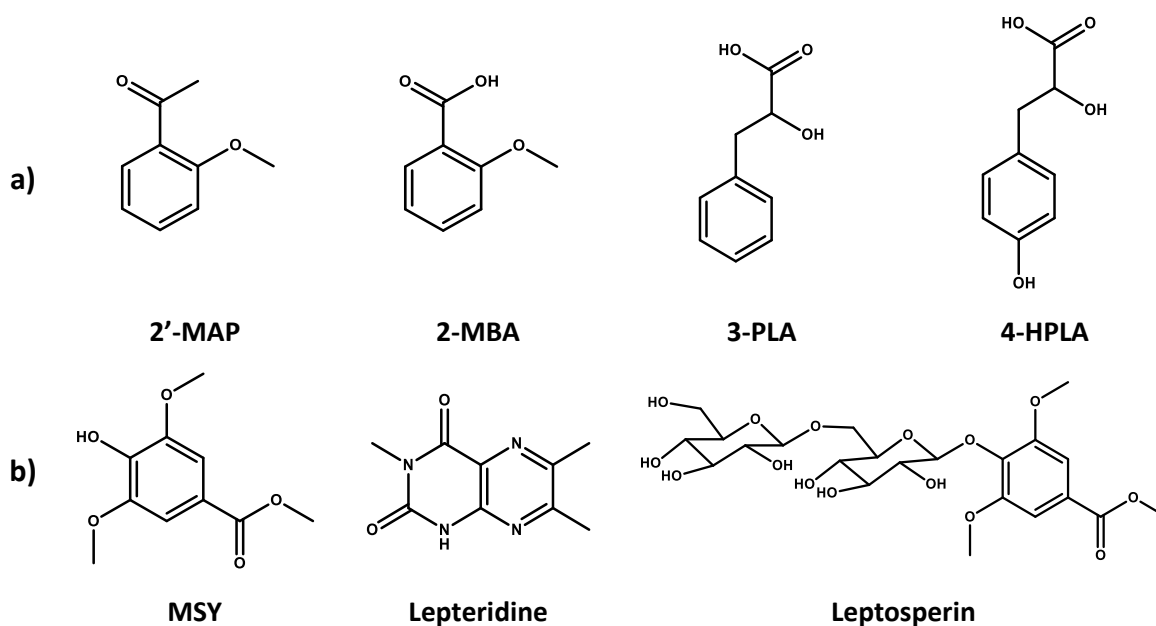
Figure 1.3. Key compounds identified in mānuka honey; a) mānuka honey markers 2'-methoxyacetophenone (2'-MAP), 2-methoxybenzoic acid (2-MBA), 3-phenyllactic acid (3-PLA) and 4-hydroxyphenyllactic acid (4-HPLA), and b) proposed markers leptosperin, lepteridine and methyl syringate (MSY).

1.5   Honey regulations

The Codex Alimentarius[12] describes various standards for quality parameters in honey, however in some cases there are differences between these and legislation in various countries, the reader is directed to a review by Thrasyvoulou *et al.* (2018)[92] which cover these differences in depth.

The Codex Alimentarus[12] states that honey sold must be in its natural form; no additives, modifications or contamination during processing and storage should change its characteristics, flavour and aroma. While foreign inorganic or organic matter can be removed, the natural honey constituents should remain the same, and should not in any way be impaired by the heating, processing or storage steps. The Codex[12] also gives recommended limits (upper or lower) for different honey types on parameters including but not limited to; moisture, sum of fructose and glucose content, sucrose content, heavy metals, pesticide residues, free acidity, electrical conductivity, 5-hydroxymethylfurfural (HMF) concentration and diastase activity. Diastase will be discussed in further detail in Section 1.6.

1.5.1 5-Hydroxymethylfurfural (HMF)

5-Hydroxymethylfurfural (HMF, 5- hydroxymethyl-2-furaldehyde) is another important quality control parameter in honey because it does not occur or occurs in very low levels in fresh honey. Cumulative heat exposure influences HMF level; with concentration of HMF increasing over time. Export requirements state that HMF should not exceed 40 mg/kg unless the honey originates from regions of tropical climate where the limit is 80 mg/kg.[12, 93] HMF is a cyclic aldehyde mainly formed from acid catalysed decomposition of monosaccharides (Figure 1.4) in honey but is also produced in the Maillard reaction from sugar degradation.[40] Rate of formation for HMF depends on temperature and time, but also pH, total and free acidity, and water content.[94] It can also be an indication of adulteration with invert syrup.[95] Low pH and water activity both promote faster HMF production, although it is primarily proportional to temperature treatment and storage conditions. Inappropriate processing and storage results in high HMF levels, although the processing of honey at 32 to 40 °C for short periods does not significantly impact HMF levels.[94] While HMF does degrade over time to levulinic acid, the formation of HMF is faster than its degradation and hence it accumulates in honey.
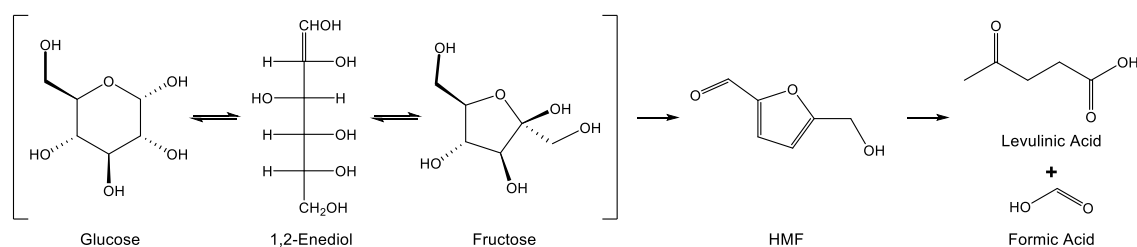
Figure 1.4. Acid catalysed decomposition of fructose and glucose to HMF and degradation to levulinic acid. Adapted from Kammoun *et al.* (2019).[96]

1.5.2 New Zealand exports

There is a strong demand for mānuka honey, especially in international markets, with premium prices paid for honey with high NPA rating. Mānuka honey is a large component of the multi-million dollar honey export industry for New Zealand; in the 2019/20 season, mānuka honey accounted for 51% (5,245 tonnes) of total export volume, yet 68% (NZ$290 million) of export value.[1, 97] As a valued export commodity, it is important that mānuka honey meets not only NZ MPI regulations but also regulations for the destination country market. Quality parameters in the above section

(Section 1.5) are generally standardised across most countries, although some countries have used different parameter values in their legislation.[92]

The price of bulk honey depends on the floral type; for the 2019/20 season light clover was worth NZ$2.50-5.50 per kg compared to mānuka which ranged from NZ$4.50-130.00 per kg depending on the NPA rating.[1] The returns demonstrate the extreme discrepancy between honey types and the effect of demand on mānuka honey due to NPA grade. Passing the criteria for the mānuka honey definition (Section 1.4.3) in conjunction with a high NPA, cultivates premium prices for mānuka honey, with almost exponential increases in retail price for higher NPA honeys.[1] As mānuka honey is sold with the price based on its current NPA, producers will try to cultivate the NPA to near its maximum before selling by accelerating MGO formation with higher temperatures. However, it is important that temperatures or storage lengths are not too excessive to ensure compliance with HMF levels (maximum limit 40 mg/kg) and diastase activity (DN ≥ 8), which respectively, are formed and degraded by heat over time.

1.6 Alpha-amylase in honey (Diastase)

An unknown fraction of high NPA mānuka honeys fail the diastase test, rendering them unable to be exported. As high NPA mānuka honeys have premium returns, this results in a large loss of prospective profit. It is crucial to identify the cause of low diastase levels in mānuka honey due to the potential impact on the export market. Determination of a cause to this issue may lead to grounds for an exemption to be made for mānuka honey so long as samples meet the other quality parameter checks, such as HMF levels.

1.6.1 Diastase overview

Diastase is the generalised term for a group of enzymes that function to catalyse the conversion of starch (polymeric carbohydrate with glucose units joined by glycosidic bonds) into maltose (disaccharide consisting of two glucose units joined with an α-bond) or maltodextrins, and is present in the majority of organisms (bacteria, fungi, plants and animals).[98-99] Specifically, alpha-amylase hydrolyses α-(1-4) glycosidic linkages of starch to varying degrees, forming a mixture of maltose (disaccharide), maltriose (trisaccharide) and maltodextrins (oligosaccharides),[100] the catalysed reaction retaining anomeric configuration. Diastase is found in honey due to bee secretions from the

hypopharyngeal glands into the nectar during collection and processing,[14] but studies have found honey to contain some diastase of vegetal origin (nectar).[101] Honey bee hypopharyngeal diastase production is age-dependent due to the development of the gland over time depending on the role of the honey bee. [14] Nurse bees do not produce diastase while forager bees do due to their foraging of pollen which contains plant starch.[14, 102] Alpha-amylase is the primary diastase in honey, however small amounts of beta-amylase have also been found.[23, 103] For this thesis, the term diastase is used when discussing the analytical testing method and results (as diastase activity), whereas alpha-amylase is used when talking about the specific enzyme and its function.

Diastase activity is expressed in Schade units or diastase number (DN) and is used in conjunction with HMF to determine the quality of a honey. Due to the sensitivity of the enzyme to heat exposure and subsequent loss of activity over time, low diastase activity is used as an indication of either long storage or inappropriate heat treatment of honey. The Codex Alimentarus states that diastase activity should not be less than DN 8, while honey with low natural enzyme content (specific floral types were not stated) should not fall below DN 3 with the stipulation of also not exceeding 15 mg/kg HMF.[12]

1.6.2 Testing of diastase activity

Diastase activity is defined as the "amount of enzyme which will convert 0.01 g of starch to the prescribed end-point in one hour at 40 °C".[12] Two main methods are used for the measurement of diastase activity in honey, the traditional Schade method and the newer commercial Phadebas method. Both methods have similar principles in that honey solution diluted with acetate buffer is mixed with starch substrate, the diastase catalytic activity on the starch results in a colour change of the solution. The Schade method utilises a potato starch solution because the substrate forms blue colouration on addition of iodine. Diastase activity diminishes the blue colour of the solution, with the absorbance measured at 660 nm in intervals to form a regression equation from which the diastase number (DN) can be converted to Schade units. The Phadebas method uses a blue-dyed artificial starch tablet that on hydrolysis by diastase, release water-soluble blue dye into solution. After reaction termination and filtering, absorbance values at 620 nm are measured, and due to good correlation between the two methods, DN in Schade Units can be determined by using Equation 1 for DN 8

through 40, and Equation 2 for DN < 8. Due to the variation of commercially available starch, the precision of the Schade method is less than half compared to that of the Phadebas method. The Phadebas method is also more efficient because there are fewer, less complex steps, making it the more attractive method to use for a large number of samples, although per sample the Phadebas method is more expensive than the Schade method.[100]

$$DN = 28.2 \times \Delta A_{620} + 2.64 \qquad \textbf{Equation 1}$$

$$DN = 35.2 \times \Delta A_{620} - 0.46 \qquad \textbf{Equation 2}$$

*Where ΔA_{620} = sample absorbance at 620 nm – absorbance of the blank

While the Phadebas and Schade methods are predominant, recent studies have found alternative, faster ways of measuring diastase activity in real time, including FT-Raman spectroscopy[104] and visible and near-infrared spectroscopy.[105] However, application to further sample sets needs to be carried out before such methods can become mainstream and used in certification for export purposes.

1.6.3 Mānuka honey and low diastase levels

In industry, it is known that mānuka honey can often fail diastase testing, especially high grade mānuka honey, however there are few publications in literature regarding this. [106] An article from Analytica Laboratories stated that in general, mānuka honey with higher MGO have lower diastase activity (Figure 1.5).[107] They imply that this is a coincidental relationship because honey with high concentrations of MGO have been stored for longer than honey with low MGO concentrations; and as previously noted, diastase degrades with heat and over time.

In addition to heat and time, it is possible that other compounds in the honey matrix may be interacting with and contributing to the inhibition of diastase activity in honey. While any one of the minor honey constituents, in concentrations unique to mānuka honey, may be involved in the inhibition of diastase activity, this thesis will primarily focus on the phenolic compounds, DHA and MGO to keep the scope of the project manageable and in line with the sponsoring company.

A more in-depth review of the alpha-amylase mechanism, structure and inhibition has been carried out in Chapter 4.
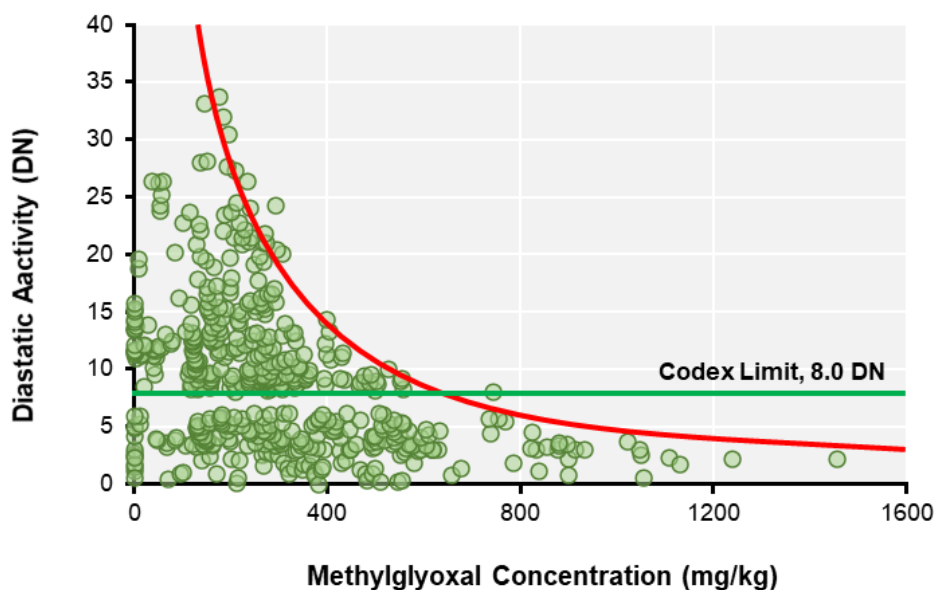
Figure 1.5. Diastase activity compared to methylglyoxal concentration (mg/kg) in mānuka honey samples analysed at Analytica Laboratories. The gap below 8.0 DN is due to the use of a different equation at DN < 8. Reproduced with permission from Analytica Laboratories.[107]

1.7 Phenolic compounds

1.7.1 Phenolic compounds overview

Phenolic compounds (also called polyphenols) are a class of secondary plant metabolites synthesised via two pathways in plant glucose metabolism: the shikimic acid and acetate/polyketide pathways. They are found in reasonably high levels in plant-based products, including honey which is derived from plant nectar (or in the case of honeydew, phloem). As the most abundant secondary metabolite in plants (over 8000 known phenolic compounds),[108] they are involved in plant growth and reproduction, pigmentation and provide defence against predators and pathogens.[109] These compounds are also responsible for colour, flavour and aroma found in plant products.[110]

Phenolic compounds are widely varied, characterised as having at least one phenol ring in their structure. As the largest class of phytochemicals, phenolic compounds are generally organised into six main groups depending on their structure: phenolic acids, flavonoids, lignans, stilbenes, tannins and coumarins (Figure 1.6). Compounds within each of these groups are derived from a specific backbone, with differentiation due to various degrees of methylation, hydroxylation and glycosylation. Phenolic acids and

flavonoids are the predominant phenolic compounds found in honey. Recently, phenolic compounds have been of interest due to their health benefits, functioning with anti-inflammatory, anti-oxidant, antiviral and anticarcinogenic activity.[111]
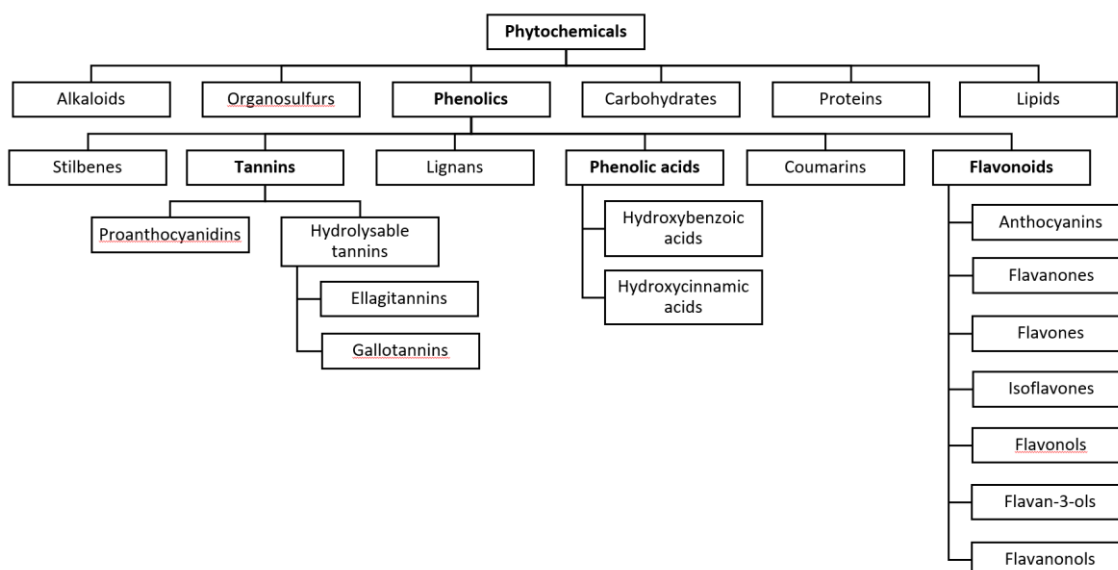
Figure 1.6. The phytochemical family tree, with expansion of the phenolic compound category.

1.7.1.1 Phenolic acids

Phenolic acids can be described as those that are derived from hydroxybenzoic acid or hydroxycinnamic acid with C_6-C_1 and C_6-C_3 backbones respectively, with methoxy and hydroxy constituents (Figure 1.7). This class contains gallic, ellagic, caffeic, ferulic and coumaric acids, all of which are synthesised from the shikimate pathway. Phenolic acids in plants are usually bound as amides, esters or glycosides and rarely occur in free form. In plants, they function to protect cells against oxidative stress conditions due to the radical scavenging activity of their phenol moiety, carrying out a similar function in the human body when ingested.[110]

Hydroxybenzoic acids	R_1	R_2	R_3	R_4	Hydroxycinnamic acids	R_1	R_2	R_3	R_4
p-Hydroxybenzoic acid	-H	-H	-OH	-H	*p*-Coumaric acid	-H	-H	-OH	-H
Vanillic acid	-H	-OCH$_3$	-OH	-H	Caffeic acid	-H	-H	-OH	-OH
Gallic acid	-H	-OH	-OH	-OH	Sinapic acid	-H	-OCH$_3$	-OH	-OCH$_3$
Syringic acid	-H	-OCH$_3$	-OH	-OCH$_3$	Ferulic acid	-H	-H	-OH	-OCH$_3$
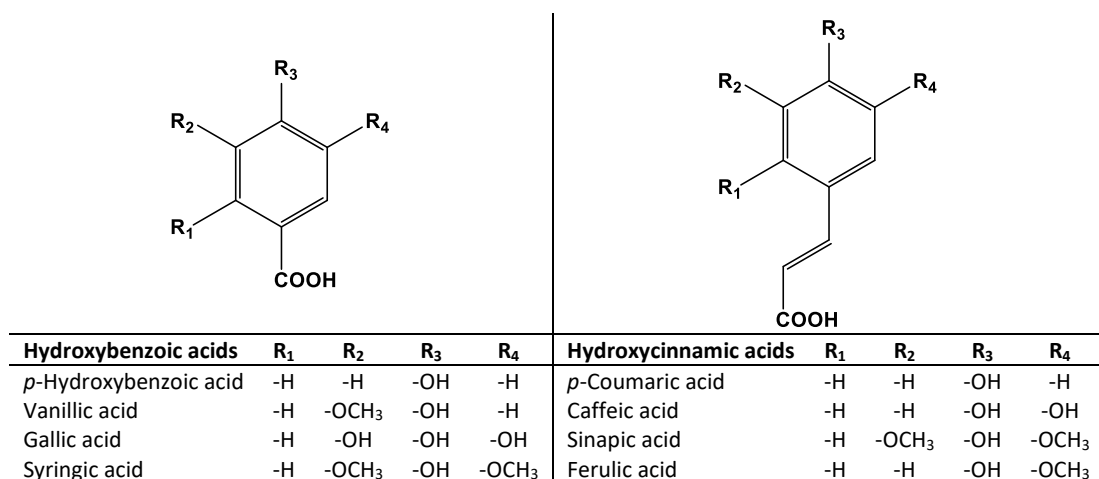
Figure 1.7. Chemical structure of some benzoic and cinnamic acid derivatives.

1.7.1.2 *Flavonoids*

The second main group, flavonoids, are heterocyclic compounds synthesized from the shikimate pathway.[111] The primary structure from which all flavonoids are derived is two benzene rings bridged by a heterocyclic γ-pyrone[*] (C_6-C_3-C_6), and based on their degree of oxidation, annularity of ring C and connection of ring B, can be further classified into; flavonols, flavones (largest group), flavanones, flavanols (catechins), anthocyanins and isoflavones (Figure 1.8).[112] For all flavonoid groups except isoflavones, aromatic ring B is connected at the C2 of the pyrone, isoflavones connects at pyrone C3. Flavones and flavonols contain a double bond between C2 and C3, while flavanones and flavanonols are saturated across C2 and C3, with flavonols and flavanonols hydroxylated at C3. The two remaining groups, flavanols (catechins) and anthocyanins, are collectively called flavans as they do not retain the pyrone ketone at C3. Anthocyanins are the stable glycosides[†] of anthocyanadins; the anthocyanidin aglycone backbone is unstable due to existing as a flavylium cation. Flavonoid levels differ significantly between plant types, but also depend on climatic conditions (geographical location) as flavonoids play a large role in plant regulation in response to biotic and abiotic stress factors. As with anthocyanins, many of the other flavonoids are found as glycosides as well as aglycones (Table 1.5).

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[*] γ-pyrone refers to the heterocyclic structure containing an oxygen and ketone functional group in para-positioning.

[†] Cyclic acetals of carbohydrates are given general class name of glycosides, while the derivatives of glucose are specifically called glucoside. (Cram & Cram, 1978 – The Essence of Organic Chemistry)
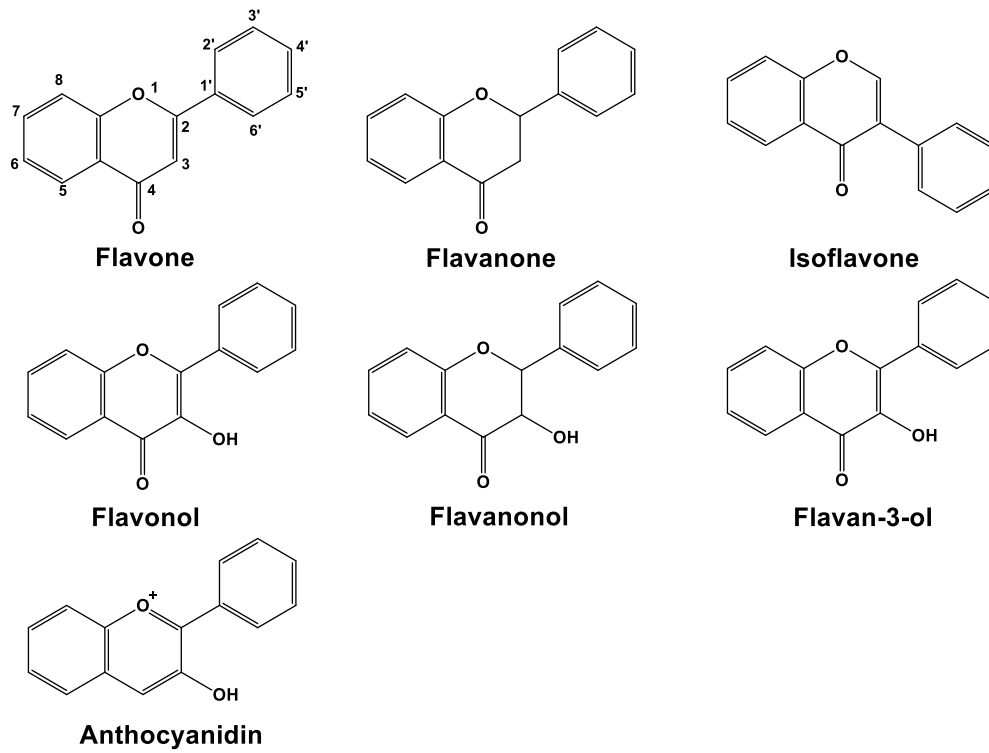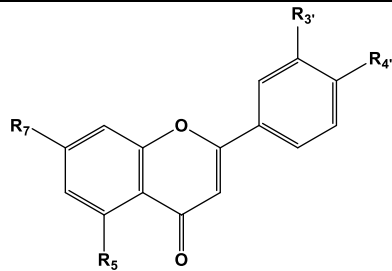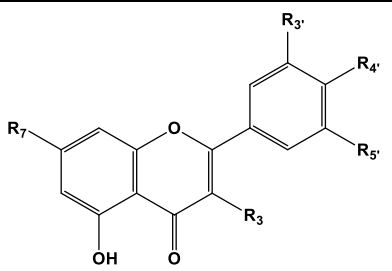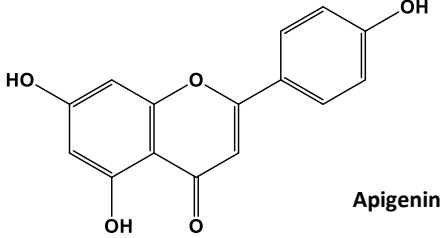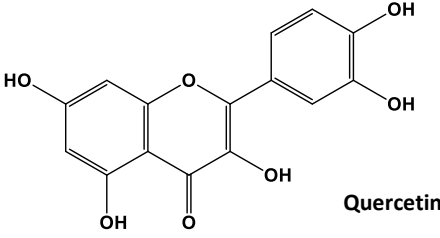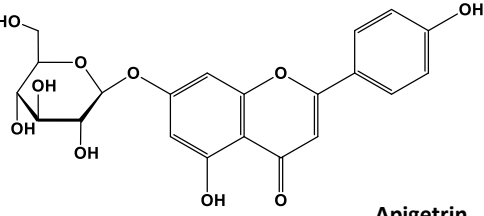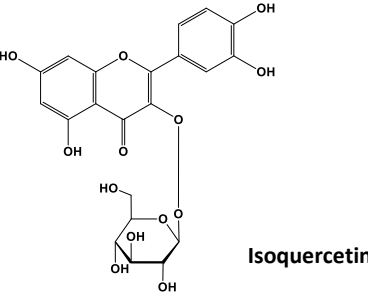
Figure 1.8. Structures of the seven flavonoid groups.[112]

Table 1.5. Structures and functional groups of various flavonoids, with examples of aglycones and simple glycosides (*-Glu = glucoside, -Rut = rutinoside). Adapted from Ahn-Jarvis *et al.* (2019).[113]

Flavone	Flavonol

Aglycones	R$_5$	R$_7$	R$_{3'}$	R$_{4'}$
Apigenin	-OH	-OH	-H	-OH
Luteolin	-OH	-OH	-OH	-OH
Chrysin	-OH	-OH	-H	-H
Glycosides*	R$_5$	R$_7$	R$_{3'}$	R$_{4'}$
Apigetrin	-OH	-Glu	-H	-OH

Aglycones	R$_3$	R$_7$	R$_{3'}$	R$_{4'}$	R$_{5'}$
Kaempferol	-OH	-OH	-H	-OH	-H
Quercetin	-OH	-OH	-OH	-OH	-H
Myricetin	-OH	-OH	-OH	-OH	-OH
Glycosides*	R$_3$	R$_7$	R$_{3'}$	R$_{4'}$	R$_{5'}$
Isoquercetin	-Glu	-OH	-OH	-OH	-H
Rutin	-Rut	-OH	-OH	-OH	-H

	Aglycone	Simple-glycoside

Apigenin — Quercetin — Apigetrin — Isoquercetin

1.7.1.3 Tannins

Tannins are of interest to the sponsor of this research as a potential inhibitor of diastase and are one group of focus within this research. Tannins occur as either hydrolysable or non-hydrolysable (condensed) tannins (Figure 1.9).[114] Hydrolysable tannins consist of phenolic acids (e.g. gallic acid) esterified to a polyol core (such as D-glucose), and are susceptible to degradation by acid, base or enzymatic hydrolysis. In contrast, non-hydrolysable tannins are oligomers or polymers of flavan-3-ols (catechin, epicatechin etc.), requiring strong oxidative and acidic hydrolysis for depolymerisation.[114] There is a large difference in their molecular weights with

hydrolysable tannins ranging from 0.5-3 kDa and non-hydrolysable tannins ranging from 1-20 kDa.[115] Tannins are produced by plants to defend against pathogen or insect attack, functioning as part of the chemical defence system.[114] Tannins are found to have a range of biological activities such as antimicrobial, antiviral, antiparasitic and antioxidant due to inhibition of microbial enzymes, interference of proteins and chelation to metal ions.[116]
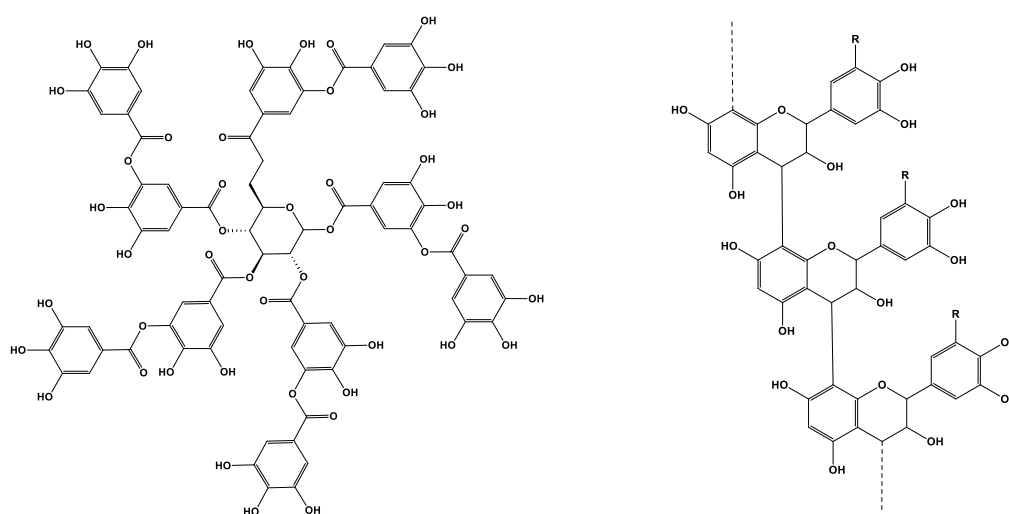
Figure 1.9. Examples of tannins: hydrolysable tannin (tannic acid, left); non-hydrolysable tannin (right).

1.7.2 Phenolic compounds in mānuka honey

In the past two decades, an increased number of publications have investigated phenolic content in honeys with two main aims; firstly to determine marker compounds or phenolic profiles specific to botanical and / or geographical origin, and secondly, to determine their contribution to the health properties of honey.[113, 117-120]

The primary phenolic compounds found in honey are from the phenolic acid and flavonoid groups, mainly flavones, flavonols and flavanones. While it is primarily flavonoid aglycones that are derived from flower nectar, glycosides have also been found,[121-122] thus are present in the honey (e.g. rutin, quercetin-*o*-rhamnoside).[53, 119, 123] The diversity of phenolic compounds, as well as some dependence of expression due to botanical origin, has led to several proposals of specific phenolic compounds to be markers for floral honey types.[124] While some marker compounds appear to be unique

(e.g. 2'-MAP in mānuka),[88] many phenolic compounds are common across different floral honey types, therefore proposed marker compounds are also those that have a much higher concentration for one floral type compared to others. Tuberoso *et al.* (2011)[125] identified high concentrations of 3-PLA (418.6 ± 168.9 mg/kg) together with lumichrome (29.4 ± 14.9 mg/kg) as potential marker compounds for thistle honey (*Galactites tomentosa* Moench). Although 3-PLA is common in many floral types, often at high concentrations,[126] lumichrome had not been reported in any other floral types. However, later publications by different authors[127-129] identified lumichrome in kānuka honey (170.5 ± 10.9 mg/kg), which also contains high levels of 3-PLA.[128]

A wide range of phenolic acids and flavonoids have been found in mānuka honey at various concentrations (Table 1.6, excluding mānuka marker compounds).[130-136] However, most of these compounds are extremely common to other floral types as well. Cheung *et al.* (2019)[131] studied phenolic acid and flavonoid profiles of various commercial honeys (*n* = 40, 39 floral sources from eight different countries) and found gallic acid and protocatechualdehyde were present in 95% of samples, while 4-methoxybenzoic acid, genistic acid and syringic acid were the next most prominent at 62.5 %, 52.5 % and 50 % respectively. Of the identified phenolic compounds in Table 1.6, none were found to be significantly higher in mānuka honey compared to other floral types.[131-133]

Tannic acid has not been reported in honey, however Mayworm *et al*. (2014) did find tannins (proanthocyanidins) in Brazilian propolis samples. Small amounts of propolis are found in honey, hence it may result in low levels of tannins in honey.[137] No literature has investigated tannins in New Zealand propolis. Interestingly, Kolayli *et al.* (2016)[118] carried out a total tannin assay, finding that there were tannins present in the honey (*n* = 17) ranging from 0.01-16.67 mg tannic acid equivalents per 100 g of honey, with chestnut honey (11.93-16.67 mg tannic acid/100 g) and buckwheat honey (6.23-6.67 mg TA/100g) containing the most. However, no other research has been done on this topic, nor were the individual tannins identified.

Interactions between phenolic compound and alpha-amylase will be discussed in Chapter 4.

Table 1.6. Phenolic compounds and concentrations found in mānuka honey.

Phenolic compound	Concentration (mg/kg)	Ref.	Phenolic compound	Concentration (mg/kg)	Ref.
Apigenin	0.4	[131]	Isorhamnetin	0.2-4.0	[130, 136]
Caffeic acid	0.05-17.9	[131-132, 136]	Kaempferol	0.3-2.0	[130-131, 133, 136]
Catechin	36.8	[132]	Luteolin	0.7-3.8	[130-131, 133-134, 136]
Chlorogenic acid	8.2-16.5	[131, 136]	Myricetin	0.7	[131, 136]
Chrysin	0-3.8	[130-131, 133, 135-136]	Naringenin	0.7	[131]
p-Coumaric acid	0-10.9	[132, 136]	Naringin	1.9	[131]
Ellagic acid	15.0	[136]	Pinobanksin	0-2.7	[130, 135]
Ferulic acid	4.8	[136]	Pinocembrin	0-1.9	[130, 133, 135-136]
Galangin	0-2.6	[130-131, 133, 135]	Protocatechualdehyde	18.2	[131]
Gallic acid	0-70.5	[79, 131, 136]	Protocatechuic acid	49.7	[131]
Genistic acid	30.4	[131]	Quercetin	0.2-4.3	[130-131, 136]
Hesperetin	1.2	[131]	Quercitrin	1.25	[131]
Hesperidin	4.7	[131]	Syringic acid	0-40.0	[79, 131, 135]
4-Hydroxybenzoic acid	9.5	[131]			

1.7.3 Methods - Determination of phenolic compounds in honey

Phenolic compounds in honey can be measured in two main ways: determination of total content through a non-specific method, or determination of individual phenolic profiles. The total content methods are simplistic and relatively universal, however, for individual determination there is great variation in the methods of analysis (discussed below). Each has its own advantages and disadvantages. Neither the IHC or Association of Official Analytical Chemists (AOAC) have proposed a standardised procedure for individual phenolic compound analysis in honey, potentially due to the wide range of methods available and the difficulty of narrowing it down to a method that covers all bases including: cost, simplicity, sustainability, precision, accuracy and sensitivity.[122]

1.7.3.1 Total content methods

Total phenolic content (TPC) and total flavonoid content (TFC) are frequently used due to being rapid, simple, and cost-effective spectrophotometric methods. However, they are not selective and generally provide an overestimated value. TPC is based on the reaction of phenolics with the colourimetric Folin-Ciocalteu reagent; electrons are transferred from the phenolic compounds to a phosphomolybdic/phosphotungstic acid

complex resulting in the formation of blue complexes, which can then be measured in the visible spectrum with a spectrophotometer.[138] Evaluation of samples is based off a gallic acid standard curve and the results expressed in mg Gallic Acid Equivalents (GAE) per unit honey (i.e., mg GAE/kg honey). It is non-specific as it determines the total reducing capacity of compounds in the sugar, which include sugars, amino acids, vitamins, and other compounds as well as the targeted phenolic compounds.

TFC is similar to TPC, however it generally uses $AlCl_3$ for the colourimetric reagent to form aluminium-flavonoid complexes. Unlike TPC, the TFC assay does not measure the total flavonoid content as it shows different specificity towards various flavonoids depending on the reaction conditions.[139] Pękal and Pyrzynska (2014)[139] showed that neutral conditions were specific for flavonols and luteolin while alkaline conditions were selective for rutin, luteolin, catechins and some phenolic acids. TFC results are generally expressed in catechin equivalents.[47, 140]

1.7.3.2 Determination of phenolic compound profile

There are three critical steps that are utilised in most of the methods for determining phenolic compound profiles and concentrations in honey: isolation and extraction, instrument analysis, and detection (Figure 1.10). A range of methods found in literature are summarised in Table 1.8 with generalised concepts discussed in the following sections.
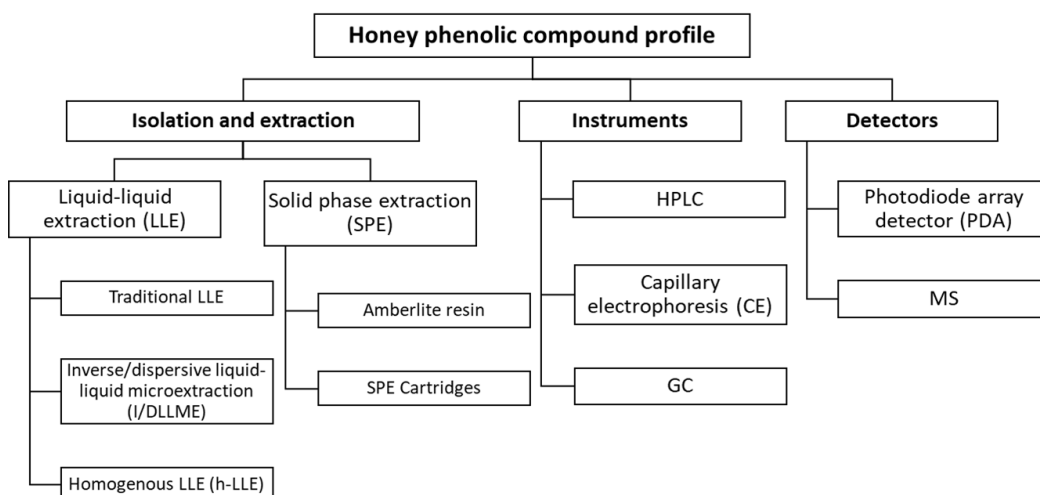
Figure 1.10. Examples of extraction methods and instrument analysis for phenolic compounds in honey.

Isolation and extraction

An important step in analysing the phenolic compound profile in honey is isolating and extracting the compounds from the complex honey matrix, as other honey constituents have the potential to greatly interfere with analysis. Some methods, like Bong *et al.* (2018)[128] and Biesaga *et al.* (2009),[141] omit this step, instead opting to dilute with acidified water and filter their samples as preparation before analysis. As extraction procedures can be arduous, this would result in quicker preparation. It also prevents the potential loss of compounds that may occur during extraction. However, simply diluting samples reduces the analyte concentration, increasing the limits of detection and quantification compared to other methods which pre-concentrate before analysis. The main extraction methods used for phenolic compounds in honey can be broadly separated into two groups: liquid-liquid extraction (LLE) and solid-phase extraction (SPE).

LLE is a process through which the analytes are transferred from a solution into an immiscible solvent through mixing. Honey samples are usually diluted with acidified water (pH 2), using various solvents for extraction, though most commonly ethyl acetate. The acidified water (pH 2) optimises extraction efficiency for phenolic acids; pH values of 3 or above significantly reduce phenolic acid extraction efficiency due to ionisation of the acids and therefore the more hydrophilic character prevents full extraction into the organic solvent.[142] The collected organic phase is generally dried down, then redissolved in methanol or a methanol:water mixture before analysis. Such conventional LLE using ethyl acetate for extraction and methanol for redissolution has been a popular method for phenolic compound extraction,[131, 143-145] with varying application and recoveries ranging from 45-112% for phenolic acids[142-144] and 69-113% for flavonoids.[142-143] Other methods have carried out serial extraction with LLE; Spilioti *et al.* (2014)[146] used butanol followed by ethyl acetate, while Can *et al.* (2015)[147] used methanol followed by a diethyl ether/ethyl acetate (50:50 v/v) extraction. Emulsion formation can cause issues with the recovery of some compounds, while high levels of solvent make traditional LLE disadvantageous compared to some newer variations of LLE or some SPE methods.

With the aim to achieve greener, easier, and faster methods, LLE has diversified into a range of different types. Dispersive liquid-liquid microextraction (DLLME) and inverse liquid-liquid microextraction (IDLLME) use complete dispersion of an extracting solvent into the aqueous honey solution via polar disperser solvent, increasing the contact between sample and extractant solutions. Centrifugation of the samples results in the extractant solution on the top (IDLLME)[148] or bottom (DLLME)[149] of the tube. However, this method has poor extraction of many polar compounds, due to their hydrophilic nature, requiring the addition of salt to improve recovery.[149] Ranjbari *et al.* (2012)[148] achieved 97.5-98.2% recovery for quercetin (only compound analysed) using IDLLME, while Campone *et al.* (2014)[149] achieved 54.9-83.9% recoveries for phenolic acids and 87.1-101.1% recoveries for flavonoids using DLLME.

Homogenous liquid-liquid extraction (h-LLE) has also recently been investigated, where water miscible solvents are used for extraction, opposed to immiscible. It has the advantage of higher extraction efficiencies of hydrophilic compounds compared to traditional LLE.[142] Adding salts or sugars to the solution triggers phase separation to occur and the transfer of analytes to the organic phase. Alternatively, cooling the samples to sub-zero temperatures can also be used for phase separation but requires a much greater energy input.[150-151] Salting-out assisted liquid-liquid extraction has not often been used for the purpose of extracting phenolic compounds in honey, although the addition of salt is sometimes used to aid in higher recoveries for traditional LLE methods.[144, 148] Advantages of h-LLE include lower cost, more environmentally friendly, and reduced sample and solvent volume.

Shi *et al.* (2019)[152] has used a derived SALLE method, counter flow salting-out homogeneous liquid–liquid extraction, to extract phenolic acids from honey samples. A mixture of aqueous honey solution with organic solvent, acetonitrile (ACN), is passed through a salt layer, collecting droplets of ACN on the salt which then aggregate as a top layer. They achieved good recoveries of seven phenolic acids (84.9-117.5%), with the benefit of low sample and extractant volumes (5 and 2.5 mL respectively) compared to traditional methods.[152]

Zhu *et al.* (2019)[142] applied a different h-LLE method to extracting phenolic compounds from honey, sugaring-out assisted liquid-liquid extraction (SULLE). This use is novel,

previously being used for biomolecules,[150, 153-154] metal ions,[155] sulfonamides[156], antiviral drugs[151], and bisphenols[154] from various matrices. Acidified honey solution (1:2, m/v of honey – water) mixed with ACN automatically separates into two phases due to the intrinsic sugars in honey, requiring no other additives, but can also be aided by centrifugation. SULLE has advantages over SALLE as salts may interfere and react with analytes or adversely affect the pH, and salting out requires high salt concentration with the potential to corrode or interfere with equipment and instrumentation.[153, 155, 157] Sugar is less reactive, cheaper and does not affect the pH of the solution.[151, 155] Zhu *et al.* (2019)[142] achieved good recoveries of eight phenolic acids (79.8-98.3%) and nine flavonoids (88.9-105.7%) using SULLE, which they also compared with traditional LLE, resulting in significantly lower recoveries for the same phenolic compounds (45.2-79.8% and 69.3-90.5% respectively).

The second major method of extraction is solid-phase extraction (SPE). Traditionally Amberlite XAD-2 resin (a non-ionic polymeric resin) is used due to its suitability for phenolic compound extraction compared to other XAD resins,[158] however there has been a recent shift towards use of commercial reverse phase (RP) SPE cartridges. Similar to LLE, honey samples are diluted in acidified water (pH 2). The samples are filtered before loading onto a column (Amberlite XAD-2 resin) or cartridge, where phenolic compounds are adsorbed to the resin while the aqueous solution passes through. Sugar and polar compounds can then be washed off with aqueous solvent followed by elution of phenolic compounds with methanol. In many cases, SPE is followed up by LLE with ethyl acetate as a clean-up step once the methanol eluent has been dried off.[136, 159-161] Other methods skip this step, directly analysing the methanol eluent.[121, 127, 129, 162] In some cases, the Amberlite resin is premixed with the aqueous honey solution to improve recovery.[136, 161] There are disadvantages to using Amberlite resin, including price, large sample and solvent volumes, the resin requires washing between samples, lowered recoveries after multiple uses, and poor extraction efficiency of phenolic acids compared to flavonoids.[160, 163-165] Compared to Amberlite XAD-2 resin, SPE cartridges are faster, simpler and less expensive,[166] although different cartridges have variable recoveries.[164, 167]

Yung An *et al.* (2016)[163] investigated extraction efficiencies, comparing Amberlite XAD-2 resin to C18 cartridge, and found that the cartridge failed to extract gallic acid but had

greater than 63% recoveries for the other eight phenolic compounds. While the Amberlite resin extracted all nine phenolic compounds, gallic acid was only 6.7% recovered, with other recoveries ranging from 25-65%. Michalkiewicz *et al.* (2008)[164] investigated different sorbents, including Amberlite XAD-2 resin and Bond Elut C18, Oasis HLB and Strata X cartridges, demonstrating the Oasis HLB obtained the best recoveries for honey samples. They found that phenolic acids were recovered in both the methanolic fraction, as well as in the effluent, due to their low affinity to the Amberlite XAD-2 resin, while flavonoids and *p*-coumaric acid were absent in the effluent. Literature also shows that there can be significant variability even though the same sorbent is used (Table 1.7), indicating that small changes in methodology can have significant impact on recovery, or that variations in honey type influence extraction efficiency.[160, 163-165]

Table 1.7. Comparison of four phenolic compound recoveries in honey using different sorbents.

SPE Sorbent	Ref.	Gallic acid	Caffeic acid	*p*-Coumaric acid	Quercetin
		Recoveries (%)			
Amberlite XAD-2	160	15	70	95	20
Amberlite XAD-2	165	0	91	55	97
Amberlite XAD-2	164	0.5	27	66	42
Oasis HLB	164	84	54	70	40
Amberlite XAD-2	163	6.7	NA	NA	20
Bond Elut C18	163	0	NA	NA	85

Another variation of SPE includes using multiwalled carbon nanotubes as the adsorbent, achieving 90-101% recoveries of 18 phenolic compounds.[168-169]

The QuEChERS method has also been utilised for extraction of phenolic compounds from honey, where LLE is followed by salting out, and the organic phase is further cleaned up by dispersive SPE.[170] Compared the majority of other SPE methods, QuEChERS uses minimal sample (5 g honey in 10 mL water) and solvent volumes (4 mL), reducing waste and cost. However, this method is not as universal as others, Silva *et al.* (2019)[170] achieved 74.3-118.5% recovery for the majority of phenolic compounds analysed, but only 58.9-67.0% recovery for rutin and 0% recovery for chlorogenic acid.

Instrument and detection methods

Liquid chromatography is the predominant method used for analysis of phenolic compounds; generally high-performance liquid chromatography (HPLC) with a recent

shift to ultra-high performance liquid chromatography (UHPLC) due to the shorter run times and better efficiency. Reverse-phase (RP) C-18 columns are used for separation, although there is great variation between column dimensions. Several studies have compared column types; Liang *et al.* (2009)[171] found the Zorbax SB-C18 column performed best, in contrast, Badjah-Hadj-Ahmed *et al.* (2016)[169] found the Betasil C18 column provided the best results against the Zorbax.

Gradient elution is the most common procedure as phenolic compounds co-elute during isocratic elution,[172] using aqueous mobile phase A acidified with formic acid, acetic acid or phosphoric acid, and organic mobile phase B, MeOH or ACN, also sometimes acidified.[53, 128, 131, 148, 167, 170] ACN generally achieves better sensitivity and peak shape than MeOH, however Zhou *et al.* (2014) determined that MeOH gives better resolution of flavonoid peaks. Chromatographic conditions (acidity, flow rate and gradients) were quite variable between studies, as they were optimised to suit the task and instrumentation used.

The most common detectors coupled to the HPLC (or UHPLC) for honey phenolic compound quantification were ultraviolet (UV) detectors (photodiode array detector, PDA or DAD), and mass-spectrometers (MS) (Table 1.8). While UV detectors are cheap and easy to use, there are several disadvantages including poor sensitivity, especially for analytes in low quantities or with weak UV absorption, and difficulty in identification of peaks that have similar UV spectra or that co-elute.[142, 171] UV chromatograms also have to be recorded at differed wavelengths for phenolic compounds to optimise detection,[142] for example 3-PLA is detected at 265 nm but not at 280 nm.

MS enables a higher sensitivity and selectivity than UV, providing more information on compound structure. However, it is more expensive to use and requires volatile mobile phase, thus phosphoric acid cannot be used. There are various ionisation techniques and mass analysers used for MS, the most common being electron spray ionisation (ESI) with either quadrupole (single or triple) or quadrupole time-of-flight (Q-TOF) mass analysers.[122] Several methods also couple UV and MS detectors together for phenolic compound analysis.[133, 162, 167-168]

Electrochemical detectors (ECD) coupled to the HPLC are also used for phenolic compound determination,[142, 171, 173-174] however they are less common than UV or MS

detectors. These detectors are very sensitive, and depending on the applied potential and working electrode material, more specific than UV [122] and are another low cost, efficient detector for phenolic compound analysis. Zhu *et al*. (2019)[142] found ECD was significantly more sensitive than UV, with limits of detection (LOD) of phenolic compounds in honey 4-83 times lower for ECD.

Another instrument method used for phenolic compound analysis in honey is capillary electrophoresis (CE), which has small sample consumption, high separation efficiency and short analysis time.[161] However, the short analysis time is somewhat negated by the required washing of the capillary between each sample, required to maintain reproducibility.[175] Delgado *et* al. (1994)[176] found that it was also difficult to separate all honey flavonoids in one run. CE can be coupled to a range of detectors,[177] primarily UV[175] and MS[161] for phenolic compounds in honey.

Gas chromatography (GC) is not often used for phenolic compound analysis, as analytes have to be volatile. Head space solid-phase micro-extraction GC-MS (HS-SPME-GC-MS) however, was used by Beitlich *et al.* (2014)[127] to determine volatiles in mānuka, kānuka and jelly bush honey, including 2'-MAP. Through derivatisation with trimethylsilyl, phenolic compounds can be analysed, however flavonoids cannot.[145]

Table 1.8. Phenolic compound extraction, instrument, and detection information from literature articles.

Samples	Honey mass in water volume	Extractant	Extraction method	Instrumentation	Stationary Phase / Mobile Phase	Phenolic Compounds	Ref
n = 28, mānuka, kānuka, clover, rewarewa	0.2 g in 20 mL	4 mL (1% Formic acid in MeOH)	SPE cartridge (Strata-X)	HPLC-MS/MS	C-18, 3 μm 150 x 2 mm A – 0.2% Formic acid (aq) B – MeOH	7 Phenolic compounds	[79]
n = 40, different floral and geographical origin	10 g in 50 mL	20 mL ethyl acetate	LLE – Ethyl acetate	HPLC-DAD	C-18, 5 μm 250 x 4.6 mm A – 2% Acetic acid (aq) B – ACN	12 Phenolic acids	[131]
n = 12 *Dendropanax dentiger* honey by *Apis cerana*	2.5 g in 10 mL	6 mL MeOH	SPE Cartridge (Oasis HLB)	UPLC-Q/TOF-MS	C-18, 1.8 μm 100 x 2.1 mm A – 0.02% Formic acid (aq) B – 0.02% Formic acid in ACN	35 Phenolic acids, 18 flavonoids	[53]
n = 16 polyfloral, 8 each of *A. mellifera* and *M. beecheii*	20 g in 100 mL	1 mL MeOH	SPE Cartridge (Sep-Pak C18)	HPLC-DAD-ESI-MS/MS	C-18, 2.7 μm 150 x 4.6 mm A – 0.1% Formic acid (aq) B – ACN	19 Phenolic compounds	[162]
n = 17 Florida monofloral (citrus, tupelo, palmetto, gallberry), 3 mānuka	10 g in 40 mL	6 mL MeOH	SPE Cartridge C18	HPLC-DAD-ESI-MS/MS	C-18, 5 μm 250 x 4.6 mm A – 0.5% Formic acid (aq) B – MeOH	10 Phenolic compounds	[133]
n = 36 Tuscany honeys (acacia, chestnut, clover, heather, marruca, sulla, sunflower)	1 g in 5 mL	3 mL MeOH	Amberlite XAD-2	HPLC-DAD HPLC-ESI-MS/MS	C-18, 2.1 μm 100 x 2.7 mm A – 0.14% Formic acid (aq) B – 0.14% Formic acid in ACN	24 Phenolic compounds	[160]
n = 10 Chinese honeys of 10 floral origins	25 g in 250 mL	2.5 mL ACN	Counter flow salting-out homogeneous LLE	UHPLC-MS/MS	C-18, 2.1 μm 100 x 1.8 mm A – 0.1% Formic acid (aq) B – ACN	7 Phenolic acids	[152]
n = 2, linden, heather	20 g in 100 mL	50 mL MeOH	SPE Cartridge (Oasis HLB)	HPLC-DAD HPLC-FLD	C-18, 5 μm 250 x 4.6 mm A – 0.2% Formic acid (aq) B – MeOH	6 Phenolic acids, 3 flavonoids	[164]
n = 11 acacia samples from China	30 g in 120 mL	5 mL (10% Formic acid in MeOH)	RP-SPE Cartridges RP-AE-SPE Cartridges	HPLC-PDA-MS	C-18, 5 μm 150 x 4.6 mm A – 2% Acetic acid (aq) B – 2% Acetic acid in MeOH	17 Phenolic compounds	[167]
n = 7, China honeys (schisandra, acacia, rape, chaste, eucalyptus, longan, litchi)	0.5 g in 1 mL	3 mL ACN	SULLE	HPLC-UV HPLC-ECD	C-18, 5 μm 250 x 4.6 mm A – 0.5% Formic acid (aq) B – MeOH	17 Phenolic compounds	[142]

Samples	Honey mass in water volume	Extractant	Extraction method	Instrumentation	Stationary Phase / Mobile Phase	Phenolic Compounds	Ref
n = 52 bracatinga honeydew honey	5 g in 10 mL	4 mL ACN	QuEChERS	HPLC-DAD	C-18, 2.7 µm 50 x 4.6 mm 0.1% Formic acid (aq), MeOH, ACN, THF	18 Phenolic compounds	[170]
n = 8 Malaysian honeys	10 mL	0.4 mL (propyl acetate: pentanol: hexanol; 1:2:1.5 v/v/v)	LLME	HPLC-DAD	C-18, 2.7 µm 100 x 4.6 mm A – 0.1% Formic acid (aq) B – ACN	14 Phenolic acids	[178]
n = 7 Italian honeys (3 acacia, 2 sulla, thistle, citrus)	10 g in 100 mL	0.45 mL CHCl₃	DLLME	HPLC-UV HPLC-MS	C-18, 1.6 µm 50 x 2.0 mm A – 0.1% Formic acid (aq) B – 0.1% Formic acid in MeOH	5 Phenolic acids, 10 flavonoids	[149]
n = 12 Greek honeys	3 kg in 1 L	3 L Butanol 3 L Ethyl acetate	Serial LLE	HPLC-DAD	C-18, 5 µm 200 x 2.0 mm A – 0.3% Formic acid (aq) B – MeOH	12 Phenolic acids	[146]
n = 20 Turkish honeys, 13 monofloral, 7 multifloral	7.5 g in 25 mL	15 mL MeOH 30 mL diethyl ether: ethyl acetate (50:50 v/v)	Serial LLE	HPLC-UV	C-18, 5 µm 150 x 4.6 mm A – 2% Acetic acid (aq) B – 80% ACN: 20% MeOH	16 Phenolic compounds	[147]
n = 2, sunflower honey	20 mL	300 µL ACN 100 µ L 1-octanol	IDLLME	HPLC-UV	C-18, 10 µm 250 x 4.6 mm A – 0.3% phosphoric acid in MeOH	Quercetin	[148]
n = 183, NZ honey including 113 mānuka, 23 kānuka	0.1 g/mL	NA	Dilute and shoot	HPLC-DAD	C-18, 3 µm 150 x 2.1 mm A – 0.1% Formic acid (aq) B – 0.1% Formic acid in MeOH	9 Phenolic compounds	[128]
n = 6, 5 Belgium, 1 Romanian	20 g in 200 mL	150 mL MeOH	Amberlite XAD-2 Resin	HPLC-DAD	C-18, 3.5 µm 150 x 4.6 mm A – 0.5% Acetic acid (aq) B – 0.5% Acetic acid in ACN	5 Phenolic acids, 3 flavonoids	[165]
n =29, 12 monofloral, 9 multifloral, 8 cactus honeys	200 g in 200 mL	300 mL EtOH 15 mL ethyl acetate	SPE with multiwalled carbon nanotubes LLE	UHPLC-ESI-MS	C-18, 1.7 µm 150 x 2.1 mm A – 0.1% Formic acid (aq) B – MeOH	9 Phenolic acids, 9 flavonoids	[168]
n = 2, rosemary honey	30 g in 150 mL	300 mL MeOH	Amberlite XAD-2 Resin	CE-ESI-MS	NA	13 Phenolic compounds	[161]
n = 11, monofloral honeys	50 g in 250 mL	300 mL MeOH	Amberlite XAD-2 Resin	CE-DAD	NA	26 Phenolic compounds	[175]

NA – not applicable

1.8   Research aims

The New Zealand honey export industry is a multi-million-dollar industry, however honey has to meet strict quality control regulations for export. It is important to identify any causes of repetitive non-regulatory results, such as the often non-compliance of high MGO mānuka honey to diastase activity requirements (DN > 8). Identification of the cause of low diastase activity in mānuka honey has implications on the future of mānuka honey exports, as sufficient evidence may lead to an exemption (or other actions) of mānuka honey to this regulation and an increase in honey exports.

This research aimed to identify correlations between diastase activity and selected bioactive compounds (MGO, DHA (MGO precursor), mānuka marker compounds, MSY and tannic acid) in fresh New Zealand honey, as well as to determine any causation of low diastase activity through perturbation of a honey matrix with these same compounds.

1.9   Thesis outline

The thesis has been divided into the following chapters to achieve the overall aims:

Chapter 2 details the experimental methodology used for this study.

Method validation for the three main analytical methods (SULLE, 3in1, and diastase) used throughout this study is described in **Chapter 3**.

Investigation of the catalysis mechanism and structure of porcine pancreatic alpha-amylase (PPA, due to lack of literature describing the honey alpha-amylase) was carried out **(Chapter 4)**. Alpha-amylase amino sequences were compared between PPA and *A. mellifera* (and seven other species) to determine conservation of important enzymatic regions. Phenolic compound inhibitors of alpha-amylase were identified.

A database (DB, *n* = 65) of various floral honey types was analysed for moisture, phenolic compounds, diastase activity and DHA, MGO and HMF (3in1) **(Chapter 5)**. Two long term storage experiments (20 °C, 161 days; 27 °C, 198 days) of a subset of DB honeys was also carried out to track changes over time in various honey types.

Clover honey spiked with MGO (2,250 mg/kg), DHA (980 mg/kg), MS (50 mg/kg), 2-MBA (30 mg/kg), 2'-MAP (30 mg/kg), 4-HPLA (30 mg/kg), 3-PLA (1000 mg/kg), and

TA (30 mg/kg) were stored at various temperatures, (20, 27, and 34 °C) and periodically tested for diastase, 3in1 and phenolic compounds to determine their influence on diastase activity over time (**Chapter 6**).

The main conclusion is summarised in **Chapter 7** with recommendations for future research on this topic.

2 Methods

2.1 Honey samples

Sixty-two honey samples were supplied by beekeepers from various locations around New Zealand, which made up the honey database. The full information for each honey is shown in Appendix A, including honey type, harvest area, and harvest date where applicable. Other honey was purchased or donated from commercial outlets. Each honey has a numeric assignment used throughout the thesis due to the commercial sensitivity of the data.

The majority of received honey was mānuka (n = 44), however also received was clover (n = 4), rewarewa (n = 2), mānuka /rewarewa blend (n = 1), kānuka (n = 3), pohutukawa (n = 1), southern rata (n = 1), beech honeydew (n = 2), kāmahi (n = 2), tawari (n = 1), thyme (n = 1), citrus (n = 1), towai (n = 1) and bush blend (n = 3) honeys.

Mānuka honeys (MGO 100+ and 900+) were supplied by Zealandia Honey as quality control samples for several of the analytical test methods. Hutton Honey clover honey was purchased for spiked trial purposes.

All honeys were stored in the freezer (–23 °C) when not used for analysis.

2.2 Solvents, standards, reagents and consumables

2.2.1 Solvents

Type 1 water (also known as MilliQ water) was obtained using a Millipore Milli Q Reference water purification system (18.2 MΩcm resistivity). Acetonitrile (ACN, gradient grade for LC) was obtained from Sigma and methanol (MeOH, ACS reagent) was obtained from VWR.

Sodium hydroxide pellets (≥99 %) were purchased from Merck. Hydrochloric acid (HCl, 36%) and formic acid (99%) were obtained from Ajax Finechem Pty Ltd.

2.2.2 Standards and reagents

2.2.2.1 Diastase – Phadebas® analysis

Sodium acetate trihydrate was obtained from May & Baker Ltd and glacial acetic acid (≥99%) from Sigma. The Phadebas® tablets were purchased from Phadebas AB.

2.2.2.2 3in1 Analysis

Methylglyoxal solution (MGO, 43.8% *w/w*) was obtained from Fluka Analytical, while 5-hydroxymethyl-2-furfuraldehyde (HMF, 99%), 1,3-dihydroxyacetone dimer (DHA, 97%), hydroxyacetone (HA, 90%), D-(+)-glucose (≥99.5%), sucrose (ACS grade), D-(-)-fructose (≥99%) and citric acid were purchased from Sigma. *O*-(2,3,4,5,6-Pentaflurobenzyl)hydroxylamine hydrochloride (PFBHA, 99+%) was purchased from Alfa Aesar.

2.2.2.3 Sugaring Out Liquid-Liquid Assisted Extraction (SULLE)

Standards used in the spiked clover trials are summarised in Table 2.1.

Table 2.1. Qualitative assignment standards.

Standard	Acronym	Chemical Name	Purity	Source
3-phenyllactic acid	3-PLA	(*S*)-2-hydroxy-3-phenylpropanoic acid	99.3%	A
4-hydroxyphenyllactic acid	4-HPLA	4-hydroxyphenyllactic acid	98%	B
2-methoxybenzoic acid	2-MBA	2-methoxybenzoic acid	NA	C
2'-methoxyacetophenone	2'-MAP	2'-methoxyacetophenone	NA	C
Methyl syringate	MSY	Methyl-4-hydroxy-3,5-dimethoxybenzoate	NA	C
Tannic acid	TA	Tannic acid	NA	C

NA – Not available. The purity of these standards was not available and therefore were used as 100%.
A. Sigma Aldrich; B. Sigma Chemical; C. Selleck Chemicals

2.2.3 Consumables

Absorbance measurements were taken of Phadebas diastase samples using disposable polystyrene cuvettes. Deep well plates (96 well, 1.2 mL) and silicon lids were used for 3in1 HPLC analysis, while 2 mL plain glass HPLC vials with 300 µL inserts, caps and septa were used for phenolic compound HPLC analysis.

The Phadebas method used Whatman #42 filter paper to filter samples. Phenolic compound samples were filtered with Minisart polytetrafluoroethylene (PTFE) syringe filters (0.45 µm) before HPLC analysis.

2.3 General methods

2.3.1 Apparatus temperature management

Water baths, incubators and ovens were used in this research for either sample preparation or storage trials. A thermocouple was used to set the temperatures accurately for each piece of equipment. Before experimentation or incubation commenced, seven temperature readings were taken at 15-minute (minimum) intervals at each spatial location (e.g., top, middle, bottom, left, right) to check for temperature fluctuations over time or spatial temperature gradients. All equipment accurately held temperature over the time tested (± 0.2 °C) and did not have a temperature gradient, ensuring that exact sample placement in the equipment was not necessary. For the incubators and ovens, temperature was checked fortnightly for the duration of sample storage.

2.3.2 Glassware cleaning

All glass vials and vial inserts were washed before use with detergent, well rinsed with tap water, then placed in 10% nitric acid baths overnight. Vials and inserts were thoroughly rinsed with tap water to remove acid, before three rinses with distilled water followed by oven drying at 100 °C for a minimum of 3 hours.

Other labware items were washed with detergent and thoroughly rinsed with tap water to remove any soap residue. Items were rinsed three times with distilled water then oven dried at 100 °C.

2.3.3 Statistical analysis and image generation

Analysis of data and graphical visualisation were carried out using Microsoft Excel (Version 2107), Minitab® (Version 21.1) and RStudio Statistical Software (Version 1.3.959). Chemical structures, diagrams and reactions were produced using ChemDraw (Version 20.0).

2.4 High performance liquid chromatography (HPLC) instrumentation

The HPLC system used for both the 3in1 and SULLE methods was a Waters Arc system with a quaternary solvent manager-R (with inbuilt degasser), Sample Manager FTN-R and 2998 PDA detector. The system was controlled using Empower™ 3 Chromatography Software.

2.5 Determination of moisture content

Samples were left to equilibrate to room temperature for a minimum of 4 hours prior to measurement by a Misco Palm Abbe PA203X digital refractometer. The refractometer was zeroed using distilled water. After placing the honey in the refractometer, the lid was closed and left to sit for at least 4 minutes to allow the temperature to equilibrate. Moisture readings were taken of homogenous honey samples at 30 second intervals until three constant consecutive readings were measured for reporting.

2.6 Determination of diastase number by Phadebas® Diastase honey test

2.6.1 Method

The diastase number of honey was determined based on the Phadebas® method.[93] Modifications include reducing the sample weight and volume, while keeping them in the same ratio, due to the limited honey sample volumes. Homogenous honey (0.200 ± 0.002 g) was dissolved in type 1 water (0.88 mL) to make the volume up to 1 mL (this solution was also used for 3in1 analysis, Section 2.7). Honey solution (0.25 mL) was transferred into a 15 mL falcon tube with acetate buffer (0.1 M, 4.75 mL, pH 5.2), mixed thoroughly and placed in a water bath (40 °C) for a minimum of 5 min. Each batch of samples required duplicate blank samples of acetate buffer (0.1 M, 5 mL, pH 5.2), which were treated the same as the honey samples. At 20 second intervals, a Phadebas® tablet was added to a sample (or blank) using tweezers, vortexed thoroughly for 10 seconds and placed back in the water bath for exactly 30 minutes. At which time, sodium hydroxide solution (0.5 M, 1 mL) was added and the sample immediately vortexed for 5 seconds to terminate the reaction. The solution was filtered (Whatman #42 paper filters) and the absorbance measured in 1 cm polystyrene cuvettes at 620 nm on the Dynamica spectrophotometer using distilled water as the blank.

The process from sample dilution to reaction termination was completed within 1 hour to provide accurate, reproducible results.

2.6.2 Calculation of results

To achieve a value for diastase number (DN) from the absorbance value, the following equations (Equation 1, Equation 2) were used (where ΔA_{620} is the sample absorbance less the average of the two blanks):

$$DN\ (for\ \geq\ 8)\ = 28.2 \times \Delta A_{620} + 2.64$$

Equation 3

$$DN\ (for < 8) = 35.2 \times \Delta A_{620} - 0.46$$

Equation 4

These relationships were determined by Bogdanov and Lischer (1993)[179], where good correlation was found between the Phadebas and Schade methods (*n* = 57 honey samples).[93] For DN ≥ 8, Equation 1 was utilised, while values that fell below 8 were recalculated using Equation 2, due to the inability of Equation 1 to give a DN value less than 2.64.[180] The results could also be determined by cross referencing the adjusted absorbance value to the DN stated on the Phadebas® instruction chart.[181]

2.7 Analysis and quantification of DHA, MGO and HMF by HPLC (3in1 method)

2.7.1 Reagents

An artificial honey (AH) was made up of glucose (34.5% ± 1%), fructose (45.7% ± 1%), sucrose (1.5% ± 1%) and type 1 water (18.3% ± 1%), mixed with short (<1 min) heating periods on a hotplate at 30 °C to aid dissolution of sugars into the saturated sugar solution. AH was stored in the freezer (–18 °C) until use. Dilute AH solution was prepared by thoroughly mixing AH (3.00 g ± 0.01 g) with type 1 water (3.2 mL) for a final volume of 5 mL.

Hydroxyacetone (HA) solution (270 mg/mL, internal standard) was prepared by making HA standard (0.25 mL, 90%) up to 100 mL in type 1 water. HA solution (5 mL) was made up to 100 mL with ACN to form a HA:ACN (1:19) solution. Citrate buffer (0.1 M, pH 4.0) was prepared; citric acid (2.1 ± 0.1 g) was mixed with type 1 water (approx. 60 mL) and NaOH solution (12.5 mL, 1 M), adjusted to pH 4.0, then made up to 100 mL with type 1 water. PFBHA derivatisation stock was prepared by dissolving PFBHA (1.00 ± 0.01 g) to 100 mL with citrate buffer (0.1 M).

2.7.2 Standards

Individual stock standards were made up of DHA (8.00 mg/mL), HMF (0.20 mg/mL) and MGO (1.25 mg/mL) in type 1 water. Six mixed calibration standards (MStd) were made up from the individual stock standards and type 1 water (Table 2.2).

Table 2.2. Concentrations of analytes (DHA, MGO, HMF) in mixed calibration standards (MS 1-6).

	Concentration of analytes (mg/mL)					
	MStd 1	MStd 2	MStd 3	MStd 4	MStd 5	MStd 6
DHA	2.40	1.80	1.20	0.60	0.80	0.01
HMF	0.030	0.020	0.012	0.006	0.003	0.0003
MGO	0.480	0.320	0.160	0.080	0.020	0.002

2.7.3 Sample preparation

Thoroughly homogenised honey (0.20 g ± 0.002 g) was dissolved in type 1 H_2O (0.88 mL), to make a final solution volume of 1 mL. The same sample solution was used for both 3in1 and Phadebas methods, with prioritisation of solution use for the Phadebas method due to the time sensitive nature of the method. On occasions where 3in1 could not be carried out on the same day as Phadebas testing, diluted samples were stored in the freezer until analysis, defrosted and vortexed to mix before analysis.

Deep well plates were used to carry out the reaction and analysis; each well contained a total of 150 µL of analyte solution, the make up of which depended on solution type (e.g., sample, quality control, standard, Table 2.3). To each analyte solution, HA:ACN (0.7 mL, 1:19) was added, followed by PFBHA solution (0.2 mL). The wells were sealed with the silicon lid and the plate inverted several times to mix thoroughly before incubation in the oven at 50 °C for 60 min ± 5 min. The plate was left to cool to room temperature and the samples were analysed by HPLC.

Table 2.3. Composition of analyte solutions used for the 3in1 method.

Analyte solution	Composition of solution
Sample	150 µL of dilute sample
Calibration standards (MStd 1-6)	50 µL AH solution, 100 µL of MStd (1-6)
AH Blank	50 µL AH solution, 100 µL T1 H_2O
Water blank	150 µL T1 H_2O
Spiked solution	100 µL of dilute QC honey sample*, 50 µL spike solution

Made up of 3 g QC honey diluted with 8.2 mL of type 1 water

2.7.4 Instrument method

Analysis was carried out using the Waters ARC HPLC system (see Section 2.4 for details); separation was performed on a Phenomex C18 150x4.6mm, 5 µm column. Mobile Phase A was 30% ACN in type 1 water (v/v) with 100% ACN for mobile phase B. Gradient conditions are shown in Table 2.4, where the flow rate was 0.8 mL min^{-1} for a total run time of 10.5 min. The injection volume was set to 10 µL, and column and sample temperatures were 30 °C and 20 °C respectively. The 3D scanning range was 210-400 nm with individual 2D channels at 260 nm (DHA, HA and PFBHA), 273 nm (HMF) and 244 nm (MGO).

Retention times (RT) for the analytes can be found in Table 2.5. Both HMF and MGO form two isomers that are observed with PDA detection when derivatized with PFBHA. Quantification of MGO was carried out using the sum of both peaks, HMF was quantified using the peak at 4.97 min only.

Table 2.4. Gradient conditions for 3in1 method.

Time (min)	% Mobile phase A 30% ACN in water	% Mobile Phase B 100% ACN
0	90	10
2.5	15	85
5.5	15	85
7.0	90	10
10.5	90	10

Table 2.5. Retention time (RT) of 3in1 analytes.

Analyte	RT (min)	Analyte	RT (min)
DHA	4.09	HA	4.80
HMF isomers	4.97, 5.06*	PFBHA	4.45
MGO isomers	7.50, 7.98		

* 5.06 min Peak not quantified

2.7.5 Data work-up

To determine the concentration of analytes in the honey, the following steps were taken. The calibration curve was made using the mass of analyte in the standard versus the corrected area ratio (Equation 5). The least squares regression slope equation of the calibration curve was used to calculate the concentration of analyte in each honey sample (Equation 6 and Equation 7).

$$Corrected\ area\ ratio = \frac{Peak\ area\ analyte_{Std}}{Peak\ area\ HA_{Std}} - \frac{Peak\ area\ analyte_{Blank}}{Peak\ area\ HA_{Blank}} \qquad \textbf{Equation 5}$$

$$Mass\ analyte\ (mg) = \frac{Corrected\ area\ ratio - intercept}{Slope} \qquad \textbf{Equation 6}$$

$$Concentration\ analyte\left(\frac{mg}{kg}\right) = \frac{Mass\ analyte\ (mg)}{Mass\ honey\ (g)} \times 1000 \qquad \textbf{Equation 7}$$

2.8   Analysis and quantification of polyphenols by HPLC - SULLE

The SULLE method is adapted from Zhu *et al.*(2019).[142]

2.8.1   Reagents

The quantified analytes for this method were 3-PLA, 4-HLPA, 2-MBA and MSY.[*] Stock standards of these were made in type 1 water to concentrations of 33.39 mg/mL, 0.99 mg/mL, 0.98 mg/mL and 1.64 mg/mL respectively. These stocks were stored in the freezer (−18 °C) until used. Both 3-PLA and MSY required sonication at 40 °C to fully dissolve once defrosted.

Due to the maximum concentration required for the calibration curves, the mixed calibration standards were made in two groups; group 1 included 2-MBA and 4-HPLA, while group 2 had 3-PLA and MSY (Table 2.6). These standards were made straight from the stock solutions (not by serial dilution); standards 1-4 were made up to 1 mL, while standards 5 and 6 were made up to 2 and 10 mL respectively. A larger volume was used for the lower concentration standards to limit any error in pipetting. All standards were made up to volume with type 1 water.

Table 2.6. Calibration standard (Std) concentrations.

	Group 1 Concentration		Group 2 Concentration	
	2-MBA	4-HPLA	3-PLA	MSY
	(mg/mL)			
Std 1	0.25	0.25	7.0	0.36
Std 2	0.15	0.15	5.6	0.29
Std 3	0.10	0.10	3.0	0.20
Std 4	0.050	0.050	1.5	0.10
Std 5	0.020	0.020	0.50	0.050
Std 6	0.0025	0.0025	0.050	0.0050

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[*] 2'MAP was originally included but could not be quantified with this method – see Method Validation Chapter

2.8.2 Sample preparation

Thoroughly homogenised honey samples (0.5000 ± 0.0020 g) were dissolved in HCl acidified water (pH 2.0) in round bottom 13 mL tubes, using an orbital shaker. Acetonitrile (1.00 mL) was added to the honey samples, and the samples vortexed for 10 seconds before orbital shaking for 10 minutes. The samples were centrifuged at 3214 RCF for 1 minute for phase separation. The upper organic phase was collected in a flat-bottomed glass vial. Extraction was repeated twice more with ACN (1.00 mL), with the organic phase from all extractions collated and dried down at 40 °C under a gentle nitrogen stream for approximately four hours then left a 40 °C overnight. Dry residue was redissolved in 10% MeOH solution (400 µL) by sonication (10 min) and filtered into a 300 µL HPLC vial insert using Minisart PDFE syringe filters (0.45 µm).

2.8.3 Instrument Method

As with 3in1 testing, analysis was carried out using the Waters ARC HPLC system (see Section 2.4 for details); separation was performed on a Phenomex C18 150x4.6mm, 5 µm column. The mobile phase A was 1% formic acid in type 1 water (v/v) with 100% MeOH for mobile phase B. Gradient conditions are shown in Table 2.7, where flow rate was 0.8 mL min^{-1} for a total run time of 48 min. The injection volume was set to 20 µL for standards and 40 µL for samples, and column and autosampler temperatures were 30 °C and 20 °C respectively. Upon run completion, the shutdown method set the autosampler temperature to 10 °C to preserve samples in case dilutions needed to be carried out. The 3D scanning range was 210-400 nm with individual 2D channels at 265 nm and 280 nm. Retention times and channels for the analytes can be found in Table 2.8.

Table 2.7. Gradient condition for SULLE method.

Time (min)	% Mobile phase A 1% formic acid $_{(aq)}$	% Mobile Phase B 100% MeOH
0	98	2
2.5	98	2
26	65	35
35	0	100
38	0	100
43	98	2
48	98	2

Table 2.8. Retention times (RT) for polyphenolic standards.

Compound	RT (min)	2D Channel (nm)
2-MBA	24.0	280
3-PLA	21.8-22.5	265
4-HPLA	13.5	280
MSY	30.2	280

2.8.4 Data work-up

Due to some issues in validation (see section 3.3), injection volume of the samples was changed from 20 to 40 μL while standard injection stayed at 20 μL. This change is taken into account in the calculations for the concentration of analyte in each sample and is demonstrated in the following equations:

$$Sample\ analyte\ conc. \left(\frac{mg}{mL}\right) = \frac{Analyte\ area - intercept}{slope} \times \frac{20\ \mu L}{40\ \mu L} \qquad \textbf{Equation 8}$$

$$Sample\ analyte\ conc. \left(\frac{mg}{kg}\right) = \frac{Analyte\ conc\ \left(\frac{mg}{mL}\right) \times 0.4\ mL}{Sample\ mass\ (g)} \times 1000 \qquad \textbf{Equation 9}$$

2.9 Storage trials

2.9.1 Database honey storage

Two storage trials were carried out with the database honeys. For the first trial of the database honeys (Database Trial, DBT), ten honey samples were chosen (Table 2.9) to analyse any affects that time had on the various tested parameters. These honeys were prepared in duplicate and stored at 27 °C for the duration of this research. From timepoint 0, the honeys were tested for 3in1, diastase and polyphenols at approximately 45 day intervals up to 198 days. The choice of honey samples was dictated by initial diastase and 3in1 results to ensure that a range of mānuka honey samples were chosen with combinations of low DN:low DHA/MGO, high DN:low DHA/MGO, low DN:high DHA/MGO, and high DN:high DHA/MGO.

Table 2.9. Database storage honey samples.

Sample	Honey Type	Sample	Honey Type
NZ269	Mānuka	NZ323	Tawari
NZ308	Mānuka	NZ327	Mānuka
NZ316	Mānuka	NZ333	Rewarewa / mānuka blend
NZ319	Mānuka	NZ335	Kānuka blend
NZ322	Mānuka	NZ337	Pohutukawa

For the second trial, all database honeys received before 05/2021 were sub-sampled and stored at 20 °C for the duration of this research (Database Snapshot, DBSH). After 161 days these honeys were tested for 3in1, diastase and polyphenols to provide information on the effect of time on these parameters on a larger dataset.

2.9.2 Clover spiked storage trials

Commercially, only a limited number of alpha-amylases are available; these are from *Aspergillus oryzae*, *Aspergillus niger*, *Bacillus licheniformis* and *Bacillus sp.*. The variation between amylase from various species means that they have the potential to behave differently than the *Apis mellifera* alpha-amylase. Due to the unavailability of honey alpha-amylase commercially to be added to an artificial honey for experiments, a clover honey was used as the doping matrix. Clover honey also prevented the added complexity of adjusting a synthetic matrix to match pH and other potentially important parameters, it makes a good pseudo matrix with no or low concentrations of the key compounds that were under investigation.

Eight compounds were chosen to be individually added into the clover honey at the higher concentrations found in literature values (Table 2.10). These compounds were chosen due to their significance in mānuka honey (DHA, MGO) or their use as mānuka markers by the Ministry of Primary Industries (MPI). The exception was tannic acid, which has not been previously found in honey, however, was of interest to the research sponsor. Also included was a control honey sample to which water was added at the same volume of other compounds.

Spiking solutions were made up in type 1 water due to the compatibility of water with honey (compared to organic solvents) and also so they could be used to make up calibration standards (limited availability of standards). For each analyte, six aliquots of honey were spiked, with the spike solution equating to 3% (v/w) of the honey to ensure that moisture content was not altered to a significant degree. Duplicate samples were incubated at three temperatures (20 °C, 27 °C and 34 °C) for storage. Testing of 3in1, SULLE and Phadebas was carried out at time zero and continued at one-month intervals up to 6 months. Aliquots for testing were removed and stored at –18 °C until analysis.

Several factors played a role in the choice of perturbant concentration. Excessive spikes would not be representative of actual honey samples, however reactions that may occur

might not be obvious if the concentration is too low. Solubility of the analytes also had to be considered, making sure to add sufficient perturbant without affecting the honey moisture levels. The concentrations were chosen to be on the upper end of values stated in literature.[31]

Table 2.10. Summary of clover spiked storage trials.

Perturbant	Concentration (mg/kg)	Perturbant	Concentration (mg/kg)
Water	Control	2-MBA	30
MGO	1000	2'-MAP	30
DHA	1000	MSY	75
3-PLA	1000	TA	30
4-HPLA	30		

2.10 Quality control

For the 3in1 and SULLE methods, steps were taken to check the quality of results. For each batch of samples analysed, three controls were used. Each instrument batch had a set of calibration standards analysed at the beginning and end of the sequence to check for any instrument drift, with randomly selected individual standards analysed every 15 to 20 samples in batches of 25 samples or greater. Blank samples were analysed each run to check for contamination. Lastly, in-house honey quality control (QC) samples with the same matrix were analysed with each batch to ensure the performance of the instrument and correct sample preparation was carried out correctly; a high and low QC for 3in1, with only the high QC analysed by the SULLE method due to the restriction of samples that could be carried out in each batch.

2.11 Alpha-amylase sequencing

Several tasks were performed to determine similarities between the structures (active sites) of alpha-amylase described in literature to the *A. mellifera* alpha-amylase, through comparison of amino acid sequences. Alpha-amylase amino acid sequences (for various animal species) were extracted from the GenBank database.[182] Multiple sequence alignment of the selected sequences was generated using the Clustal Omega program (version 1.2.4)[183] with the signal peptides identified using the SignalP program (version 5.0).[184]

3 Method Validation

The methods used for this research were validated prior to their utilisation on the research samples. Methods chosen from literature were modified to suit the current research, therefore validation was essential to ensure that reliable analytical data was produced. Validation was carried out on the Phadebas® method (diastase), the 3in1 method (HMF, DHA, MGO) and the SULLE method (2-MBA, 4-HPLA, 3-PLA, MSY).

3.1 Phadebas® Method – Diastase

3.1.1 Method development for Phadebas®

Before method validation, several aspects of the method had to be altered from the Phadebas® Diastase Honey Test[181] supplied instructions to optimize the procedure for this research. The first modifications were the mass of honey and the volume of solvent used. The instructions called for 1.00 g of honey diluted to 100 mL with acetate buffer, of which 5 mL was used for the diastase test. This volume is not practical nor feasible with large sample numbers, thus the sample size was altered to 0.2 g of honey diluted to 1 mL with distilled water, where 0.25 mL of honey solution was mixed with 4.75 mL of the acetate buffer for the diastase test, resulting in the same concentration (0.01 g/mL) as the original method (Section 2.6). This alteration was one supplied by the project sponsor, allowing the same prepared solution to also be used for analysis by the 3in1 method (DHA, MGO, HMF).

After initial trials of the method, the centrifugation step (3,500 rcf for 5 min) stated by Phadebas® in order to clean up the sample before reading the absorbance was not adequate. Longer centrifugation time was applied (10 min), however this did not produce a clean sample. After centrifugation, most of the particulates from the Phadebas® tablet had settled to the bottom of the tube, however there were some low density, medium sized (approximately 1 mm diameter) particles floating at the top with small particles suspended in the rest of the solution which caused high absorbance readings due to the scattering of light from the particles. Variation between readings of the same sample was observed due to the turbidity causing absorbance fluctuations. The alternative option was filtration by either filter paper or syringe filters. Whatman #42 filter paper was chosen as it was cheaper and created less waste than the plastic

syringe filters. However, syringe filters may have been able to provide more precise results (less variation) as well as faster filtration.

3.1.2 Validation of the Phadebas® method

Validation of the Phadebas® method was carried out using a mānuka (NZ296) and a clover (NZ297) honey, which were chosen for their low and high DN respectively. Using the method stated in Section 2.6.1, ten replicate samples of each honey type were analysed on day 1, with three replicates carried out on the following two days to give a total of 16 replicates. Repeatability (intra-day and inter-day precision) was acceptable with RSDs of 5.7% and 5.8% respectively for NZ296, and 3.4% and 3.0% respectively for NZ297 (Table 3.1). The standard deviations for both honeys are similar, however the RSD for NZ296 is elevated compared to NZ297 due to a lower diastase activity (DN).

Table 3.1. Intra-day and inter-day precision of diastase activity for mānuka (NZ296) and clover (NZ297) honey samples.

	Intra-day (n= 10)		Inter-day (n= 16)	
	NZ296	NZ297	NZ296	NZ297
Average diastase activity (DN)	11.8	18.4	10.9	18.4
Standard deviation	0.6	0.6	0.6	0.5
RSD (%)	5.7	3.4	5.8	3.0

During the validation, the effect of two other variables on absorbance readings was also investigated. The first variable was the spectrophotometer used for measurements; there was a Dynamica and an Agilent Cary spectrophotometer available for use, with the Dynamica most accessible. Using the ten replicates from the first validation day of both honey types, comparison of the A_{620} values recorded by each spectrophotometer model was carried out through a student t-test (95% CI), demonstrating that there was no significant difference between the instruments (p = 0.16). The Dynamica was chosen for use in this method due to its simplicity and the ease of access.

The second variable was the influence of time between filtration and absorbance measurement. Using the day 1 replicate samples (n = 10) for both honey types, absorbance was measured with the Dynamica within 1 hour of filtration, then at 2, 6, 24 and 48 hours. Between analysis, samples were kept in their cuvettes in a covered box at room temperature. Using the student t-test (95% CI) it was found that there was no significant difference (p > 0.13) between the absorbance reading within 1 hour of

filtration and measurements at 2, 6, 24 and 48 hours after filtration. This allows for more flexibility when carrying out this method, as there is less time constraint after filtration.

3.2   3in1 Method – HMF, DHA, MGO

There have been various methods developed and published in peer-reviewed journals for the quantification of HMF, DHA and MGO individually,[94, 185-189] however the 3in1 method based off Windsor *et al*. (2012)[190] using PFBHA derivatisation has the advantage of analysing all three compounds simultaneously with UV detection. The rapid derivatisation allows for high throughput of samples, although the high cost of PFBHA compared to other methods is a disadvantage. This disadvantage is offset by the simultaneous analysis of three analytes opposed to individual analysis, reducing time and consumables. Modification of the original literature method[190] has reduced solvent and sample volume, thus reducing cost, while the use of deep well plates (DWP) simplifies the method, reducing preparation time and glassware clean up.

Derivatisation of the analytes, as well as the internal standard (hydroxyacetone), occurs through the bonding of the PFBHA hydroxylamine group to a carbonyl group on the analyte, forming the corresponding oxime. Due to the C=N double bond, rotation is restricted and therefore *E* and *Z* isomers are formed of both the MGO and HMF molecules, as they are asymmetrical. While two isomers occur with HMF (Figure 3.1),[191] the two carbonyl groups on MGO allow for the formation of four different isomers, *E+E*, *E+Z*, *Z+Z*, *Z+E* (example of *E+Z* shown in Figure 3.2),[192] although only two peaks are observed in the chromatogram at lower concentrations. This could be due to possible co-elution of the *E+Z* and *Z+E* isomers (very similar structures), as well as preference for formation of isomers based off steric hindrance of the PFBHA structure or concentrations of sterically hindered isomers falling below the limit of detection (LOD). Examination of the two observed MGO peaks across six days (*n* = 176) demonstrated that the ratio is relatively consistent with an RSD of 7.81%, the large peak accounting for approximately 96% of the total MGO peak area. The sum of both peaks was used for determination of MGO concentration in the honey samples. Unlike MGO, the ratio between the HMF isomers was not consistent. At lower concentrations (approx. <40 mg/kg), HMF peak 2 was found at a higher ratio to peak 1, while at higher concentrations (>40 mg/kg), the ratio was relatively consistent (7.7% RSD, *n* = 30).

Grainger (2015)[192] determined that HMF peak 2 co-eluted with an interfering compound, and hence used only peak 1 to calculate the concentration of HMF. The use of only HMF peak 1 for quantification of HMF was also used for this research.
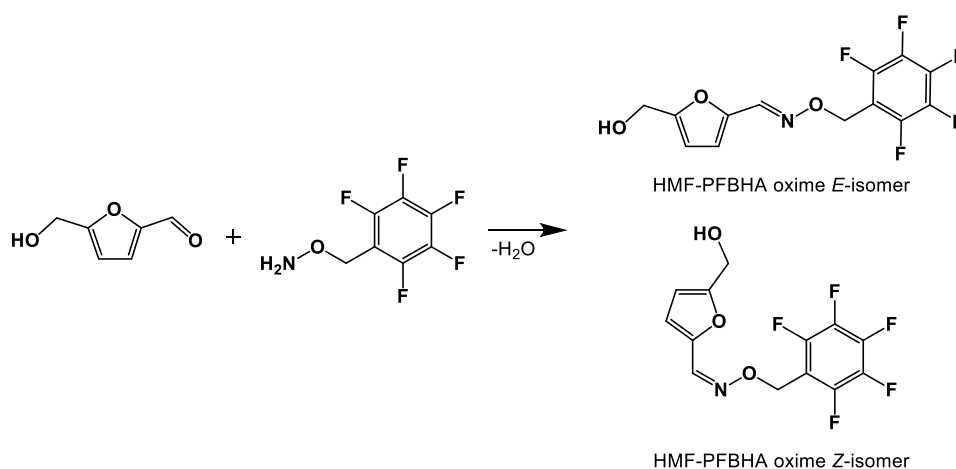
Figure 3.1. PFBHA derivatisation of HMF to form the *E* and *Z* isomers. Adapted from Bao *et al.* (2014).[191]
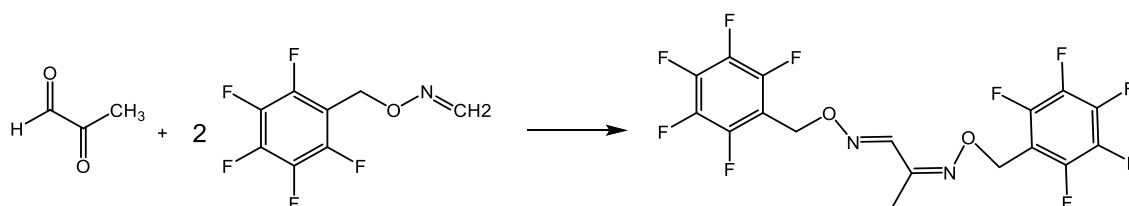
Figure 3.2. PFBHA derivatisation of MGO to form *E,Z* isomer of MGO-bisPFBHA. Adapted from Grainger (2015).[192]

Due to the low solubility of the MGO-bisPFBHA compound, it crystallises out in aqueous solution, thus ACN is required to solubilise the derivative.[190] Large variation in MGO content of honey samples may require the addition of more ACN than the method states for samples with very high MGO concentration. This change in volume is accounted for due to the use of the analyte to internal standard (HA) ratio in calculating all results (Section 2.7.5). Dilution of samples with ACN to dissolve MGO-bisPFBHA crystals will proportionally decrease the peak area of all analytes and HA.

As PFBHA also reacts with sugars in the honey, it is very important to have it well in excess for analysis. The PFBHA peak in the chromatogram is an indicator of whether analytes have been fully derivatised. If the PFBHA peak is too low or absent, it indicates the compounds of interest are unlikely to be completely derivatised, requiring reanalysis with either a diluted sample or with addition of more PFBHA. Example chromatograms

are shown in Figure 3.3 for the analytes, internal standard and derivatising agent at their optimum wavelength; HMF and MGO isomers are visible.
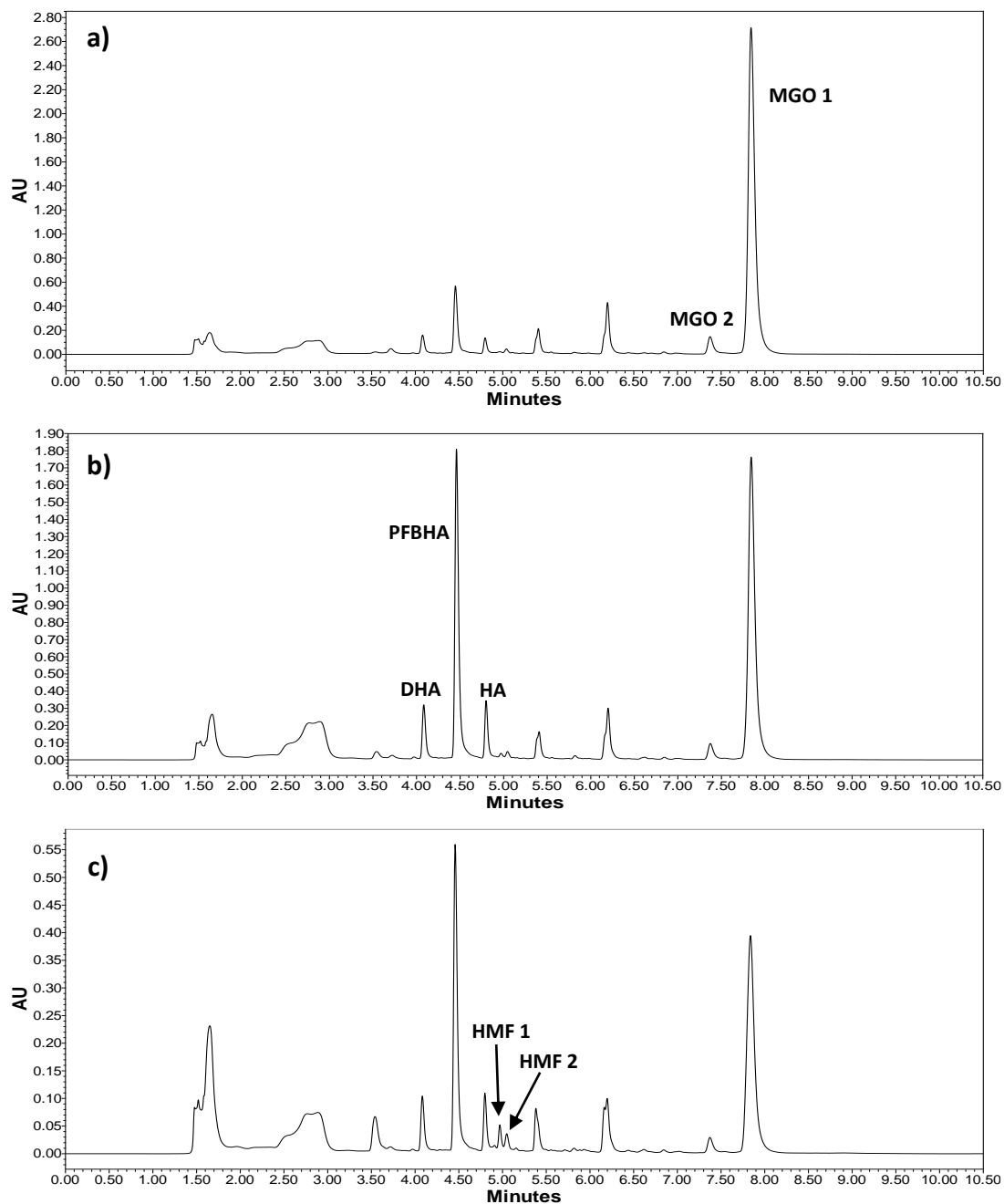
Figure 3.3. Typical 3in1 chromatogram of mānuka honey extracted at a) 244 nm (λ_{max} for MGO); b) 260 nm (λ_{max} for DHA, HA and PFBHA) and c) 273 nm (λ_{max} for HMF).

3.2.1   Method validation for DHA, MGO and HMF

Validation was carried out to evaluate the quantitative performance of the 3in1 method for DHA, HMF and MGO. The examined parameters include linearity, accuracy, precision (repeatability and intermediate precision) limit of detection (LOD) and limit of

quantification (LOQ). To determine these parameters, testing was carried out across three days. Each day, triplicate samples of three mānuka (NZ296) treatments (endogenous/non-spiked, high spike and low spike) and two clover (NZ297) treatments (endogenous and low spike) were analysed alongside calibration standards.

3.2.1.1 Linearity

For analysis, the working range should encompass the analyte concentrations found in honey samples to prevent extra sample dilution steps. The working ranges for DHA and MGO (Table 3.2) were chosen based on the expected concentrations found in literature (Section 1.4).[68, 70, 74, 79] Although the limit for HMF is 40 mg/kg for exports,[12] a range up to 100 mg/kg was chosen due to the potential of the storage trial samples within this research to exceed 40 mg/kg. The working range should be linear, with linearity evaluated by least-squares regression analysis. The calibration curve parameters for each of the analytes is found in Table 3.2. The working range for each analyte was determined to be linear, with coefficient of determination values (R^2) greater than 0.9990, showing strong correlation with little deviation. The regression equation for each analyte also intercepts the y-axis close to zero, indicating low background noise.

The linearity of each compound exceeded the working range for this method. Grainger (2015)[192] reported a linear range of up to 11640, 4237 and 525 mg/kg for DHA, MGO and HMF respectively using PFBHA derivatisation, demonstrating the large working range for this method.

Table 3.2. Calibration curve parameters for 3in1 analytes. Values for the regression equation and R^2 are calculated as the average of the three validation days (n = 3).

Analyte	Linear Range (mg/kg)	Regression equation*	Regression slope RSD (%)	Average coefficient of determination (R^2)
DHA	53.5-8030	$y = 31.55\,x - 0.04$	4.6	0.9995
HMF	1-100	$y = 437.123\,x - 0.004$	4.2	1.0000
MGO	5-1500	$y = 1097.76\,x + 0.36$	3.6	0.9996

* y is the corrected area ratio and x is the analyte mass (mg)

3.2.1.2 Accuracy

As there is no certified reference material (CRM) for DHA, HMF or MGO in honey, accuracy was measured via spike recovery. Two different honey types were used to determine accuracy, with only a low spike being carried out for NZ297 (clover) as DHA and MGO are not expected in non-mānuka honey types, and both a high and low spike carried out for NZ296 (mānuka) to ensure that analyte measurement is accurate at both

the top and bottom of the expected concentration range. High and low mixed spike solutions were prepared using the individual DHA, HMF and MGO stock standards in Section 2.7.2. As described in Section 2.7.3, spike solution (50 µL) was added to dilute honey solution (100 µL, 3 g honey diluted in 8.2 mL water) before sample preparation, resulting in the analyte concentrations in Table 3.3. In future, this could be improved by adding the spike directly to the honey before dilution to give it a chance to interact with the matrix, as this would show if the matrix were binding any analytes (lowered recovery). The clover honey was used as a pseudo mānuka honey matrix, since it contained low concentration of DHA and MGO (Table 3.3) and allowed for low concentrations to be analysed for help assessing the LOD.

Table 3.3. Spike concentration and endogenous sample concentration.

	High spike concentration (mg/kg)	Low spike concentration (mg/kg)	NZ296 endogenous concentration (mg/kg)	NZ297 endogenous concentration (mg/kg)
DHA	2000	100	230 ± 10	34 ± 1.6
HMF	60	10	14 ± 0.7	30 ± 1.8
MGO	1290	100	85 ± 5.5	22 ± 5.3

With such a large difference between the minimum and maximum expected concentrations for DHA and MGO, a mid-range spike would also be beneficial, however with an acceptable recovery achieved at both the low and high concentrations, it was deemed unnecessary to add a third. Recovery was calculated as in Equation 10.

$$Recovery = \frac{[MGO]_{spiked\ sample} - [MGO]_{endogenous}}{[MGO]_{theoretical\ spike}} \times 100\% \qquad \textbf{Equation 10}$$

The acceptable recovery range is 80-120%,[193] which all values occurred within (Table 3.4), except for the mānuka low spike on day 3 of validation (121.1%). This may have occurred due to a pipetting error; Dixon's outlier test was applied and this result was deemed to be an outlier ($p<0.001$) and was removed from analysis. The recoveries for DHA (84.5-88%) and MGO (88-91.9%) in this method were lower than those obtained by Grainger (2015)[192] of 111% and 109% respectively, while HMF recovery was similar, using the same derivatisation method (PFBHA). However, the PFBHA method is less accurate than methods using OPD derivatisation, which achieved 95.3% for DHA[194] and 98.3-101.5% for MGO.[195]

Overall, this method is accurate for the quantification of DHA, HMF and MGO in honey. HMF had the best recoveries, falling in the ideal range of 90-110%.[193]

Table 3.4. DHA, HMF and MGO recoveries for the spiked samples. Recoveries are calculated as the average across the three validation days.

	Recovery (%)		
Sample	DHA (*n* = 9)	HMF (*n* = 9)	MGO (*n* = 9)
Mānuka High Spike	88.0 ± 1.2	93.3 ± 1.0	91.9 ± 1.6
Mānuka Low Spike	84.5 ± 1.0*	95.1 ± 3.0	90.6 ± 2.5
Clover Low Spike	87.3 ± 0.6	99.9 ± 2.8	88.0 ± 3.8

* *n* = 6, as this excludes the erroneous recovery of 121.1% for validation day 3.

3.2.1.3 Precision

Two levels of precision were examined for the 3in1 method: intra- and inter-day precision. Intra-day precision was determined as the RSD of triplicate sample results within one day (*n* = 3); as there were five different concentration treatments (spikes and endogenous samples) across three days, fifteen RSD values were calculated for each analyte and the range for these values is shown in Table 3.5. Inter-day precision was calculated as the RSD of the results of each treatment across the three validation days (*n* = 9), describing the variation in results over time. Although there is variation in the intra- and inter-day precision, the highest RSD value for intra-day (MGO endogenous - 7.84%) and for inter-day precision (DHA endogenous - 7.71%) fall within the acceptable threshold of less than 10% RSD.[193] This indicates that the method is reproducible over time and at different concentrations. Although precision has been presented as a range for each of the analytes, the lower end range values for each are from the spiked samples which are not representative of a real sample, due to the added error of pipetting the spike. These spike samples give lower RSD though, due to the higher analyte concentration with the larger %RSD from the endogenous samples. The precision for this method is comparable to literature values for the same derivatisation method,[192] lower than the OPD method for DHA (7.8% RSD)[194] and higher than the OPD method for MGO (0.43% RSD).[195]

Table 3.5. Intra-day and inter-day precision ranges for DHA, HMF and MGO using both the spiked and non-spiked sample data.

Analyte	Intra-day precision (%RSD, *n* = 3)	Inter-day precision (%RSD, *n* = 9)
DHA	0.01-5.83	0.69-7.71
HMF	0.33-4.52	1.53-5.81
MGO	0.14-7.84	1.30-6.83

3.2.1.4 Limit of detection (LOD) and limit of quantification (LOQ)

There are numerous ways to determine the LOD and LOQ of an analyte for any given method.[196-199] For this research, LOD and LOQ were calculated as 3.3 and 10 times the standard deviation respectively,[198-199] of either the mānuka or clover low spike results across 3 days (*n* = 9). LOD is the lowest concentration of analyte that can be reliably detected but not necessarily quantified (precise not accurate), and LOQ is the lowest concentration of analyte that can be determined with suitable precision and accuracy. Ideally, the LOD and LOQ should have been obtained by spiking each analyte at concentrations close to the expected LOQ. The concentration that is both precise and accurate at this low level should be carried out with a minimum of six replicates to determine the standard deviation.[196] Due to the constraint of finding a suitable matrix and given the aim of the research, this more accurate method to determine LOD and LOQ was not undertaken.

The determined LOD and LOQ are found in Table 3.6. For DHA, the LOQ falls below the working range of the calibration curve, however both HMF and MGO LOQ values fall within calibration range. In this research, concentrations of MGO less than 23 mg/kg are not of consequence as these concentrations are unlikely to impact diastase activity (56 mg/kg MGO = NPA 5). It would have been ideal to obtain a lower LOQ for HMF, as this is also an indicator of heat and storage conditions with export regulations requiring <40 mg HMF per kg honey. It would give confidence in HMF concentrations reported in the 1-5 mg/kg region, which is the likely range for fresh honey, samples stored at room temperature up to several years[200] or several days at up to 70 °C.[201]

The MGO LOD for this research was higher than most literature (0.04-2.19 mg/kg) using both the PFBHA[192] and OPD methods,[195, 202] however it is much lower than the LOD reported by Adams *et al*. (2008)[68] who used a direct dilution method (50 mg/kg). The LOD and LOQ for DHA was approximately four times lower than those reported by Grainger (2015)[192], while for HMF these values were approximately four times larger.

Table 3.6. LOD and LOQ for DHA, HMF and MGO using the 3in1 method.

	LOD (mg/kg)	LOQ (mg/kg)	Data used
DHA (clover low)	2.8	8.4	Clover low spike
HMF (mānuka low)	1.8	5.5	Mānuka low spike
MGO (mānuka low)	7.5	23	Mānuka low spike

The validation data shows that the 3in1 method is suitable for the quantification of DHA, HMF and MGO in honey with good accuracy (84.5-99.9%), precision (<7.84% RSD) and capability to detect all three analytes at low concentrations.

3.3 Sugaring out assisted liquid-liquid extraction (SULLE) – phenolic compounds

The method chosen for phenolic compound analysis in honey for this research was based off the sugaring out assisted liquid-liquid extraction (SULLE) method by Zhu *et al*. (2019)[142] and used HPLC-PDA detection. Compared to the other methods discussed previously (Section 1.7.3.2), SULLE was determined to be the most suitable for the task required. The benefits of this method include:

- Reduced solvent volume
    - More suitable for the large number of samples in this research
    - Reduces cost
    - More environmentally friendly, greener method
- Reduced sample volume
    - More suitable for repeated analysis of storage trials
- Relatively rapid method with the capability to do multiple samples at once
    - Larger throughput
- Good recoveries of analysed compounds (79.8% to 98.3% recoveries of eight phenolic acids and 88.9 to 105.7% recoveries of nine flavonoids with HPLC-ECD)[142]

Although Zhu *et al*. (2019)[142] determined that the electrochemical detection (ECD) had better sensitivity and selectivity than the UV detector, HPLC-PDA was utilised for this research as the instrumentation was readily available.

3.3.1 Method development

3.3.1.1 Instrument method optimisation

Using the instrument method stated by Zhu *et al.* (2019)[142] the first priority in developing the method was to determine the retention times of the phenolic compound standards and check the suitability of the method. Unfortunately, this method was not suitable for the detection of tannic acid as originally planned; no tannic acid was observed in the

chromatogram on injection of a 100 mg/L standard and thus tannin could not be quantified in this research. As tannic acid has maximum absorption at approximately 280 nm,[203] it should be detectable, however it may not be retained on the C18 column due to its high water solubility. Originally, this method was going to be used for the quantification of 2'-MAP as well as 2-MBA, 3-PLA, 4HPLA and MSY. However, 2'-MAP is volatile[204] and was not stable in the standards over time. It was also completely lost during the extraction procedure dry down phase at 40 °C under nitrogen, hence 2'-MAP was not quantified in this research.

Zhu *et al*. (2019)[142] used gradient analysis starting at 50% aqueous solution (0.5% formic acid) and 50% MeOH, which resulted in the quick elution of the phenolic acids compared to the flavonoids. A 2% starting MeOH concentration was chosen instead, to improve retention and resolution of the phenolic acids. The aqueous phase composition was also altered from 0.5% formic acid to 1% to ensure the phenolic acids were present in neutral rather than ionised form and were retained better on the C18 column. Due to the alteration in the composition of the starting gradient, the solution in which the phenolic compound residue was redissolved was also altered from 50:50 water:MeOH to 90:10 water:MeOH. Ideally, the dissolution solution composition should match the starting mobile phase concentration, however some compounds, including 3-PLA and MSY, are less soluble in water and 10% MeOH was decided as the optimum concentration. A lower MeOH concentration may reduce phenolic compound recovery due to precipitation, while a higher concentration would result in the band broadening of some compounds on the chromatogram due to the difference between sample and starting gradient compositions.

Once the full method (sample preparation and instrument analysis) described by Zhu *et al*. (2019)[142] had been applied to several honey samples, the chromatograms were reviewed and adjustments to the HPLC gradient were applied to achieve the best separation between all peaks in the samples. Complete peak resolution was difficult to achieve; SULLE and PDA are non-specific extraction and detector method causing numerous peaks to be detected in the chromatogram (Figure 3.4). The gradient was optimised to obtain the best resolution for the quantified analytes, although full resolution could not be achieved (an example is shown with 2-MBA, Figure 3.5). While for some samples, analyte peak resolution was good, variation in phenolic compounds

between floral honey types (and even within a single floral type) often resulted in less-than-ideal resolution. This research focused on mānuka honey, therefore separation of peaks within the mānuka matrix was prioritised over samples of other botanical origin.

The use of the 3D channel in PDA detectors allowed for some differentiation between peaks, however many phenolic compounds have similar UV profiles. Also, overlapping peaks cause skewed UV profiles and made identification difficult. For data analysis, the UV profiles of analyte peaks were compared back to those of the standards to ensure correct peak designation, or to confirm the absence of an analyte in the presence of other peaks with similar retention times. The ideal detector for this method would be MS, as it is more selective and informative than UV, but was not available for this research.
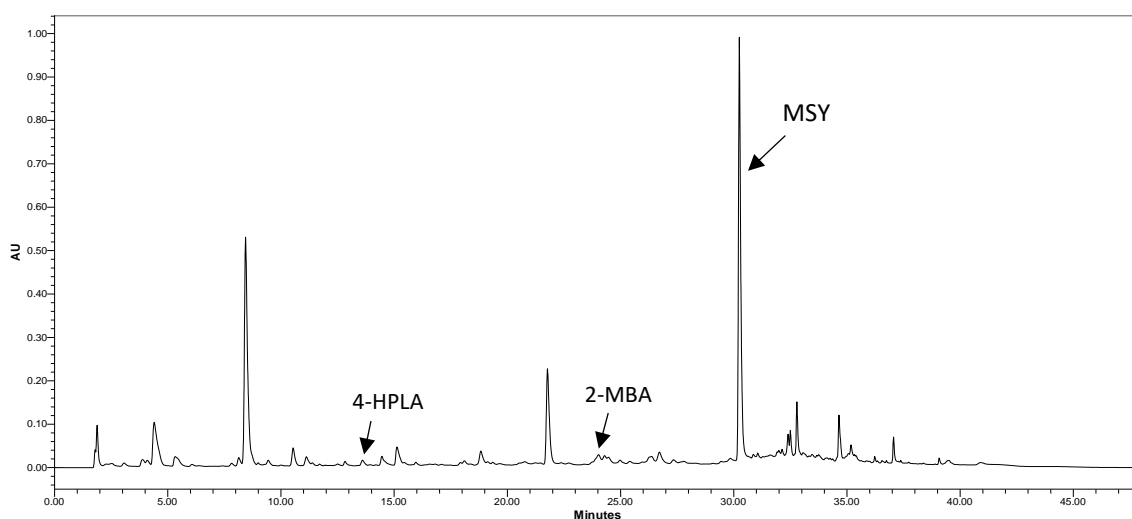
Figure 3.4. Typical SULLE mānuka (NZ316) chromatogram extracted at 280 nm, demonstrating the large number of extracted compounds and positions of quantified analytes. The 3-PLA peak is not observed at 280 nm.
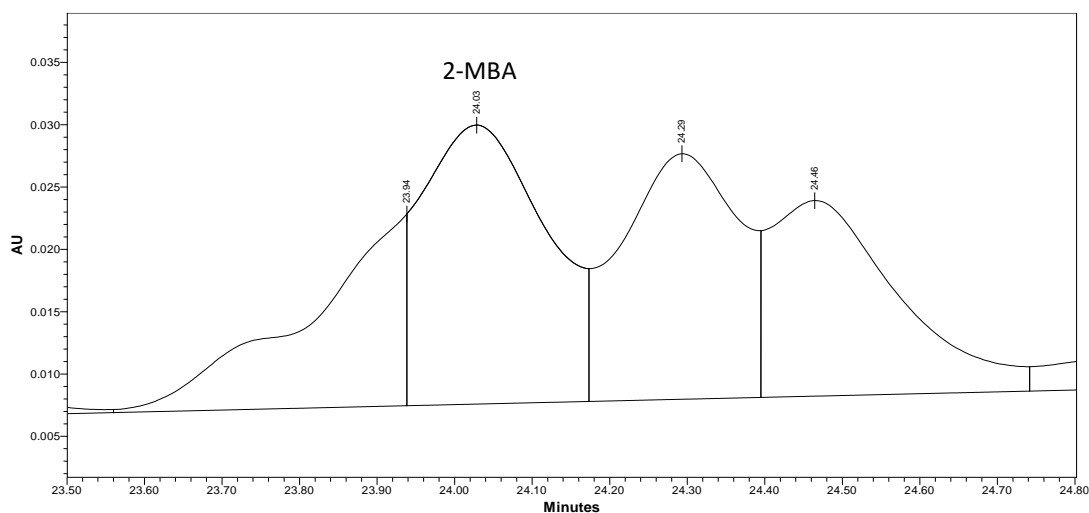
Figure 3.5. Zoomed in chromatogram exhibiting poor resolution of 2-MBA peak (24.03 min, 280 nm) in a mānuka honey (NZ316).

3.3.1.2   Preparation - Dissolution

Due to the lengthy duration required to dissolve honey into solution at a 1:2 (w/v) ratio, various attempts were made to reduce the time spent on this preparation stage. Addition of more acidified water at 1:3 and 1:4 (w/v) ratios did improve dissolution rate, however this also decreased the aqueous sugar concentration and prevented phase separation, thus the original ratio of 1:2 (w/v) was kept. When weighed into a falcon tube ('V' bottom), honey collected in the 'V'; to increase the surface area of honey exposed to the water, the sample vessel was changed from falcon to round-bottomed tubes which allowed honey to be more easily dissolved. Vortex mixing, sonication and orbital shaking were trialled to determine the optimal procedure for dissolution. While vortex mixing was the fastest procedure for individual samples due to its vigorous nature, for high sample numbers it was the most time consuming as samples could only be mixed individually, requiring active involvement by the researcher. Sonication was not efficient due to the low surface area to volume ratio of the phase interface. Orbital shaking, rigged to hold a rack of sample vials at an almost horizontal position, was determined as the optimal method. This provided vigorous agitation without the requirement of active involvement, reducing the overall time spent on dissolution.

As UV detectors are less sensitive than the ECD detector used by Zhu *et al*. (2019),[142] initially the mass of honey and acidified water was doubled while keeping the volume of ACN in the extractant steps the same, to obtain a more concentrated final sample.

However, recoveries were poor (<75%); method adjustments were made to increase the volume of extracting ACN and the number of extractions (Table 3.7). However, these did not improve recoveries.

Table 3.7. SULLE extraction combinations of ACN.

Number of extractions	ACN extraction volume combinations (mL)				
2	2+1	2+2	4+3	4+4	
3	2+1+1	2+2+1	2+2+2	4+2+2	4+4+2
4	2+2+2+1	2+2+2+2			

After trialling these adjustments with no gain in recovery, the method was partially reverted back to the original,[142] using 0.5 g honey diluted in 1 mL acidified water, with three extractions of ACN (1 mL) to reduce preparation time and the volume of sample and solvent used.

Compared to the Zhu *et al*. (2019)[142] method, the volume of 10% MeOH solution used to redissolve the dried extraction residue was increased from 200 μL to 400 μL to reduce any error (increase method precision) that may have occurred, either due to retained moisture in the dried extract or extracted residue volume variation between samples. This volume increase was compensated for by doubling the injection volume from 20 μL to 40 μL. The phase partition between the honey solution and ACN is not 100%,[153, 157] therefore some water will end up in the extracted organic phase. Hydrogen bonding between water molecules makes it more resistant to drying down at 40 °C under N_2 and therefore some moisture is likely to be retained in the residue. The increased volume could also potentially improve recovery of the less soluble compounds (however, these were not the focus of this research and were not investigated) as this dilutes the very soluble compounds in solution that may be affecting solubility of less soluble compounds. Syringe filtration was also made easier by increased volume.

3.3.1.3 Sugaring out

The separation of aqueous and ACN phases via sugaring out relies on the sugar molecules interacting and forming hydrogen bonds to the water molecules, replacing and "pushing" out the ACN.[153, 157, 205] Wang *et al.* (2008)[205] showed that at 1 °C, glucose (50 g/L) was the most effective phase separator (0.51 phase ratio, 95.4% upper phase ACN conc.) followed by sucrose (0.43 phase ratio, 90.4% upper phase ACN conc.) and fructose (0.40 phase ratio, 89.1% upper phase ACN conc.), at the same concentration.[205]

Using this method at temperatures higher than 1 °C requires a greater sugar concentration to achieve phase separation; 6 °C and 24 °C require 90 and 165 g/L of glucose respectively.[153] Increasing temperature causes a reduction in the phase ratio, although this is increased with higher sugar concentration until a certain concentration is reached, upon which the ratio plateaus (e.g. the phase plateau of 0.5 occurred after 165 g/L glucose at 18 °C). The upper phase ACN enrichment at 18 °C was found to be approximately 85% with 165 g/L glucose.[153]

Glucose, fructose and sucrose are the main sugars in honey, and utilising the SULLE method (0.5 g honey dissolved in 1 mL water) the accumulative concentration is approximately 260 g/L (adjusted for density of honey and average concentration of sugar in honey)[206]; consisting of fructose (145 g/L), glucose (104 g/L) and sucrose (12 g/L). Although the extractions are carried out at room temperature, this concentration is sufficient to achieve phase separation with a good phase ratio and upper phase ACN concentration. Observations of the phase ratio for SULLE extraction appeared to match measurements made by Dhamole *et al.*(2010),[153] although no exact measurement was carried out.

Wang *et al.* (2008)[157] found that addition of perturbants (e.g. syringic acid, furfural) to a glucose, water and ACN solution reduced the concentration of glucose need for phase separation at 1 °C from 25 g/L to 15 g/L. It is likely the compounds in honey display a similar effect. The addition of more glucose to the system increased the phase ratio (V_{upper}/V_{lower}) and the distribution co-efficient, where more perturbant was "pushed" into the ACN.[157] While this would work for the less polar compounds in honey, the water solubility of the polar analytes are less affected. Thus Zhu *et al.* (2019)[142] added salt in addition to the sugaring out effect, to reduce the solubility of these polar compounds (discussed further in Section 3.3.2.2).

3.3.2 Validation

The validation procedure for SULLE was carried out similar to 3in1, examining linearity, LOD, LOQ, accuracy and precision for 2-MBA, 3-PLA, 4-HPLA and MSY. Testing was carried out across three days using a clover honey (NZ324) with no spike (endogenous) and spiked with high and low concentrations of the analytes alongside calibration curves.

3.3.2.1 Linearity

The linear range of the analyte calibration curves were chosen based on expected concentrations as determined from the literature.[128] Six calibration standards were initially made up to cover a range from 50% of the lowest result (minimum) to approximately 150% of the highest result (maximum) from Bong *et al*. (2018)[128] for each of the analytes, except MSY. Due to the alterations in the method, changing the honey mass from 1 g back to 0.5 g, the standard concentrations converted into mg/kg were twice as much as originally planned. However, as it still covered the range of interest, no alterations were made.

Compared to the other three analytes, the instrument method is more sensitive for MSY, shown by the steep linear regression slope (Table 3.8). Due to this, MSY saturated the detector with the two most concentrated calibration standards, resulting in a non-linear calibration curve that plateaued (Figure 3.6). MSY standard concentrations were thus reduced to obtain a linear calibration curve. Samples with high MSY concentrations fell outside of the reduced working range; these samples were re-run using a smaller injection volume, either 20 or 10 μL depending on the concentration, effectively reducing the amount of MSY to be detected. Another method which quantified MSY using HPLC-DAD had a working range of only 0.4-20 mg/kg which corresponds to 0.008-0.4 μg/mL,[207] a much lower concentration than is used in this research (4-360 μg/mL). The authors stated that this peak was the most intense peak, aligning with what was observed in this research.[207]

The plot shows Peak area (y-axis, 0 to 30000000) versus Concentration (mg/mL) (x-axis, 0.000 to 0.700) with equation $y = 4E+07x + 2E+06$ and $R^2 = 0.9641$.
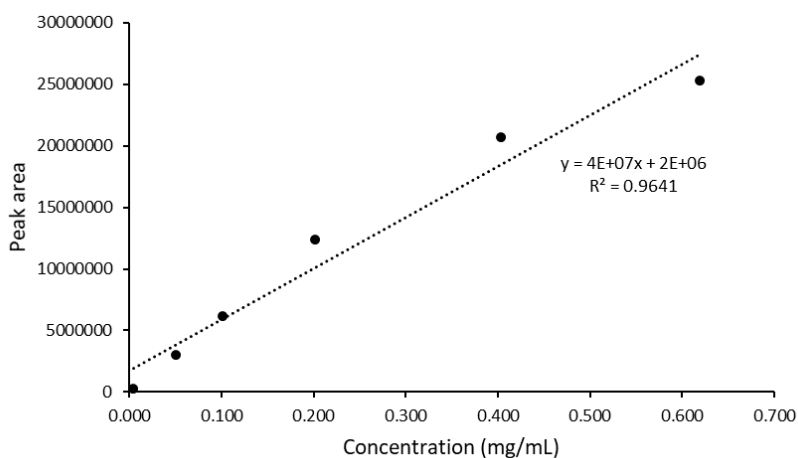
Figure 3.6. Calibration curve of methyl syringate (MSY) demonstrating saturation of the detector response for highest calibration points.

The calibration curve parameters for each of the analytes is found in Table 3.8. Each of the analyte working ranges is determined to be linear, with coefficient of determination values (R^2) greater than 0.9988, showing strong correlation with little deviation.

Table 3.8. Calibration curve parameters for SULLE analytes. Values for the regression equation and R^2 are calculated as the average of the three validation days.

Analyte	Linear Range (mg/kg)	Regression equation*	Regression slope RSD (n = 3, %)	Average coefficient of determination (n = 3, R^2)
2-MBA	0.98-98	y=19812616 x − 22867	0.4%	0.9997
3-PLA	19-2780	y=1114760 x − 12206	0.8%	0.9997
4-HPLA	0.99-99	y=9800263 x − 13770	1.2%	0.9995
MSY	1.7-144	y=58174809 x + 6895	0.6%	1.0000

* y is the peak area (AU) and x is the analyte concentration (mg/mL)

3.3.2.2 Accuracy and precision

As with the 3in1 method, accuracy for SULLE was determined with spike recovery. A clover honey (NZ324) was used as the spike recovery honey matrix due to its use in the clover spiked storage trial. It was also not expected to contain high levels of 2-MBA, 3-PLA, 4-HPLA or MSY, therefore allowing for low concentrations to be analysed. Unfortunately, due to the adjustments made in the method development step and a dilution error (neither of which were noticed before analysis of samples had started), compensations were not made to the original calculations resulting in spikes that were much higher than planned (Table 3.9 and Table 3.10).

High and low mixed spike solutions were prepared using the individual 2-MBA, 3-PLA, 4-HPLA and MSY stock standards in Section 2.8.1. Spike solution (50 µL) or T1 water (50 µL, endogenous) were added directly to each honey aliquot (0.500 ± 0.002 g), then prepared as described in Section 2.8.2, resulting in the analyte concentrations in Table 3.10. For 3-PLA and MSY, this was still representative of the samples (just not at a low concentration), however for 2-MBA and 4-HPLA, the low concentration spike was roughly around the upper range of the results for the database samples and clover perturbed samples.

Table 3.9. Original planned spike concentrations.

	High spike concentration (mg/kg)	Low spike concentration (mg/kg)
2-MBA	25	2
3-PLA	750	100
4-HPLA	25	2
MSY	35	5

Table 3.10. Spike concentration and endogenous sample concentration.

	High spike concentration (mg/kg)	Low spike concentration (mg/kg)	NZ324 endogenous concentration (mg/kg)
2-MBA	49.2	15.7	0
3-PLA	1390.3	772.4	43.7
4-HPLA	49.5	15.8	0
MSY	64.8	28.8	3.4

The acceptable recovery range is 80-120%,[193] however all analytes except MSY fell below this range, with 4-HPLA low spike having the lowest recovery at 71%. The recoveries for 2-MBA, 3-PLA and 4-HPLA are lower than ideal, however they are consistent between the high and low spike for each of the analytes. Zhu *et al*. (2019)[142] achieved recoveries of 79.8-98.3% for the phenolic compounds they analysed, much higher than the recoveries for 2-MBA, 3-PLA and 4-HPLA in this study. Due to the polar nature of these analytes and their hydrogen bonding capabilities, a portion is being retained in the aqueous phase during SULLE. Addition of salt may have improved the recoveries as salt would interact with the water molecules, reducing interactions between analytes and water molecules. Zhu *et al*. (2019)[142] found that adding salt had the potential to increase analyte recoveries up to 15% higher; phenolic compounds, which are more polar than flavonoids, were the most impacted by this addition, while flavonoids were less affected and had high recoveries (>80%) without the addition of salt. However, this salt addition was only discovered post validation and sample analysis as it was not stated in the methods section of the article, therefore was not incorporated into this research. If future analyses were carried out, it is recommended to redo the method validation utilising salt to obtain more accurate recoveries. Oelschlaegel *et al*. (2012)[129] achieved higher recoveries (95-105%) for 2-MBA, 3-PLA and MSY, compared to this method, however they used SPE cartridges to extract the phenolic compounds.

Table 3.11. 2-MBA, 3-PLA, 4-HPLA and MSY recoveries for the spiked samples. Recoveries are calculated as the average across the three validation days.

Sample	Recovery (%)			
	2-MBA (*n* = 9)	3-PLA (*n* = 9)	4-HPLA (*n* = 9)	MSY (*n* = 9)
High spike	72 ± 5	75 ± 1	71 ± 2	83 ± 4
Low Spike	74 ± 2	71 ± 1	72 ± 3	82 ± 3

Intra-day precision was determined as the RSD of triplicate sample results within one day (*n* = 3), with three RSD values (endogenous, high and low spike) calculated for each analyte and the range for these values is shown in Table 3.5. Intermediate precision was calculated as the RSD of the results for endogenous, high and low spike each across the three validation days (*n* = 9), describing the variation in results over time. The RSD values are all less than 8.5% (Table 3.12), indicating that the method is reproducible over time.[193]

Table 3.12. Repeatability range and intermediate precision for 2-MBA, 3-PLA, 4-HPLA and MSY.

Analyte	Intra-day precision (%RSD, *n* = 3)	Inter-day precision (%RSD, *n* = 9)
2-MBA	0.3-6.1	3.1-5.1
3-PLA	0.3-2.7	0.8-6.7
4-HPLA	0.3-6.3	4.0-8.5
MSY	0.2-6.6	3.5-5.9

Although the recoveries for the analytes were lower than the acceptable range, there was good precision (RSD<10%) and little variation between result recoveries. The results for this research have not been adjusted to account for the lower recovery, as without trialling the method incorporating salt, the percent increase is unknown and would have to be loosely based off the increases stated by Zhu *et al*. (2019)[142]. The low recoveries also have less consequence on the results as this research is primarily focused on relative differences between samples, and less so on the absolute concentrations.

3.3.2.3 *Limit of detection (LOD) and limit of quantification (LOQ)*

As with the 3in1 method, LOD and LOQ for SULLE were determined as 3.3 and 10 times the standard deviation respectively,[198-199] of either the low spike or endogenous results across 3 days (*n* = 9, Table 3.1). The LOD and LOQ results are higher than expected for the analytes. For 2-MBA, 3-PLA and 4-HPLA, their LOQ values (2.9, 31, 2.8 mg/kg respectively) are above the minimum value stated by MPI for the mānuka honey

definition (>1.0, >20.0, >1.0 mg/kg respectively), reducing confidence in results which fall under these values. Determination of LOD and LOQ using the standard deviation of analytes in a honey matrix gives a good indication of the limits of this method. In comparison, other studies use either the calibration curve deviation[129] or analysis of blanks[141, 208] to determine LOD and LOQ, which does not account for the background introduced by the sample matrix. It appears that the HPLC method is not suitable for detection at low concentrations of analyte; an alternative detector (e.g., MS), or change in column and/or method gradient may increase analyte separation from background interferences and improve the LOQ of the method.

Using HPLC with a triple quadrupole mass spectrometer, Oelschlaegel *et al*. (2012)[129] achieved LODs that were at least a magnitude lower for 2-MBA (0.04 mg/kg), 3-PLA (0.08 mg/kg) and MSY (0.15 mg/kg), demonstrating the effect of a more sensitive detector. Zhu *et al*. (2019)[142] carried out LOD and LOD determination for their phenolic compounds on both a UV detector and ECD detector, showing the ECD detector was much more sensitive than the UV, with LOD and LOQ values 4-40 times lower for the analysed phenolic compounds. However, the LOD and LOQ values for phenolic acids using UV detection were ranged from 3.87-36.88 µg/kg and 12.95-122.96 µg/kg respectively, calculated from the concentration that produced a signal-to-noise ratio of 3.[142] Based on the chromatograms from this research, these values (µg/kg) would not be quantifiable due to the large number of analytes extracted and occurring in the baseline, especially as they used a comparatively short instrument run time (37 min) for the number of compounds extracted, overlapping peaks would occur.

It is likely the LOD and LOQ for the analytes in this research are conservative, especially compared to literature methods,[129, 142] hence calculated values for results in the following chapters have been retained if they fall between LOD and LOQ; samples with results below the LOD have been removed.

Table 3.13. LOD and LOQ for 2-MBA, 3-PLA, 4-HPLA and MSY using the SULLE method.

Analyte	LOD (mg/kg)	LOQ (mg/kg)	Data used
2-MBA	0.88	2.9	Low spike
3-PLA	9.3	31	Endogenous
4-HPLA	0.85	2.8	Low spike
MSY	1.9	6.4	Low spike

The validation data shows that the SULLE method is suitable for the quantification of 2-MBA, 3-PLA, 4-HPLA and MSY in honey with average accuracy (70.9-83.2-%), precision (<8.5% RSD) and capability to detect the analytes at low (though not ideal) concentrations.

4 Alpha-Amylase Catalysis and Inhibition

4.1 Alpha-amylase overview

Diastase collectively refers to a group of enzymes, including amylases (alpha-, beta- and gamma-amylase), which break down starch into shorter units or maltose. The occurrence of diastase in honey (although there is no starch in honey) has allowed for it to be used as a quality control parameter through an assay which measures honey diastase activity on starch. Diastase in honey is primarily alpha-amylase (EC 3.2.1.1) originating from the honey bee (*Apis mellifera*),[23, 103] and belongs to the glycoside hydrolase 13 (GH13) family.[209] *A. mellifera* alpha-amylase has not been structurally characterised in literature, while other species have, including porcine (pig, *Sus scrofa*) pancreatic alpha-amylase (PPA, P00690.3). PPA is used as an example in this chapter to describe the general structure of alpha-amylase and its mechanism of action. Although the entire amino acid sequence between PPA and *A. mellifera* alpha-amylase will not be the same, important active and auxiliary site residues on the enzyme and the mechanism of action are generally conserved between various species of the animal kingdom.[210] Comparison of the amino acid sequences will identify conservation (or lack of) of important residues and give an indication of how alpha-amylase activity in honey might be reduced (low diastase activity).

4.2 Alpha-amylase structure and mechanism for action

Amino acid sequences of alpha-amylase vary depending on the organism of origin,[211-212] however the three core domains (domains A, B and C) that determine the three-dimensional protein structure are conserved. Domain A is an α/β 8-barrel where eight parallel β strands form the core of the barrel, surrounded by eight α-helices. A loop between the β3 strand and α3 helix of domain A forms domain B, with domain C consisting of the C-terminal extension; a C-terminal β-sheet domain in a Greek key β-barrel motif (Figure 4.1).[212-214]
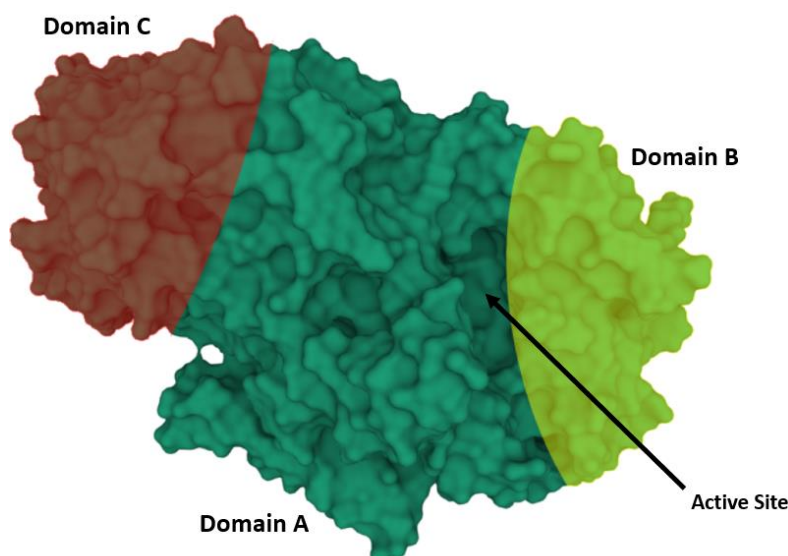
Figure 4.1. Structure of porcine pancreatic alpha-amylase modified from Gilles *et al.* (1996)[215] using Mol* Viewer.[216]

There are five important sites found on alpha-amylase, enabling its hydrolytic action:

- The active site

- Two surface carbohydrate binding sites

- A chloride binding site

- A calcium binding site

The cleft between domain A and domain B contains the active site, consisting of a catalytic triad of residues which hydrolyse the glycosidic starch bond.[212] These residues are Asp197, Glu233 and Asp300 (Asp – aspartic acid, Glu – glutamic acid) which act as the proton donor (Glu233)) and the base/nucleophile (Asp197) for the acid hydrolysis mechanism,[217] while the surrounding cleft is formed by numerous residues; charged, aromatic and non-polar.[214] Due to the configuration of the cleft active site, random binding of the substrate is promoted as opposed to specific binding and catalysis to the non-reducing end of starch molecules.[218] The generalised mechanism can be seen in Figure 4.2; protonation of the glycosidic oxygen by the acid catalyst and subsequent nucleophilic attack on the anomeric carbon allow for cleavage of a maltose unit (or maltodextrin) from the main starch chain, hydrolysis of the glycosyl enzyme by a water molecule follows, generating a dextrin with the same anomeric configuration as the substrate, and regenerating the acidic residue on the enzyme.[218]
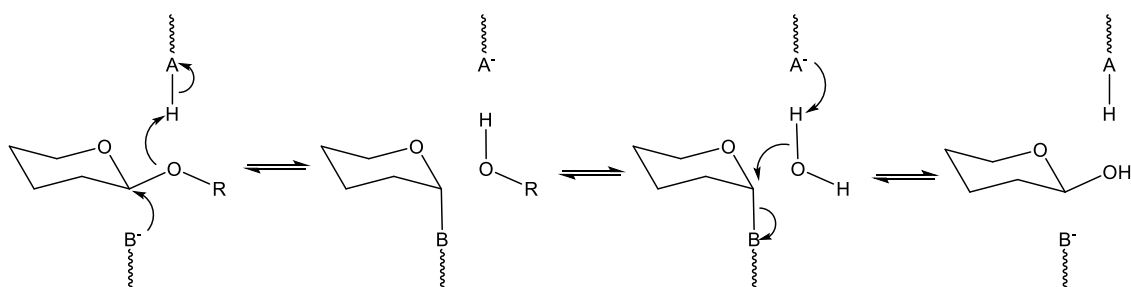
Figure 4.2. Generalised mechanism for the acid hydrolysis of a hexose chain by a glycosyl hydrolase, where A and B denote the catalytic residues at the active site of the enzyme and the anomeric configuration is retained (beta-configuration). A similar mechanism will be carried out by alpha-amylase on starch (alpha-configuration). Adapted from Davies and Henrissat (1995).[218]

As well as the active site, several studies have found two surface carbohydrate binding sites,[98, 219-220] though initially there was only one found, which was hypothesized as being involved in the alpha-amylase mechanism action.[214] Alkazaz *et al.* (1996, 1998)[98, 220] confirmed the presence of the two binding site in their investigation to determine the mechanism of acarbose inhibition on PPA using the substrates maltopentaose, reduced DP18-maltodextrin (DP – degree of polymerisation, 18 glucose units) and amylose. They concluded that these binding sites were required for product processing but have not fully elucidated their function. These binding sites were not found to be required for small saccharide molecules (DP<5), while short chain maltodextrins (5>DP<18) required one site, and long chain maltodextrins (DP>18) required both sites for substrate processing and product release. Non-competitive inhibitors to these sites prevented hydrolysis of the DP>5 maltodextrins (Figure 4.3a/b).[98, 220] Koukiekolo *et al.* (1999)[219] described a similar result, proposing a different mechanism of inhibition based on the predominant formation of enzyme-inhibitor$_2$(EI$_2$) and enzyme-substrate-inhibitor$_2$(ESI$_2$) complexes over EI or ESI formation (Figure 4.3c) and concluded that inhibition is a mixed non-competitive type.
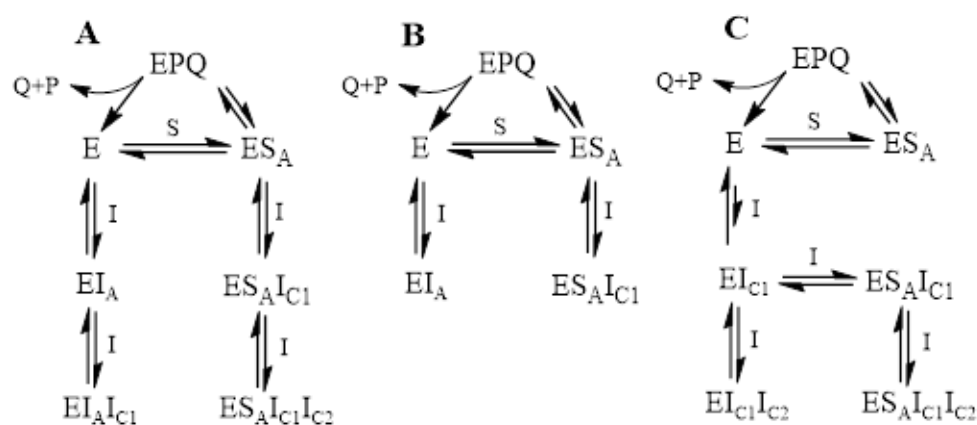
Figure 4.3. Proposed mechanism for substrate processing and inhibition - A) long chain and B) short chain maltodextrins inhibited by acarbose (adapted from Alkaziz *et al.* (1996)[220]), and C) amylose and maltopentose inhibition by *Phaseolus vulgaris* inhibitor (adapted from Koukiekolo *et al*. (1999)[219]). Labels are enzyme (E), substrate (S), inhibitor (I) and products (P, Q) followed by a subscript indicating binding to the active site (X_A), carbohydrate site 1 (X_{C1}) or site 2 (X_{C2}).

Many alpha-amylase are chloride dependant; while they have basal levels of catalytic activity without Cl^-,[221] Cl^- is required to substantially enhance levels.[213] Chloride induces a subtle conformation change in the enzyme, encouraging the calcium cation to bind to the calcium binding site (Domain A: Asn100, His201; Domain B: Asp159, Asp167), both sites lie close to the active site cleft on domain A.[214, 221-222] Levitzki *et al.* (1974)[221] found that binding affinity of Ca^{2+} to PPA increased by 240-fold on addition of chloride anions binding to the anion site (Arg195, Asn298, Arg337).[214, 217, 221] Qian *et al*. (2005)[217] found that chloride also played an important role in optimising the orientation of Glu233. While other small monovalent anions can substitute for Cl^- (some of which have greater binding affinity, e.g., NO_3^- or ClO_3^-), binding affinity is independent of activation capacity and their activation levels are lower.[213, 221-223]

Calcium ions are a crucial co-factor for maintaining the structural integrity of the catalytic site and therefore catalytic activity of alpha-amylase.[209] Binding of Ca^{2+} to Asn100, His201, Asp159, and Asp167 induces an ionic bridge between domain A and domain B to stabilise the cleft region, keeping it open and accessible to substrates.[214, 222] In all organisms, the calcium binding site is conserved on the alpha-amylase enzyme. While Ca^{2+} is crucial for enzyme structural stability, excess Ca^{2+} can be detrimental, directly inhibiting enzyme function by binding to Asp197 and Glu233 in the active site.[224] Chloride binding helps negate the effect of excess calcium, protecting against inhibition.[223]

4.3   Alpha-amylase sequence alignment

Although the main aim of this chapter was to compare the amino acid sequence between *A. mellifera* and *S. scrofa* pancreatic alpha-amylase and determine catalytic similarities, seven other alpha-amylase sequences were included from across the animal kingdom to achieve a better understanding of conserved sites (Table 4.1).

Table 4.1. Scientific / common names for alpha-amylase sequences. Sequence length (amino acids) refers to the functional protein sequence, which excludes the signal peptide.

Scientific name	Common name	Accession number	Amino acids	Reference
Apis mellifera	European honey bee	BAA86909.1	476	[225]
Drosophila melanogaster	House fly	AAA92231.1	476	[226]
Penaeus japonicus	Karuma shrimp	AHN91844.1	495	[227]
Scyliorhinus canicula	Small spotted catshark	XP_038649517.1	497	[228]
Xenopus laevis	African clawed frog	NP_001079910.1	496	[229]
Gekko japonicus	Schlegel's Japanese gecko	XP_015271586.1	497	[230]
Chaetura pelagica	Chimney Swift	XP_010004971.1	497	[231]
Sus scrofa	Pig	XP_020945783.1	496	[232]
Homo sapiens	Human	BAA14130.1	496	[233]

The selected amylases had similar length amino acid sequences (excluding the signal peptides), although both insect species were shorter compared to the other sequences investigated by approximately 20 amino acids (Table 4.1). Figure 4.4 shows the sequence alignment of these amylases, highlighting the active site, and chloride and calcium binding sites based off the PPA amino acid positions. With the exception of Asp159 (calcium binding site), there is conservation of important amino acid residues across all species. Asp159 is replaced with Asn in four of the investigated species, including *A. mellifera*, however this is a conservative replacement as the chemical properties of Asp and Asn are quite similar and are likely not to make any significant changes to the functionality of the protein.[234] The high conservation of the active and auxiliary site amino acids shows that the basic functionality between the various alpha-amylase have remained the same, due to selective pressure over millions of years of evolution.[235]

```
Apis_mellifera           -MMPAIVLLLALLTLAAGEIAHNDPHFAPGHDAIVHLFEWKWNDIAKECEQFLGPVGFGGVQVSPVQENIVI----DKRPWWERYQPISYKWITRSGTREQFIDMVARCNKAGVRIYVDV
Drosophila_melanogaster  MFLAKSIVCLALLA---VANAQFDTNYASGRSGMVHLFEWKWDDIAAECENFLGPNGYAGVQVSPVNENAVK----DSRPWWERYQPISYKLETRSGNEEQFASMVKRCNAVGVRTYVDV
Penaeus_japonicus        -MLRV---VPLVVLLAAASLAQWDPNSSNG-QAIVHLFEWKWPDIAAECENFLGPRGFAGVQVSPPNEYVEVYQGEVKRPWWERYQPVSYKLVTRSGDENAFKDMVTRCNNVGVKIYVDI
Scyliorhinus_canicula    --MKV---L-LLLAFLGGCLAQYDPHMVPGRTSIVHLFEWKWKDIAAECERYLAPNGFGGVQISPPNEHIVL--NDPWRPWWERYQPISFKLCSRSGNLEAFRDMVTRCNNVGVRIYADV
Xenopus_laevis           --MKL---L-LLLVTIGLCSAQYNPNTQSGRTSIVHLFEWKWVDIAAECERYLGPNGFGGVQISPPNENIVV--TNPYRPWWERYQPISYKLCTRSGNEQQFRDMVTRCNNVGVYIYVDA
Gekko_japonicus          --MRI---F-LLLVAVELCWAQYNPNTKPGKTSIVHLFEWRWADIAVECERYLAPNGYAGVQISPPNENLII--TNPMRPWWERYQPISYKLCTRSGNESEFQDMVARCNAVGVHIYVDA
Chaetura_pelagica        --MQV---L-LLLAAVGLCWAQYNPNTLPGRTSIVHLFEWRWADIALECERYLAPNGFGGVQISPPNENIVI--TNPNRPWWERYQPISYKLCTRSGNENEFRDMVTRCNNVGVRIYVDA
Sus_scrofa               --MKL---F-LLLSAFGFCWAQYAPQTQSGRTSIVHLFEWRWVDIALECERYLGPNGFGGVQISPPNENSII--TNPSRPWWERYQPISYKLCTRSGNETEFRDMVTRCNNVGVRIYVDA
Homo_sapiens             --MKF---F-LLLFTIGFCWAQYSPNTQQGRTSIVHLFEWRWVDIALECERYLAPKGFGGVQVSPPNENVAI--HNPFRPWWERYQPVSYKLCTRSGNEDEFRNMVTRCNNVGVRIYVDA
                            :       ::        *:   :    *   .:*******:*  ***  ***.:*.*  *:.***:** .:*             *********:*:*  :***  .  *  .** *** .** *.*

Apis_mellifera           IMNHMSGDRN--DAHGTGNSRANTYNFDYPQVPYTVKNFHPR------CAVNNYNDPSNVRNCELVGLHDLDQSQEYVRSKLVDFLNDLVAIGVAGFRVDAAKHMWPSDLRTIYSRVRNL
Drosophila_melanogaster  VFNHMAADGG---TYGTGGSTASPSSKSYPGVPYSSLDFNPT------CAISNYNDANEVRNCELVGLRDLNQGNSYVQDKVVEFLDHLIDLGVAGFRVDAAKHMWPADLAVIYGRLKNL
Penaeus_japonicus        VMNHMSGGW-PQGTGGSGGSSFDSGAQSYPGVPYSAFDFNDGNCHTGSGNIENYGDANQVRNCKLVGLNDLNQGTDYVRGKIRDYLNTLIGYGVAGFRIDASKHMWPGDMKAIFDSLNNL
Scyliorhinus_canicula    VINHMCGASGGSGTHSSCGSCFDAGSESFPAVPYSSGDFNDGKCKTASGNIENYNDINQVRDCRLVSLLDLALQKDYVRGKIAEYLNHLIDLGVAGFRVDAAKHMWPSDMEAIFSQLKKL
Xenopus_laevis           IINHMCGSGGGGAGTHSTCGSYFNAGSRDFP-VPYSGLDFNDGKCRTGSGEIENYGDANQVRNCRLVGLLDLAMEKDYVRGKIAEYMNNLINIGVAGFRLDAAKHMWPGDLKAISDRLNNL
Gekko_japonicus          VVNHMCGAAAGSGSYSTCGSYFNAMTRDFPAVPYSAWDFNDGKCRTGSGEIENYNDPDQVRDCRLVSLLDLALEKDYVRSKVAEYLNHLIDIGVAGFRIDASKHMWPGDMKAFLDKLNNL
Chaetura_pelagica        VVNHMCGAAGSGTHSTCGSYFNAGNRAFPAVPYSAWDFNDDKCRSGSGEIENYGDMYQVRDCRLVGLLDLALEKDYVRSKVAEYMNYLIDTGVAGFRIDAAKHMWPGDMRAFLDKLKDL
Sus_scrofa               VINHMCGSGAAAGTGTTCGSYCNPGNREFPAVPYSAWDFNDGKCKTASGGIESYNDPYQVRDCQLVGLLDLALEKDYVRSMIADYLNKLIDIGVAGFRIDASKHMWPGDIKAVLDKLHNL
Homo_sapiens             VINHMSGNAVSAGTSSTCGSYFNPGSRDFPAVPYSGWDFNDGKCKTGSGDIENYNDATQVRDCRLVGLLDLALEKDYVRSKIAEYMNHLIDIGVAGFRLDASKHMWPGDIKAILDKLHNL
                          :.***.           :  : .*.    :* ***  :*:          :..*.*  :*.:.**.*.* . .**:. : :::: *: ******:*:*.*****.*: .. .. .:.*

Apis_mellifera           NRTHGFPNDAQPYIFQEVIDYGNEAISKREYNGIGAVIEFKYSYEISNAFRG--NNNLKWLVNWGGFLPSKDSLVFVDNHDTQRDN----PQILTYKYSRRYKMAVAFMLSHPFGTP
Drosophila_melanogaster  NTDHGFASGSKAYIVQEVIDMGGEAISKSEYTGLGAITEFRHSDSIGKVFRG--KDQLQYLTNWGTAWGFAASDRSLVFVDNHDNQRGHGAGGADVLTYKVPKQYKMASAFMLAHPFGTP
Penaeus_japonicus        NTD-FFKAGSRPFIFQEVIDLGGEAISGQEYVGNGRVTEFRYGKYLGEAFRG--NNQLQYLKNFGEDWGMMDRANALVFIDNHDNQRGHGAGGDMILTFRVSKWYKMANAFMLGWPYGYT
Scyliorhinus_canicula    NAR-WFSKDSRPFIYQEVIDLGGEAIQSSQYFGLGRVTEFKYSAKLGTVVRRWNGEKMAYLRNWGEGWAFMPTDRALVFVDNHDNQRGHGPGGASVLTFWDSRLYKVAVAFMLAHPYGQT
Xenopus_laevis           NTK-WFPAGARPFIFQEVIDLGGEAISVNEYFGVGRVTEFKYGAKLGGVIRKWNGEKMAYLRNWGEGWGFMPNDRALVFVDNHDNQRGHGAGGASILTFWDARLYKMGVGFMLAHPYGFT
Gekko_japonicus          NTQ-WFAEGTRPFIYQEVIDLGGEGIKTSDYFGNGRVTEFKYGAKLGKVIRKWDGERMAYLKNWGEGWGFMPSDRALVFVDNHDNQRGHGAGGASILTFWDARLYKMAVGFMLAHPYGFT
Chaetura_pelagica        NTT-WFPAGTKPFIYQEVIDLGGEPIKGSDYFGNGRVTEFKYGAKLGTVIRKWNGERMAYLRNWGEGWGFMPSDRALVFVDNHDNQRGHGAGGSSILTFWDARLYKMAVGFMLAHPYGFT
Sus_scrofa               NTN-WFPAGSRPFIFQEVIDLGGEAIQSSEYFGNGRVTEFKYGAKLGTVVRKWSGERMSYLKNWGEGWGFMPSDRALVFVDNHDNQRGHGAGGASILTFWDARLYKVAVGFMLAHPYGFT
Homo_sapiens             NSN-WFPAGSKPFIYQEVIDLGGEPIKSSDYFGNGRVTEFKYGAKLGTVIRKWNGEKMSYLKNWGEGWGFMPSDRALVFVDNHDNQRGHGAGGASILTFWDARLYKMAVGFMLAHPYGFT
                          *   *.     :: * ****.*:*. .*  * .* * **:. :. ..*   ::: :*:* *:  *.   :***;****.**:.          :**:   . :**. . **:. .***.*:.*

Apis_mellifera           RIMSSFDFQS---------KDQGPPNDGNGNILSPSI-HDNICSNGWICEHRWRQIYNMVRFRNLVKGTKIDNWWDNGSNQIAFSRGCSGFVAFNGDQYDLKKNLKVCLPPGQYCDVISG
Drosophila_melanogaster  RVMSSFSFTD---------TDQGPPTTDGHNIASPIFNSDNSCSGGWVCEHRWRQIYNMVAFRNTVGSDEIQNWWDNGSNQISFSRGSRGFVAFNNDNYDLNSSLQTGLPAGTYCDVISG
Penaeus_japonicus        RVMSSYYWDQWWENGQDKNDWIGPPQDDSFNIISPSFNADGTCGNGWICEHRWRQIYNMVEFRNVAHGTDMNDWWDNGSNQIAFCRGDKGFLAINNDGWDLKETLQTCLPAGTYCDVISG
Scyliorhinus_canicula    RVMSSFRWDRYFVNGKDVNNWMGPPSNSDGSTKAVTLHADTTCGNGWVCEHRWRQIRNMVIFRNVVDGQPFANWWDNESNQIAFGRGNRGFIAINCDNWNMGVTLQTGLPAGTYCDVISG
Xenopus_laevis           RVMSSYRWTRNISNGKDQNDWIGPPTNSDGSIKSVPINADATCGDNWVCEHRWRQIKNMVIFRNVVNGQPFSNWWDNGSNQVAFGRGNKGFIVFNNDDRYLDATLNTGLPSGTYCDVISG
Gekko_japonicus          RVMSSFRWPRYFENGKDVNDWVGPPSNADGSIKTVTINPDTTCGNDWVCEHRWRQIRNMVIFRNVVDGQPFTNWWDNGQNQVSFGRGNKGFIVFNNDDWNLSQTLQTGLPAGTYCDVISG
Chaetura_pelagica        RVMSSFRWPRYFENGRDVNDWVGPPSDSDGSIKPVTINPDTTCGNDWVCEHRWRQIRNMVIFRNVVDGQPFSNWWDNNSNQVAFGRGNKGFIIFNNDDWDMNVYLQTGLPAGTYCDVISG
Sus_scrofa               RVMSSYRWARNFVNGQDVNDWIGPPNN-NGVIKEVTINADTTCGNDWVCEHRWRQIRNMVWFRNVVDGQPFANWWANGSNQVAFGRGNRGFIVFNNDDWQLSSTLQTGLPGGTYCDVISG
Homo_sapiens             RVMSSYRWPRQFQNGNDVNDWVGPPNN-NGVIKEVTINPDTTCGNDWVCEHRWRQIRNMVNFRNVVDGQPFTNWYDNGSNQVAFGRGNRGFIVFNNDDWTFSLTLQTGLPAGTYCDVISG
                          *:***: :        ***    .*  :  : .*.:*:******* *** ***  . . .: :*: *.**::* ** **:.* ** :*  :      *:. ** * ******

Apis_mellifera           NLEKGRCTGKIVTVGSDGNANIEIGAGEEDGVLAIHVKAKMA
Drosophila_melanogaster  SKSGSSCTGKTVTVGSDGRASINIGSSEDDGVLAIHVNAKL-
Penaeus_japonicus        SKEGGSCTGKSVTVGGDGKAYIEITTMEDDGVLAIHANSKL-
Scyliorhinus_canicula    QKEGDRCTGLQVAVGPDGRVSLQISNTAEDPMLAIHVAKL-
Xenopus_laevis           QKEGSRCTGRQINVDGNGFARFQISNTDEDPFAAIHVNAKL-
Gekko_japonicus          QKEGDRCTGITIQVNNDSTAHFQISNHAEDPFVAIHIDAKL-
Chaetura_pelagica        QKEGDKCTGKQVYVSRDGMANFQISSSAEDPFVAIHVNAKL-
Sus_scrofa               DKVGNSCTGIKVYVSSDGTAQFSISNSAEDPFIAIHAESKL-
Homo_sapiens             DKINGNCTGIKIYVSDDGKAHFSISNSAEDPFIAIHAESKL-
                          .  . *** :*. :. . .:.*   :* . *** .:*:
```

Figure 4.4. Multiple sequence alignment of alpha-amylase found in Table 4.1. Identical (*), very similar (:) and slightly similar (.) amino acids are identified with the active site (orange), chloride binding site (green) and calcium binding site (blue) highlighted.

4.3.1 Implications of sequence similarities and differences

Sequence similarity (exact conservation) between *A. mellifera* and *S. scrofa* is 51%, with a further 16% amino acid conservative replacements; the remaining 33% of aligned amino acids showing poor or no conservation. Although there are similarities between these two organisms it does not mean that both amylases will have the same properties. Non-active site amino acids are also extremely important to the functionality of the enzyme, contributing to the 2-D and 3-D structures and physico-chemical properties.[236] As the active sites are highly conserved, these structural changes caused by varied amino acids are likely to be minor, however even minor changes can affect functionality and enzyme stability.[237] Changes in non-active site amino acids may result in variance between optimal reaction conditions and also degree of susceptibility to inhibition by various compounds or to denaturing in sub-optimal conditions.[237] Therefore, the mechanism of action for alpha-amylase in honey is likely to be the same as described in section 4.1 for PPA, but it is unlikely that inhibitors or environment conditions will affect both enzymes the same.

4.4 Factors affecting alpha-amylase activity

Enzymatic activity is affected by a variety of factors including temperature, pH, co-factors (Section 4.1, Cl^-, Ca^{2+}) and inhibitors. Enzyme structure and activity depends on the interactions of peptide amino acids to form the secondary and tertiary structures (also quaternary) and thus the active sites. These structures are stabilised by weak non-covalent interactions including: van der Waals interactions, hydrophobic interactions, electrostatic interactions and hydrogen bonding.[238]

Increasing temperature will increase the enzymatic activity (reaction rate) to a maximum (at the optimal temperature) as reacting molecules gain more kinetic energy. However, past this optimal temperature, the activity will decrease as enzymes become inactivated, then denatured through a breakdown of amino acid interactions from the excessive kinetic energy. Enzymes also have an optimal pH where they are most active. They are generally only active over a limited pH range as pH affects the ionisation of amino acids and thus their interactions with other amino acids (within the protein) or compounds (at the active site or auxiliary sites). Slight pH changes away from the optimum may cause inactivation of the enzyme, while larger changes may cause

irreversible denaturing due to affecting the enzymes stability. Several studies have isolated alpha-amylase from honey and determined that it has optimal activity between pH 4.6-5.9 and 39-55 °C.[239-240] Hence the Phadebas diastase honey test is carried out in pH 5.2 buffer at 40 °C.[181]

Inhibition of enzymes can either be reversible (non-covalent) or irreversible (covalent) with three classes of inhibitors.[241] Competitive inhibitors will compete with the substrate to bind at the active site. Non-competitive inhibitors will bind to a different site, either allowing the substrate to bind to the active site but inhibiting catalysis (pure non-competitive) or preventing the substrate from binding at all due to a conformational change in the active site (mixed non-competitive). Lastly, uncompetitive inhibitors bind to the enzyme after the substrate binds to the active site, preventing catalysis. Inhibitors can greatly decrease enzymatic activity, however the extent of inhibition varies between compounds due to their compatibility to specific sites on the enzyme.[241]

4.4.1 Reduced diastase activity in mānuka honey

Temperature is the main contributor to loss of diastase activity over time, hence why it is used as a heat treatment indicator for quality control. However, low diastase activity occurs in fresh mānuka honey and temperature is not likely to be the cause. The literature information discussed in this chapter gives basis for some theories on potential factors that could cause low diastase activity in mānuka honey (Table 4.2).

Table 4.2. Hypothesised factors for reduced diastase activity in mānuka honey.

Factors	Reason	Plausibility
pH	Non-optimal conditions reduce enzymatic activity.[239]	Honey pH is variable (3.4-6.1)[20, 22]
High sugar / low moisture nectar	Requires less manipulation by bees to optimise moisture levels (<20%).	Nectar composition can vary.[11, 24, 242]
Low Cl⁻ concentration	Lower affinity of Ca^{2+} to binding site, destabilising the enzyme.	Cl levels are variable.[20, 23]
Other monovalent anions	Other anions (e.g., NO_3^-, ClO_3^-) have higher binding affinity to anion site, prevent Cl⁻ binding causing a lower affinity to Ca^{2+}.	No data on other anions found in honey.
Low Ca^{2+} concentration	Destabilises the active site.	Ca levels are variable.[38, 243-244]
High Ca^{2+} concentration	Binds to the active site.	Ca levels are variable.[38, 243-244]
Reactive compounds	A wide range of compounds exist in honey, any one of these has potential inhibitory action.	Phenolic compounds and MGO have been proven to inhibit enzymes and exist in varying levels in honey.[23, 82, 245]

Although several potential causes have been identified (Table 4.2), the primary objective of this thesis is to examine the effect of bioactive compounds (phenolic compounds, MGO and DHA (MGO precursor)) on diastase activity in honey. Low diastase activity has not been reported in other floral honey types, hence if a compound is the cause of low of diastase activity due to inhibition, it should, theoretically, be unique or much higher in mānuka honey than in other floral types. Manuka honey sells for a high price and is subject to fraudulent activity, so is more often tested than other New Zealand floral honeys. Therefore, any issues (such as low diastase activity) in other floral types may get less recognition. The investigated compounds (DHA, MGO, 2'-MAP, 2-MBA, 3-PLA, 4-HPLA and MSY) were chosen as they are indicative of mānuka honey.

There are several possible mechanisms by which bioactive compounds could inhibit diastase based on the above literature. These have been listed below:

- Reversible or irreversible bonding to active site (competitive inhibition)
- Binding to residues not within the active site causing conformation changes that result in loss of activity (non-competitive inhibition) or sterically hindering access of maltodextrins to the active site
- Binding to auxiliary sites, preventing long chain starch (DP > 5) from being hydrolysed (non-competitive inhibition)

- Binding to Ca^{2+} or Cl^- sites preventing enzyme activation/stabilisation (non-competitive inhibition)

- Interaction with Ca^{2+} or Cl^-, preventing their binding to the enzyme

- Binding to other molecules (e.g., oligosaccharides) to form complex which may instigate covalent bond with enzyme active site (competitive inhibition)

4.4.2 Alpha-amylase inhibition by phenolic compounds

This section identifies potential inhibitor compounds for *A. mellifera* alpha-amylase through investigation of PPA inhibitors, as there are a number of studies on PPA and no publications for *A. mellifera.* As stated prior (Section 4.3.1), there are some difference between PPA and *A. mellifera* alpha-amylase thus interactions with inhibitors may not be the same, however this investigation may allow mechanisms to be better understood and provide suggestions on the types of compounds that may cause inhibition.

Interactions between phenolic compounds and proteins (not just bee enzymes) are discussed extensively in literature, with varying degrees of interaction leading to the reversible or non-reversible binding of phenolics to proteins. For reversible bonding, non-covalent interactions such as ionic pairing, hydrogen bonding, π-bonding and hydrophobic interactions occur.[246] For irreversible bonding, the interaction is covalent; oxidation of phenolic compounds leads to the formation of electrophilic quinones which conjugate to protein moieties (lysine, methionine, cysteine and tryptophan) through nucleophilic attack of the residue, resulting in a derivatised protein.[247-248]

Most protein derivatisations with phenolic compounds occur in oxidising alkaline conditions,[247-248] which is opposite to the acidic conditions in honey. Under derivatising conditions (pH 9.0, 24 °C), Rawel *et al.* (2002)[248] found flavonoids (kaempferol, quercetin and myricetin) and phenolic acids (chlorogenic acid, caffeic acid and gallic acid) bound to tryptophan residues in soy protein, linking the increased reactivity in the flavonoids to a higher number of hydroxyl substituents and also hydroxyl positioning. These derivatisations resulted in protein secondary structure changes and alterations in the functional properties of the protein. Their results also indicated that caffeic acid and quercetin were involved in inter- and intramolecular cross-linking of proteins.[248]

Due to the obesity and diabetes mellitus pandemic, there is ongoing research on diastase inhibition by phenolic compounds. Different therapeutic approaches are being investigated as to the best processes for diabetes management; currently drugs are used, enhancing insulin secretion and sensitising insulin receptor organs.[249] However, another approach is considered through the inhibition of digestive enzymes, which includes alpha-amylase as part of the gut microbiome, preventing break down of complex sugars into digestible monosaccharides. A variety of flavonoids, phenolic acids and tannins have all been found to have inhibitory action against alpha-amylase, although IC_{50} values are variable, in part due to different experimental conditions (Table 4.3).[2-3, 245, 250-254]

Kinetics experiments by Narita and Inouye (2011)[2] found mixed type inhibition indicating the potential presence of an inhibitor binding site as well as the substrate binding site. They also concluded that free chain restriction with the double bond contributed to the inhibitory action of phenolic compounds, as caffeic acid inhibitory action was five times great than dihydroxycaffeic acid.[2, 255] For flavonoids, Lo Pipari et al. (2008)[254] reported direct binding to the active site. For all phenolic compounds, the degree of hydroxylation has shown to effect inhibition activity, as more hydroxyl groups result in a greater number of hydrogen bonds to the protein residues and therefore stronger bonding.[4, 252, 254-255] As such, flavonoids with methoxy groups demonstrate lower inhibitory action against alpha-amylase due to reduced hydrogen bonding capacity.[254-255] Hydroxylation of C7 and C4' are important for inhibition as these initiate hydrogen bonding to Asp197 (C7), Glu233 (C4') and His305 (C7).[4, 254] Hydroxyl groups at C3 and C3' were found to be less important.[254] Tadera et al. (2006)[4] found that a hydroxy at C3 was unfavourable for inhibitory activity while a hydroxy group at C5 enhanced inhibition.

Lo Pipari et al. (2008)[254] investigated the structural requirements of flavonoids for inhibiting human alpha-amylase. Differentiation of flavonoids is determined by ring C heterocyclic formation, functional groups on ring A and ring B (hydroxy or methoxy) and the positioning of ring B off ring C. Flavones and flavonols have the strongest inhibitory action against alpha-amylase, due to the double bond between C2 and C3 on ring C forming a highly conjugated π-systems which stabilises the interactions of the phenolic compounds with the active site through π-π interactions with indole Trp59 (human

alpha-amylase).[4, 254] The configuration of ring B off C3 was found to enhance inhibition,
[4, 254] however, contrary to Lo Pipari *et al.* (2008)[254] who stated isoflavones had weak
inhibitory action, Tadera *et al.* (2006)[4] found that isoflavones had the greatest inhibitory
action, followed by flavone and flavonol.

Table 4.3. Phenolic compound inhibitions of alpha-amylase, where mixed is both competitive and non-competitive inhibition. HAS – human salivary amylase, PPA – porcine pancreatic amylase, NA – not available.

Phenolic compound	IC$_{50}$	Amylase type	Competition type	Reference
Quercetin*	55.2%	PPA	NA	251
	21.4 µM	HAS	NA	254
	0.5 mM	PPA	NA	4
Anthocyanins	0.68 mg/mL	NA	Competitive	252
Proanthocyanins	3.61 µg/mL	PPA	Mixed	256
Tannic acid	0.86 mg/mL	PPA	NA	253
	0.301 mg/mL	PPA	Competitive	3
	0.14 mM	PPA	NA	255
Chlorogenic acid**	8.63 mg/mL	PPA	NA	253
	0.08 mM	PPA	Mixed	2
	1.96 mg/mL	PPA	Mixed	3
	1.71 mg/mL	PPA	NA	257
	1.4 mM	PPA	NA	255
	0.498 mg/mL	PPA	Mixed	6
Myricetin*	30.2 µM	HAS	NA	254
	0.38 mM	PPA	NA	4
Luteolin**	18.4 µM	HAS	NA	254
	0.36 mM	PPA	NA	4
	0.17 mM	PPA	NA	255
Dihydrocaffeic acid	1.94 mM	PPA	Mixed	2
	>14 mM	PPA	NA	255
Caffeic acid**	0.40 mM	PPA	Mixed	2
	3.69 mg/mL	PPA	Mixed	3
	1.81 mg/mL	PPA	NA	257
	4.8 mM	PPA	NA	255
Ferulic acid**	5.45 mM	PPA	Mixed	2
	>5.0 mM	PPA	NA	255
	0.622 mg/mL	PPA	Mixed	5
Isoferulic acid*	4.27 mM	PPA	Mixed	2
p-Coumaric acid **	4.86 mM	PPA	Mixed	2
Catechin*	>0.5 mM	PPA	NA	4
Epicatechin	>0.5 mM	PPA	NA	4
	9.321 mg/mL	PPA	NA	3
Kaempferol*	>0.5 mM	PPA	NA	4
Apigenin**	>0.5 mM	PPA	NA	4
Naringenin**	>0.5 mM	PPA	NA	4
Hesperitin**	>0.5 mM	PPA	NA	4
Sinapic acid*	>6.7 mM	PPA	NA	255

*Phenolic compounds found in honey[43, 131]
** Phenolic compounds found in mānuka honey[130-133, 136]

The interactions of phenolic compounds have been shown to interfere with the active
site, effectively blocking the substrate (starch) from binding, however the interactions

also cause other effects. Miao *et al.* (2021)[252] found that anthocyanins result in a structure change of the secondary configuration of the amylase (specifically the α-helix and β-sheet), this effect was also observed by Liu *et al.* (2017)[256] for proanthocyanins. Due to this structural change, the enzyme properties (solubility etc.) were also altered. Zheng *et al.* (2020)[5-6] found a similar occurrence with ferulic and chlorogenic acid, where the secondary structure of PPA was altered through hydrogen bonding.

Many of the compounds in Table 4.3 are found in honey (annotated by *). While some of these also occur in mānuka honey (annotated **), there are several reasons why they were not included in this study. The concentration in mānuka is over 100 times weaker than the lowest IC_{50} concentration for each (some were 1000 times weaker). Also, as this was directly comparing mānuka honey phenolic compound concentration (mg/g) to the IC_{50} (mg/mL), it does not take into account the viscosity of honey which would further prevent inhibition (density also has not been considered). These compounds are also found in other floral honey types: comparison of mānuka concentrations to other floral honey types did not find any that were unique to mānuka nor any that were significantly high.

4.5   Summary of alpha-amylase inhibition

There are similarities between the PPA and *A. mellifera* amylases, enough to show the general mechanism is the same, although the physico-chemical properties and susceptibility to inhibition may not be. There are several factors that could cause low diastase activity in mānuka honey, including inhibiting compounds such as phenolic compounds which can inhibit PPA. Although some of these inhibiting compounds are found in mānuka honey, they were not chosen for this research as the endogenous concentrations are likely too low to observe inhibitory action and were not unique or significantly high compared to other floral types.

5 Database Results and Discussion

The database honeys were those supplied by beekeepers from around New Zealand, with three different experiments carried out on them (Table 5.1), which are described by the abbreviations DB (database), DBSH (database snapshot) and DBT (database trials). The initial testing of the DB honeys was carried out on receipt of the honeys to give an overview of the honey profile and to determine differences between mānuka and other New Zealand floral type honeys. Storage of a sub-set of these honeys (n = 28) at 20 °C for 161 days was carried out to determine the changes in parameters after extended storage (i.e., snapshot view) of the DB honeys (DBSH). Ten of the DBSH honeys were also stored at 27 °C in duplicate and tested at regular 1.5 month intervals over the period of approx. 6 months (198 days); this data was to provide a more in-depth view on how the examined parameters changed over time at a slightly elevated temperature (compared to room temperature). Due to the shortage of Phadebas tablets from the supplier, testing of diastase activity for all samples was not possible. As such, there are discrepancies between the number of samples in the experiment and the number of samples analysed for DN. For example, there were 65 DB honey samples analysed for 3in1, SULLE and moisture content, but only 51 samples were analysed for diastase.

Table 5.1. Database honey experiments and testing.

Experiment	Abbreviation	Samples	Storage conditions	Tests
Database	DB	65 Honeys	N/A – analysed on receipt	Moisture, DN, 3in1, SULLE
Database snapshot	DBSH	28 DB honeys	20 °C, tested at 161 days	Moisture, DN, 3in1, SULLE
Database trials	DBT	10 DB honeys in duplicate	27 °C, tested at 69, 117, 166 and 198 days	DN, 3in1, SULLE

Honey was obtained from donation of beekeepers, who specified the age and floral variety. However, on analysis of the DB HMF data (discussed in more detail in Section 5.1.2), it was found that the HMF levels were higher than expected for some samples, potentially indicating that they were not fresh. While the data was analysed holistically, the purpose of this research was to investigate low diastase activity in fresh mānuka honey, thus a subset of the DB, comprising only fresh honey was used for data analysis, where fresh honey was defined as < 10 mg HMF per kg honey. It should be noted that with a HMF concentration of 10 mg/kg the honey had not recently been harvested,[258] but for the purpose of this work, it was used as the cut off threshold. Additionally, the

mānuka honey classification supplied by the beekeeper was also checked by comparing three of the four mānuka honey chemical marker compounds in the MPI criteria[*] for mono- and multi-floral mānuka honey. In this chapter, where data is labelled as mānuka, it is the subset taken from the database honeys that fall in the category of either mono- or multi-floral mānuka honey as defined by their 2-MBA, 3-PLA and 4-HPLA concentrations (greater than 1, 20 and 1 mg/kg respectively). As only five out of 37 mānuka categorised by the beekeepers were determined to be multi-floral, they were not separated into multi and mono-floral categories. It is also possible that if diastase activity is inhibited by a compound found in mānuka honey, the lower concentrations of these compounds in multi-floral mānuka honey may be sufficient for inhibition. During data analysis, these multi-floral mānuka samples were monitored to ensure they were not outliers in the results. It is important to note that 2'-MAP was not able to be quantified using SULLE, and thus the designation is not accurate according the MPI export standard, but for the purpose of this study it is a suitable classification tool. 2'-MAP it the only chemical marker that MPI assessment determined to only be found in mānuka plants.[87]

Where non-mānuka honeys (as classified by the beekeepers) fulfilled the criteria for either mono- or multi-floral mānuka, DHA and MGO concentrations were reviewed. If the honey had the potential to reach an NPA rating of 5 or greater (134 mg/kg MGO) alongside meeting the MPI criteria, it was reclassified. This was determined based off the DHA to MGO stoichiometry of 2DHA:1MGO and the MGO formation plateau when the concentration ratio between DHA and MGO is 2:1.[28] The reclassified samples are listed in Table 5.2. It is likely that the honeys reclassified to mānuka also contained some pohutukawa (NZ337) and rewarewa (NZ333) (as specified by the beekeeper) as both have similar flowering periods as mānuka;[259] pollen or DNA analysis would be required to confirm this and determine which was the primary floral type.

Table 5.2. Reclassifications of DB honeys and their MPI mānuka marker concentrations.

Honey sample	Original classification	New classification	2-MBA (mg/kg)	3-PLA (mg/kg)	4-HPLA (mg/kg)
NZ358	Mānuka	Non-mānuka	0	1.2	82.0
NZ359	Mānuka	Non-mānuka	0	1.5	78.3
NZ337	Pohutukawa	Mānuka (mono)	3.3	4.2	849.2
NZ333	Mānuka / rewarewa	Mānuka (mono)	3.1	3.8	471.9

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[*] Note that the MPI criteria also use DNA for classification

5.1   Analysis of the honey database (DB) samples

For the following data analysis, honey was grouped by floral type if there were three or more of the specific type. However, it is important to note that *n* = 3 is not a large enough sample size to be representative of a floral type; instead, it gives an indication of where that floral type might lie with respect to other types, provided that neither sample is an outlier of its group (which is not possible to determine without a larger number of samples). Floral types of *n* ≤ 2 were placed in the "non-mānuka" grouping. Averaged values for each grouping are shown in Table 5.3; Appendix B contains all information and test results for each sample.

5.1.1   Moisture content

Moisture content for the database honeys was quite varied, ranging from 15.1 to 23.1% (Figure 5.1). Mānuka honey moisture content ranged from 17.3 to 23.1%, which all other honey types fell within, except for the two honeydew honeys (NZ360-361) which had 15.1-15.8% moisture. This lower moisture content for honeydew honey aligns with literature (15.7 ± 0.18%)[39]. While other honeys are expected to fall within the range of 16.5-18.5%,[39, 260-261] only three honeys (NZ333 – mānuka 17.3%, NZ343 – rewarewa 17.6%, NZ345 – multifloral 18.5) fall within this bracket. The remainder have 18.7-23.1% moisture content, with mānuka honey containing 19.9 ± 1.0 % moisture. The mean of mānuka honey (*n* = 44) moisture falls just below the acceptable threshold for moisture of exported honey (20%), with over half of these honeys (*n* = 24) failing to meet the requirements for exports.[12] The higher moisture content of the honey could be due to premature harvesting of the honey, before the honey bees have dried and capped the honey.[19] Alternatively, it may be related to storage conditions or treatment before they were received for this study; honey is hygroscopic and thus will absorb water when stored incorrectly. Once the honey was received for this research, it was stored in the freezer until analysis, thus would not be likely to gain moisture content during this time.

Table 5.3. Average values for each of the examined parameters for DB experiment.

Floral type	Moisture (%)	DN	DHA (mg/kg)	HMF (mg/kg)	MGO (mg/kg)	2-MBA (mg/kg)	3-PLA (mg/kg)	4-HPLA (mg/kg)	MSY (mg/kg)
Mānuka (n = 44)	19.9 ± 1.0	10.9 ± 7.8	1306.9 ± 996.8	6.7 ± 5.6	318.8 ± 225.4	6.5 ± 3.3	796.8 ± 405.1	6.3 ± 3.0	79.9 ± 47.0
Kānuka (n = 3)	20.1 ± 0.6	10.5 ± 2.5	80.8 ± 85.1	11.9 ± 11.3	20.1 ± 15.9	0.0 ± 0.0	1191.3 ± 396.2	7.4 ± 1.4	299.5 ± 48.5
Non-manuka (n = 21)	19.3 ± 1.5	12.9 ± 5.5	73.6 ± 68.2	16.9 ± 26.6	20.6 ± 23.1	0.5 ± 1.2	217.4 ± 429.5	2.0 ± 2.4	54.6 ± 104.6
All (n = 65)	19.7 ± 1.2	11.6 ± 7.2	908.5 ± 1003.4	10.0 ± 16.3	222.5 ± 232.5	4.6 ± 4.0	609.6 ± 492.4	4.9 ± 3.5	71.7 ± 71.0
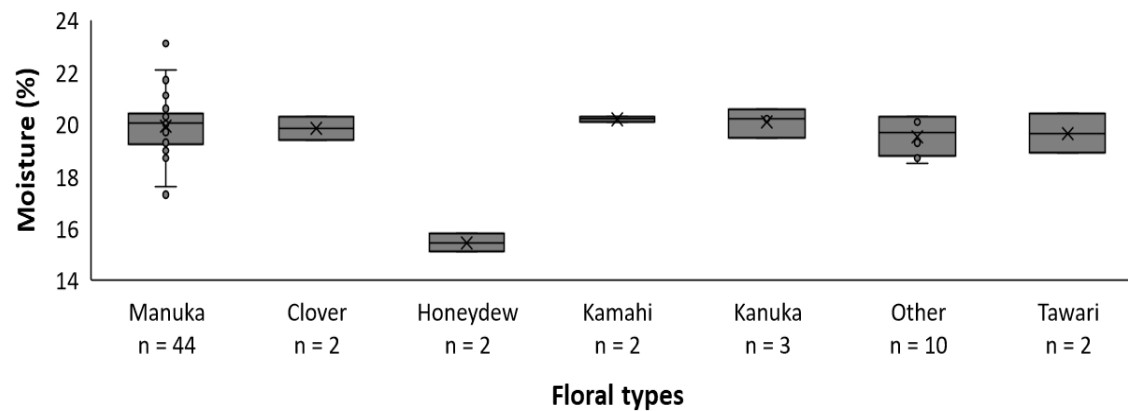
Figure 5.1. Box and whisker distribution plots for moisture. Other = citrus, thyme, towai, rewarewa, southern rata.

5.1.2 HMF and diastase activity

HMF levels varied considerably between DB samples (Figure 5.2). Of 65 samples, 61 had HMF concentrations less than 30 mg/kg (Figure 5.2). A rewarewa (NZ365), tawari (NZ364), citrus (NZ363) and clover (NZ324) had concentrations greater than 30 mg/kg, with 32.9, 34.8, 47.1 and 117 mg/kg respectively, with NZ363 and NZ324 both failing to meet the export criteria of 40 mg/kg.[12] A common factor for all these honeys with high HMF is that they are commercial purchases as opposed to direct supply from the beekeepers. As discussed previously, HMF concentration increases over time due to the Maillard reaction occurring in honey.[40] This occurs faster at higher temperatures and is also used to indicate poor honey treatment or storage (alongside diastase activity). Based on the "fresh" definition for this research (HMF < 10 mg/kg), 49 out of 65 meet the criteria, with 16 considered aged. Eight honeys had HMF concentrations greater than 20 mg/kg. Of all the honeys analysed, NZ324 (clover) has an extremely high HMF concentration (116.6 mg/kg). As this is the honey that was used for the clover spiked trials, it will be discussed further in Chapter 6.

The DB honey HMF values mostly align with the literature for fresh honey (<LOD-14.6 mg/kg).[28, 262-263] The higher HMF concentrations match honey which has been stored for a period of time (0.5-4 years), potentially at higher temperatures (20-30 °C).[263-264]
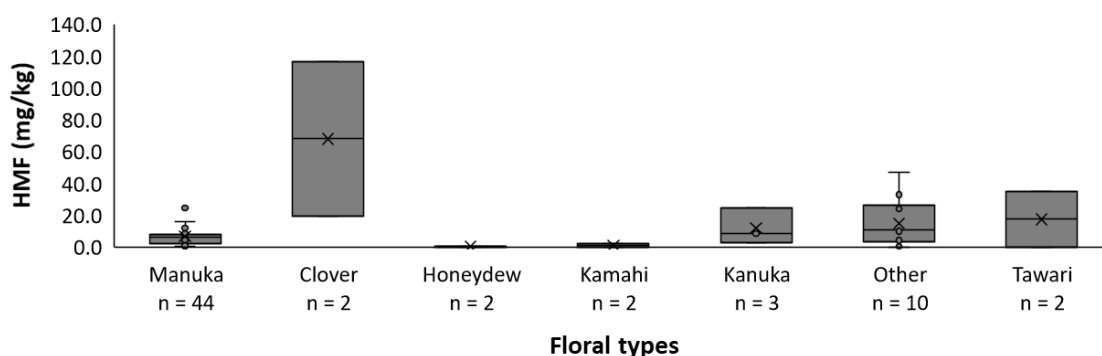
**Figure 5.2. Box and whisker distribution plot of HMF (mg/kg). Other = citrus, thyme, towai, rewarewa and southern rata.**

The diastase activity of the DB honeys covered a wide range from 0.9-28.9 DN (Figure 5.3), with both extremes of 0.9 and 28.9 DN occurring for mānuka honey samples (NZ340 and NZ316 respectively). Of the 51 honeys tested for diastase, 14 samples did not meet

the exportation criteria of >8 DN. Eleven of the failed samples were mānuka, while the other three were rewarewa (NZ343), tawari (NZ323) and pohutukawa (NZ337). Two of these samples had HMF levels greater than 10 mg/kg, eight samples greater than 5 mg/kg and two samples between 4-5 mg/kg, potentially indicating longer storage time or higher temperatures during processing to cause decreased diastase activity and elevated HMF. Interestingly, the rewarewa and tawari samples had 0.0 mg/kg HMF, indicating that the low diastase activity for these samples may not be due to extended storage time or elevated temperature. Some samples do not form HMF as quickly as others,[201, 265] and may also have had low diastase initially. At the opposite end of the spectrum, several samples, including five mānuka honeys, had high HMF content (>10 mg/kg) but also high diastase activity (>8 DN), again potentially due to variances in the rate of HMF formation.[201, 265]

While it has been stated in literature that New Zealand honeys of high MGO concentration are known to have low diastase activity,[106] there is no literature showing this specific data for New Zealand honeys. Several studies carried out overseas with commercially brought mānuka of unspecified MGO concentration found two different result, high diastase activity (DN = 19.0 ± 2.1)[266] and low diastase activity (DN = 8.0 ± 1.0).[267] The fresh honey subset (< 10 mg/kg HMF) was not statistically different from the aged set for either the full sample set (p = 0.32) or the mānuka honey subset (p = 0.27).
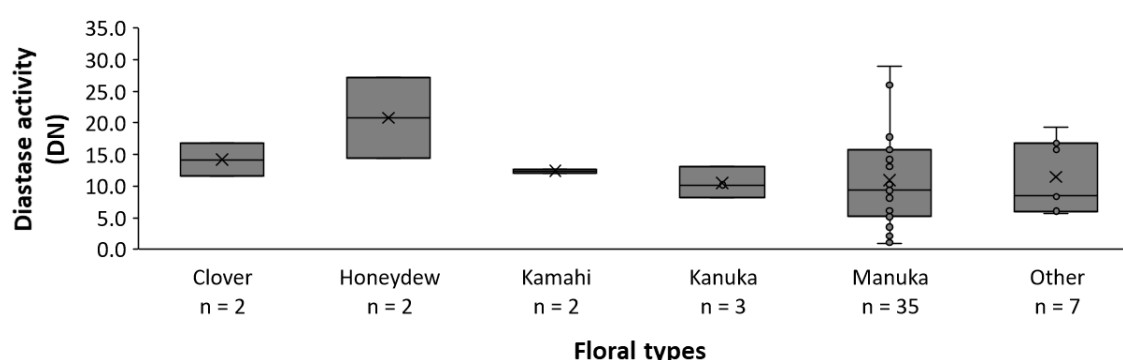
Figure 5.3. Box and whisker distribution plots for Diastase activity (DN). Other = citrus, thyme, towai, rewarewa, southern rata, and tawari.

As diastase activity and HMF concentration are both used to determine honey freshness and exposure to detrimental storage conditions, it is expected that there would be a moderate negative correlation between these two parameters. However, this was not

observed with the DB honeys (R^2 = 0.010, Figure 5.4), subset of mānuka honeys (R^2 = 0.095, Figure 5.5), non-mānuka honeys (R^2 = 0.016), fresh (R^2 = 0.171) or aged (R^2 = 0.012) (figures not shown). The weak correlations for these plots highlight the variability of diastase activity in fresh honey (<10 mg/kg HMF), ranging from below DN 5 up to almost DN 30. A similar lack of correlation was observed by da Silva Sodré *et al*. (2011).[263]
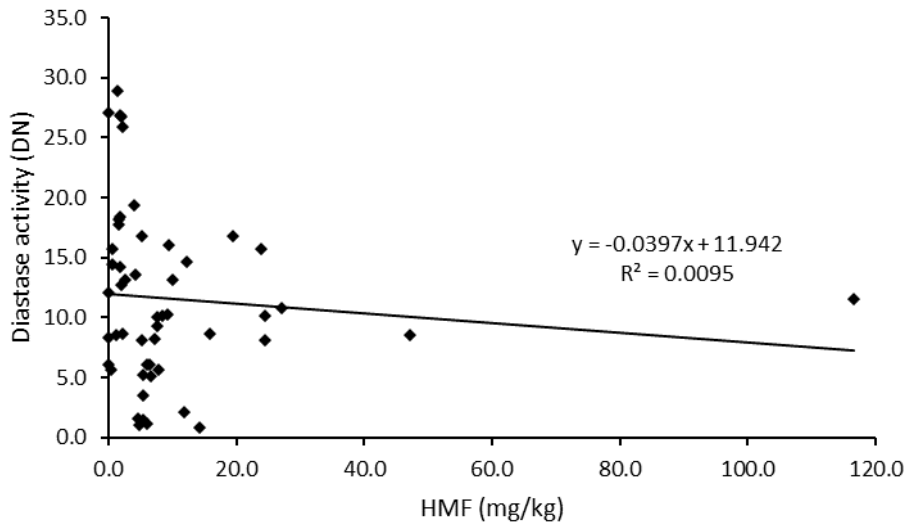
Figure 5.4. Diastase activity vs. HMF (mg/kg) concentration for all DB honeys.
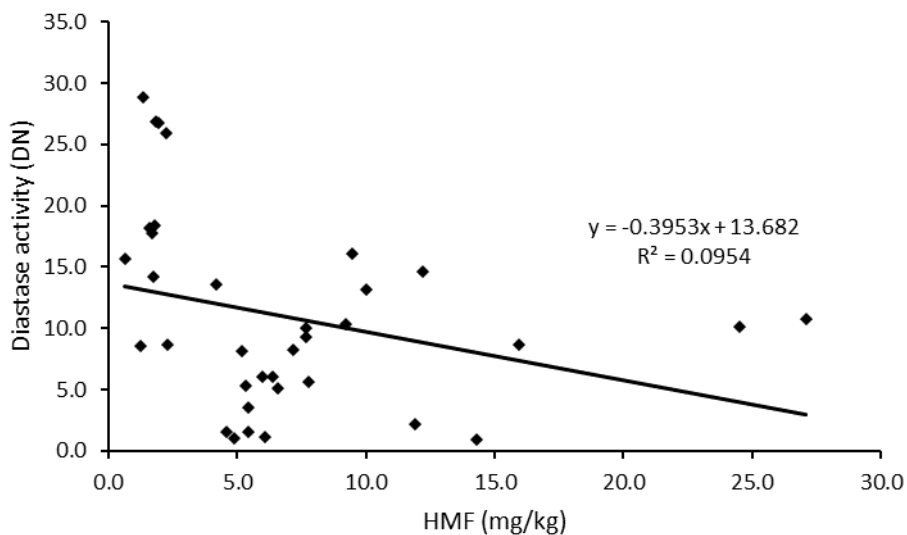
Figure 5.5. Diastase activity vs. HMF (mg/kg) for the mānuka honey database subset.

For some samples, an extraction date was provided by the beekeeper. Plots of HMF and DN compared to the time since extraction were created (Figure 5.6) to investigate the correlation of both these parameters with time. There was a negative trend in the DN for samples, although with a weak correlation (R^2 = 0.213), while HMF had a positive trend and a slightly stronger correlation (R^2 = 0.333). Outliers weakened the correlation; an example is NZ314 (mānuka) which was extracted 32 days prior to receipt at which point it was stored in a freezer (−18 °C) yet had DN 2.1 and HMF 11.9 mg/kg. These outliers could be due to variance in the natural occurring diastase levels or due to the method used to process the honey as well as the temperature the honey was stored at prior to and after extraction. No details were provided by beekeepers regarding the processing and storage temperatures that may have occurred after harvest. Conventional processing preheats the honey to 40 °C for straining and filtering, followed by indirect heating for 25-30 minutes at 60-65 °C for pasteurisation, then rapid cooling.[27] Tosi *et al*. (2008)[268] found rapid heating of six honeys up to 60 °C followed by isothermal heating for 20 minutes resulted in a loss of 2.1-4.1 DN, with the greatest decrease of diastase activity occurring during the temperature increase. While there will be variances in the honey processing procedure (temperatures and times) for beekeepers and commercial processors, it shows that diastase activity can decrease a substantial amount during processing.

Overall, there appears to be a lot of natural variation between honey samples, with no particular trends linked to mānuka or non-mānuka samples for this database of honeys. This variation is likely due to honey extraction, processing and storage conditions, but is also attributed to the chemical parameters and botanical origin of the honey.[265]
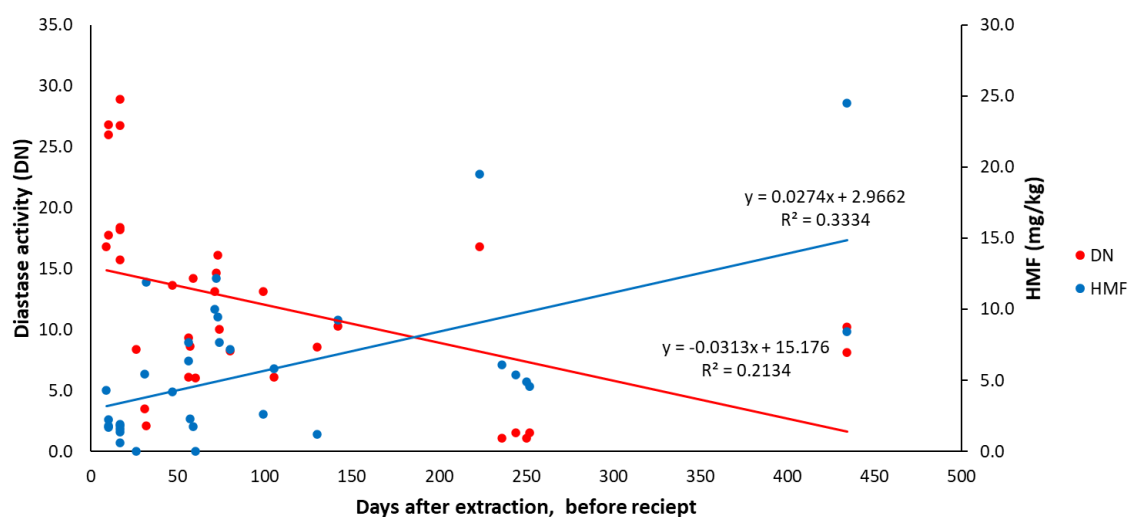
Figure 5.6. Diastase activity (red) and HMF (mg/kg) concentration (blue) vs. time after extraction. Diastase decreases overtime, while HMF increases.

5.1.3 DHA and MGO

Mānuka honey has the highest concentration of DHA and MGO, as expected. The DHA for the mānuka classified samples ranges from 91.3 mg/kg to 3960 mg/kg, while MGO ranges from 55.8 to 995 mg/kg. Literature has shown that DHA concentrations, and therefore MGO, can vary quite widely within the mānuka honey classification, due to the genotype of mānuka trees and dilution by other floral types.[71-72] Concentration of MGO usually ranges from 0-800 mg/kg, with some being recorded up to 1,500 mg/kg.[68, 74, 79] Lin *et al*. (2020)[269] examined 27 mānuka honey samples from various regions around New Zealand, finding DHA and MGO concentrations covered a large range, 957-4570 mg/kg and 61-862 mg/kg respectively, aligning with the DB honey results. The higher concentrations of MGO are found in mature mānuka honey due to the time it takes for chemical conversion of DHA to MGO. Once the DHA to MGO concentration ratio is approximately 2:1, MGO concentration plateaus, due to equal formation and loss of MGO and then subsequently decreases due to side reactions of MGO becoming more prominent.[28]

The ratio of DHA to MGO can also be used to indicate honey freshness due to this process. A low ratio of DHA to MGO indicates mature mānuka honey, as these honeys will have been stored either for a long time or at higher temperatures to facilitate the DHA to MGO reaction.[28] The 2DHA:1MGO ratio indicates the maximum MGO

concentration that will be reached for a given honey, hence the closer it is to this ratio, the longer it has been stored. For honey with lower initial DHA concentrations, this is less indicative of storage as less DHA to react will result in it reaching this maturity point sooner. Indeed, this is observed with the DB honeys. The [DHA]:[MGO] ratio for all DB honeys range from 1.2-26.2, with the mānuka subset ranging from 1.6 – 11.0. At a ratio of 2 or less, MGO side reaction are more prominent than MGO formation.[28] For the mānuka honey subset, there is a moderate positive correlation (R^2 = 0.336) between [DHA]:[MGO] and the concentration of DHA (Figure 5.7a) and a strong positive correlation (R^2 = 0.729) between [DHA]:[MGO] and the inverse of HMF concentration (1/[HMF]) (Figure 5.7b).

All non-mānuka honey samples also contain DHA, although this has a much lower range of 7.5-191 mg/kg, falling just within or below the lower quartile of the mānuka honeys. Only 28.6% of the non-mānuka samples have DHA concentrations greater than 100 mg/kg (NZ344 bush blend, NZ338 towai, NZ335 kānuka blend, NZ362 kāmahi, NZ363 citrus, NZ330 bush blend). The presence of DHA, especially at levels between 100-200 mg/kg, demonstrates the promiscuous nature of bees which prevents formation of fully mono-floral honeys, as although these non-mānuka honey samples do not meet the mānuka criteria, they contain enough mānuka nectar to contain quantifiable DHA. Of the non-mānuka honeys, two do not contain any MGO (NZ341 thyme, NZ324 clover), while the remainder range from <LOD-92.9 mg/kg. Examination of 26 non-mānuka samples by Lin *et al*. (2020)[269] showed similar results of <20-568 mg/kg DHA and 5-155 mg/kg MGO, with the higher DHA and MGO concentrations attributed to samples containing some mānuka honey.
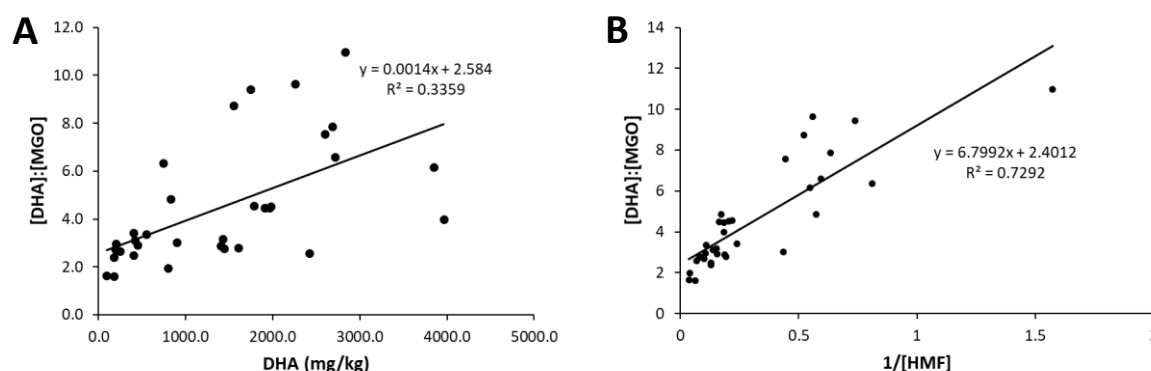
Figure 5.7. [DHA]:[MGO] vs. (a) DHA concentration for the DB mānuka honey subset, and (b) HMF concentration for mānuka and non-mānuka DB honey.

Least squares regression analysis was carried out to find the correlation of DHA and MGO in mānuka honeys to diastase activity (Table 5.1, Table 5.4). The datasets were separated into aged and fresh groups (Figure 5.8). While there is little correlation observed between either DHA or MGO to diastase activity for the fresh subset (n = 37), in the aged subset (n = 7) diastase activity has a strong negative correlation to both DHA (R^2 = 0.807) and MGO (R^2 = 0.782). Due to the very small sample size of the aged subset, this data should be treated with caution, however due to the known reactivity of MGO with proteins,[80] these results may also reflect what would be observed within a larger dataset; further samples should be analysed to confirm this trend. The correlation of DHA in the aged subset is most likely due to the higher concentration of MGO, and hence may be why no correlations were observed to diastase activity in the fresh subset.

Table 5.4. Regression statistics of DHA and MGO to diastase activity for the mānuka honey subset, fresh and aged data.

	DHA	MGO
Aged	DN = −0.00526 [DHA] + 12.77 R^2 = 0.807	DN = −0.01378 [MGO] + 13.04 R^2 = 0.782
Fresh	DN = 0.00208 [DHA] + 8.323 R^2 = 0.071	DN = −0.00882 [MGO] + 14.25 R^2 = 0.048
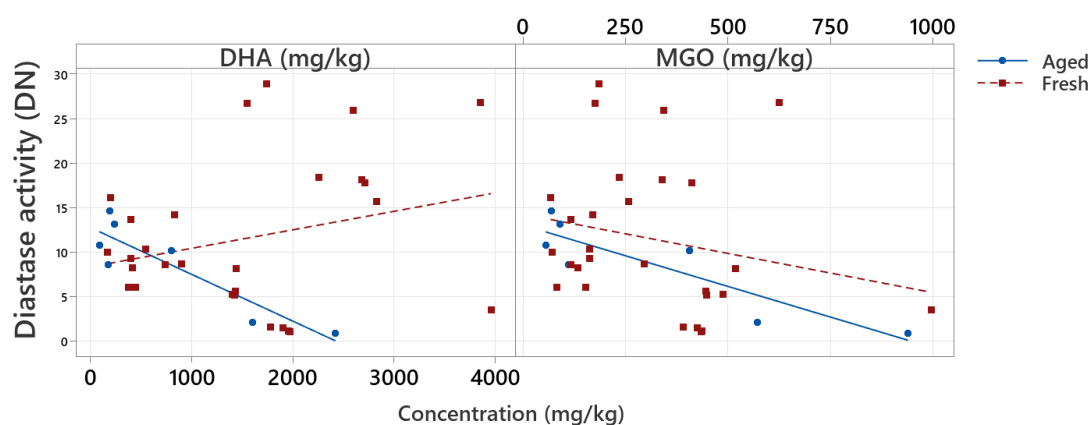
Figure 5.8. Correlation of DHA and MGO concentration in mānuka honey samples to diastase activity, with data separated into fresh (square) or aged (circle) honey.

5.1.4 Phenolic compounds

Out of the phenolic compounds analysed, 2-MBA occurred the least in the samples analysed (Figure 5.9a); 28% of samples did not contain 2-MBA, including two samples that were classified as mānuka honey by the beekeeper but did not meet the mānuka

honey definition of >1.0 mg/kg 2-MBA (Table 5.2). Several non-mānuka honey samples contained 2-MBA; both honeydews (2.6 mg/kg; NZ361 and 3.2 mg/kg; NZ360) and a kāmahi (NZ362) with 4.1 mg/kg. Due to the higher level of DHA and MGO in NZ362 (although less than NPA 5), as well as containing the marker compounds, this honey likely contains a reasonable portion of mānuka, but this classification would have to be determined using mānuka 2'-MAP and DNA marker analysis to confirm. While 2-MBA is used in the MPI mānuka marker definition, it is not unique to mānuka honey, however the concentration of 2-MBA can be used to differentiate between non-, multi- or mono-floral mānuka.[87] The concentration of 2-MBA in the mānuka honey subset ranged from 1.8 to 19.2 mg/kg, averaging 6.9 ± 3.4 mg/kg. This aligns with literature values for 2-MBA in mānuka honey of 1.2-52.1 mg/kg (n = 19)[79] and 1.8-55.2 mg/kg (n = 20),[128] although the upper range is higher in literature. There were five honeys with 2-MBA above 10 mg/kg, four of these were provided by the same beekeeper and had levels between 12-13 mg/kg while the other was significantly higher than all other samples at 19 mg/kg.

The non-mānuka samples generally had the lowest 3-PLA, with 86% containing less than 200 mg/kg and five of these containing no 3-PLA (NZ341 thyme, NZ343 rewarewa, NZ342 kāmahi, NZ323 tawari, NZ364 tawari). The three exceptions were the kānuka samples (NZ334, NZ335, NZ336) at 1640, 905 and 1030 mg/kg respectively, which aligns with literature. Bong et al. (2018)[128] reported kānuka samples (n = 3) to have an average 3-PLA concentration of 725 mg/kg, lower than that found in this research. Mānuka honey samples varied from 82.2 to 1760 mg/kg 3-PLA (Figure 5.9b), with an average concentration of 850 ± 409 mg/kg. This is slightly lower than values stated in the literature (1130 ± 436 mg/kg, n = 19;[128] 1556 ± 264, n = 20[79]), due to natural variation between within $L.$ $scoparium$ varieties, but potentially also because of seasonal difference in honey production.[20]

The 4-HPLA distribution profile between the floral types was quite similar to 3-PLA albeit on a different scale (Figure 5.9c). The non-mānuka honeys had 4-HPLA concentrations of 2.5 mg/kg or less, with three exceptions of the kānuka honey samples at 5.8, 8.0 and 8.3 mg/kg for NZ335, NZ334 and NZ336 respectively. Neither clover (NZ324, NZ329), nor rewarewa (NZ343) or thyme (NZ341) samples contained 4-HPLA. Only two mānuka honey samples had 4-HPLA levels below 2.5 mg/kg, at 1.1 and 2.2 mg/kg respectively for NZ345 and NZ310. All other mānuka samples ranged from 2.7 to 13.5 mg/kg with an

average of 6.7 ± 3.0 mg/kg. The 4-HPLA results for kānuka and mānuka matched those reported by Bong *et al.* (2018).[128]

Of the 21 non-mānuka honey samples, 18 had MSY concentrations of less than 50 mg/kg (Figure 5.9d). Unlike the other three phenolic compound analytes, MSY is not a mānuka marker. Although it is found in high levels in mānuka honey, it is not unique, and MPI determined that it was not stable over time and temperature, nor could concentrations be used to differentiate between multi- and mono-floral mānuka honey.[87] Analysis of the DB honeys showed that kānuka honeys (NZ334-6) had the highest MSY concentrations at 300 ± 49 mg/kg. Mānuka samples ranged between 44.6 to 117 mg/kg of MSY, with two exceptions; NZ345 had only 6.8 mg/kg MSY, while NZ322 contained 305 mg/kg. This high result for NZ322 (classified as a mānuka blend by the beekeeper) that falls within the kānuka sample concentrations indicates that it is a mānuka / kānuka blend, as there is a large gap in MSY concentration between the highest mānuka sample and the kānuka samples. The three mānuka marker concentrations indicate NZ322 is a mono-floral mānuka honey, however this high level of MSY indicates a significant portion of this honey could be kānuka and therefore would require analysis of 2'-MAP and DNA to affirm classification.
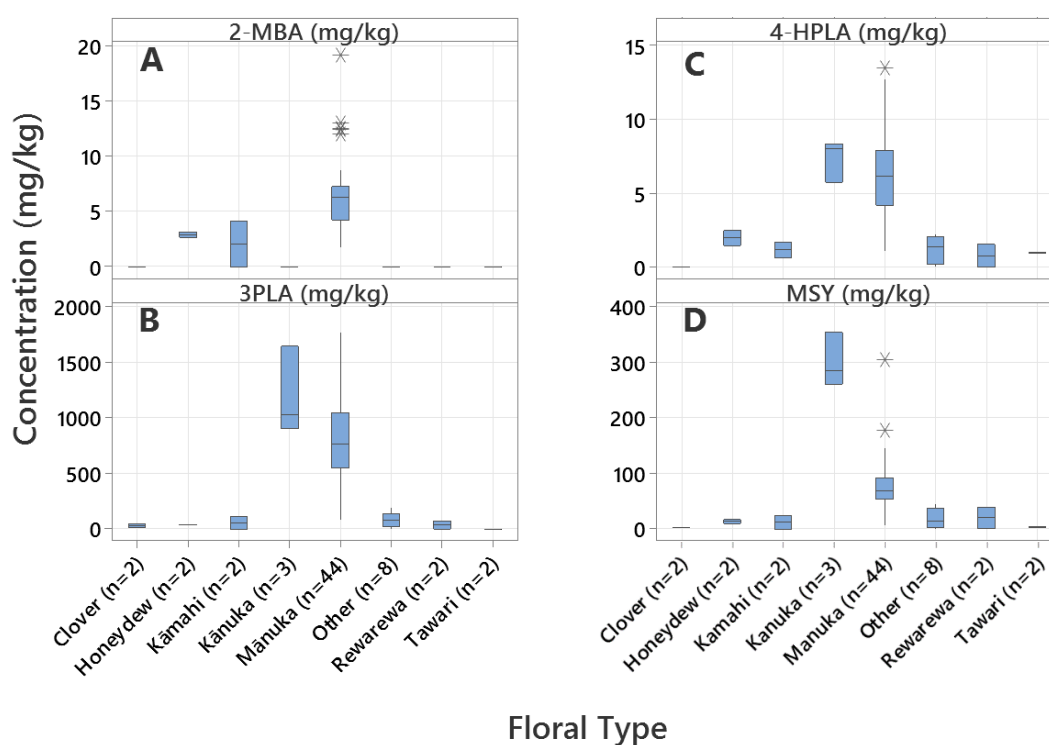

Figure 5.9. Box and whisker distribution plots for phenolic compounds; a) 2-MBA, b) 3-PLA, c) 4-HPLA and d) MSY. Other = citrus, thyme, towai, southern rata, and poly-floral (unknown).

Least squares regression (LSR) analysis was carried out between the phenolic compounds and the other investigated parameters to determine any relationships and correlations for both the overall database (Table 5.5) and the mānuka honey subset (Table 5.6). There were only small differences between the overall DB and the subset.

Positive correlations between DHA, MGO and the mānuka chemical markers were moderate to strong for both the database (R^2=0.44-0.73) and the mānuka subset (R^2=0.38-0.71), as expected. A moderate positive correlation (R^2=0.39) was also observed between 3-PLA and MSY for the DB samples, but this was weak (R^2=0.23) for the mānuka subset due to the removal of the kānuka samples which contain high MSY. A moderate positive correlation was observed between MSY and HMF for the mānuka subset (R^2=0.48), but is most likely due to chance, not causation.

LSR indicates that these parameters may not influence diastase activity, or that any influence is too small for correlation to be observed. Alternatively, any interaction of compounds with diastase may require an intermediate compound which forms over time (rate limited). Therefore, the effect may not be as prominent in fresh samples and is simultaneously disguised by the natural variation in DN between samples rendering these results inconclusive.

Table 5.5. Phenolic compound relationships to other parameters and their correlation for all DB samples. Moderate correlations (>0.25) are in bold font.

		DN	DHA	HMF	MGO	2-MBA	3-PLA	4-HPLA	MSY
2-MBA	Relationship	–	+	–	+				
	Correlation	0.08	**0.61**	0.05	**0.62**				
3-PLA	Relationship	–	+	–	+	+			
	Correlation	0.01	**0.57**	0.02	**0.44**	**0.40**			
4-HPLA	Relationship	–	+	–	+	+	+		
	Correlation	0.07	**0.56**	0.05	**0.46**	**0.62**	**0.73**		
MSY	Relationship	–	+	+	+	+	+	+	
	Correlation	0.03	0.00	0.13	0.00	0.00	**0.39**	0.20	

Table 5.6. Phenolic compound relationships to other parameters and their correlation in only the mānuka designated group. Moderate correlations (>0.30) are in bold font.

		DN	DHA	HMF	MGO	2-MBA	3-PLA	4-HPLA	MSY
2-MBA	Relationship	–	+	–	+				
	Correlation	0.16	**0.43**	0.00	**0.45**				
3-PLA	Relationship	–	+	–	+	+			
	Correlation	0.01	**0.71**	0.00	**0.46**	0.38			
4-HPLA	Relationship	–	+	–	+	+	+		
	Correlation	0.10	**0.56**	0.00	**0.40**	**0.64**	**0.58**		
MSY	Relationship	–	+	+	+	+	+	+	
	Correlation	0.06	0.00	**0.43**	0.01	0.02	0.23	0.06	

5.1.5 Samples of interest

There were several samples in the database with results that did not fit the trends of other samples, either when compared to the entire database or within their floral type.

NZ323 (tawari), NZ321 (southern rata) and NZ343 (rewarewa) all had no or low (0.5 mg/kg for NZ343) HMF levels, indicating that they had not been subject to poor storage conditions and were very fresh. In contrast to the low HMF results, each of these three honeys also had low diastase activity, with NZ323 and NZ343 falling below the export threshold at DN 6.0 and 5.6 respectively, and NZ321 just above at DN 8.4. Either DN is low due to unknown causes / mechanisms or HMF formation is prevented by the honey composition. The tawari sample was harvested 60 days before receipt, the southern rata 26 days before, and the harvest date for rewarewa is unknown. Comparison of the tawari sample to other honeys that were received within the same timeframe from harvest (Figure 5.10) showed no relationship between HMF and DN and time from harvest. This is somewhat expected as time is only one component to consider for loss of DN and increase of HMF; the temperature treatment history of these samples is not known. However, the variability could also be influenced by differences in the initial diastase activity (amount that comes from the bee) or honey composition influencing diastase enzyme stability, degradation or inhibition, or influencing HMF formation.[265]
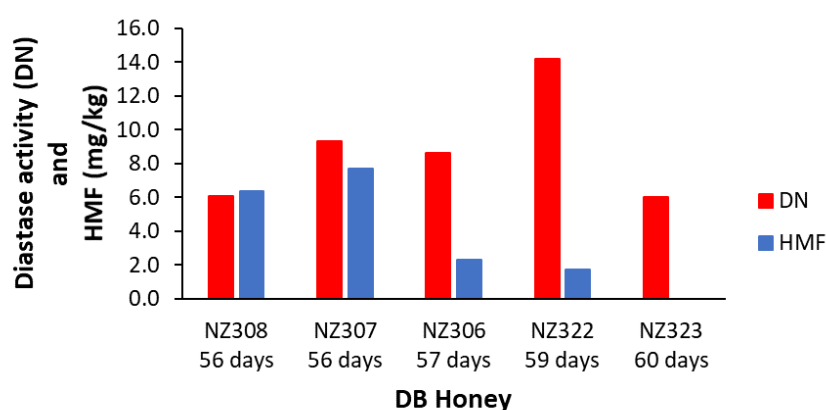
Figure 5.10. Comparison of diastase activity and HMF levels for database honeys that were received within the same timeframe from harvesting (56-60 days). NZ323 is tawari, the other four honeys are mānuka. There is no relationship between DN and HMF.

In contrast to the above samples, two honeys had high DN as well as HMF greater than 10 mg/kg. NZ312 (mānuka) had DN 14.7 with 12.2 mg/kg HMF and NZ329 (clover) had DN 16.8 and 19.5 mg/kg HMF. Levels higher than 10 mg/kg HMF indicate the samples

are not fresh, however the DN are still above the threshold for diastase export criteria. These two honeys may have originally had very high DN; or alternatively, components in the honey may influence the stabilisation of DN or promote HMF formation.[270]

5.1.6 DB summary

Analysis of the database honey samples highlights the large variation of the analysed parameters between samples, especially within the mānuka honey subset. Diastase activity varied greatly between samples and no strong conclusive correlations were observed between DN or any of the measured parameters. As expected, differences between mānuka and the other floral honey types, specifically DHA, MGO and 2-MBA were observed. The other two mānuka markers and MSY were also generally higher in mānuka than other honeys, with the exception of kānuka.

5.2 Database snapshot (DBSH) experiment

Twenty-eight DB honeys were stored for 161 days at 20 °C (DBSH experiment) and were analysed on Day 0 and 161 to observe changes in chemical and physical parameters overtime (only 21 were tested for DN due to Phadebas tablet shortage). To compare changes between samples, the data was normalised to the value recorded on Day 0 (Appendix B); hence the increase/decrease in concentration at Day 161 is discussed.

The analysed samples were chosen to ensure they covered the breadth of samples in the database, including a variety of floral types (19 mānuka, 3 kānuka and 1 each of clover, kāmahi, tawari, thyme, towai and southern rata) from different regions, with a range of initial diastase activity (0.9-28.9), DHA (7.5-3960 mg/kg), HMF (0-24.5 mg/kg) and MGO (0-995 mg/kg) content.

5.2.1 Moisture

Moisture content changed very little after 161 days of storage, varying from the initial values by −0.5% to 1%. Although honey is hygroscopic, proper storage prevents moisture accumulation.

5.2.2 HMF

Changes in HMF concentration over time for the DBSH samples were varied for each sample (Figure 5.11), with six mānuka and four non-mānuka samples showing a loss of

HMF (loss of 0.2 to 9.7 mg/kg) contrary to expectations. Although HMF is expected to increase overtime due to accumulation from the Maillard reaction, HMF formation sometimes has a lag phase (induction phase) where the formation stalls before increasing, although it is not certain what causes this. The remaining seventeen samples had HMF increases of 0.3 to 4.4 mg/kg. Korkmaz *et al.* (2017)[271] found larger HMF increases (6.1 to 9.2 mg/kg increases) for five floral honeys stored at 22 °C for six months. The differences in HMF concentrations for some honeys are larger than for others after 161 days of storage at 20 °C. Although temperature and time are known to be major factors in the increase in HMF concentration, other factors include the chemical properties of the honey including pH, total acidity and mineral content. [201] These results show that HMF is not a reliable indicator, in fact it may prove that the segregation of non-fresh to fresh honey subsets using HMF <10 mg/kg is not reliable, however, it is the only other age tool (commonly used in industry) on hand to attempt order on the database.

There was no significant difference between mānuka and non-mānuka HMF losses and gains after 161 days. No correlation was observed between DN and HMF concentration at T=161 days, nor was correlation observed between the change in DN vs. the change in HMF after 161 days (not shown).
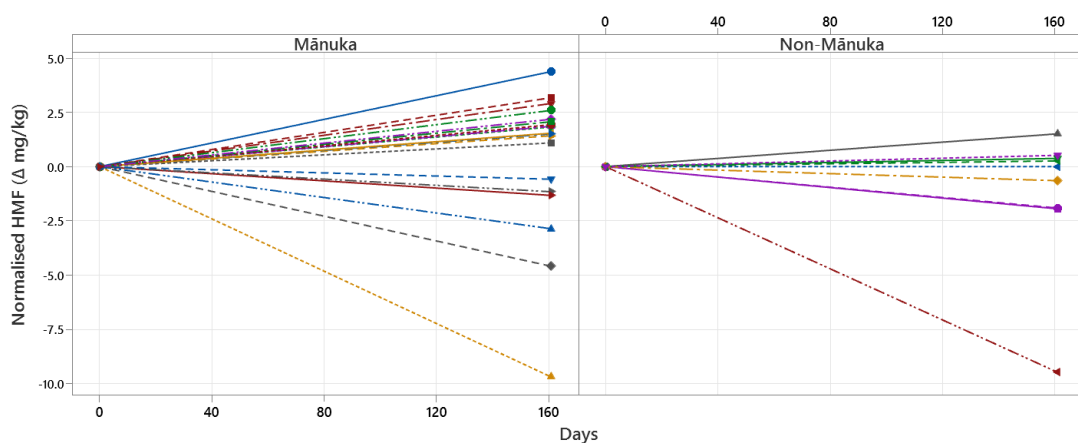
Figure 5.11. Normalised HMF concentration vs. time for mānuka and non-mānuka samples stored at 20 °C.

5.2.3 DHA and MGO

As expected, DHA and MGO changed over time (decreased and increased respectively), with the largest changes observed in the samples with the highest initial DHA concentration (Figure 5.12). Compared to the mānuka samples, the degree of change in non-mānuka samples was much smaller (from −49.5 mg/kg DHA and +16.1 mg/kg MGO) due the lower initial DHA concentration.
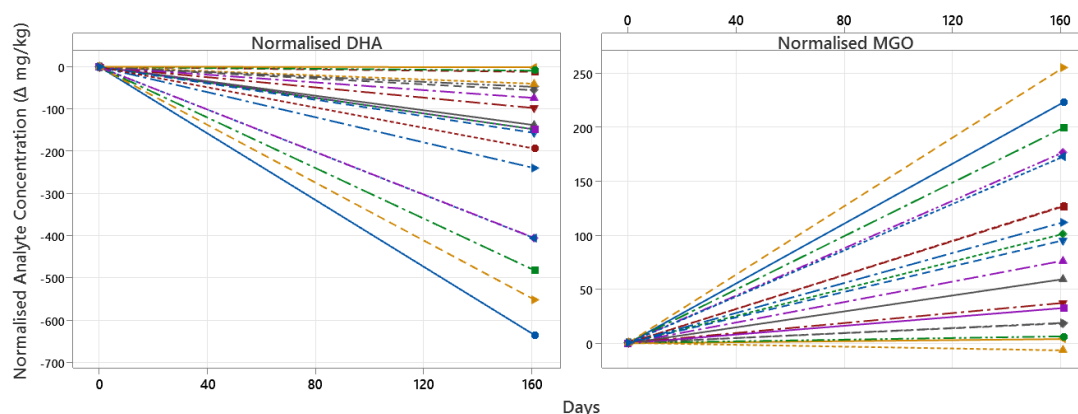
Figure 5.12. Normalised DHA and MGO concentration vs. time for mānuka and non-mānuka samples at 20 °C.

5.2.4 Phenolic compounds

For the mānuka marker compounds, little change is observed in the non-mānuka samples. For mānuka samples, 2-MBA changed by −1.8 to +2.7 mg/kg (within the method error) with two outliers of 8.9 and 10.9 mg/kg increases for NZ314 and NZ331 respectively. These two measurements are likely due to experimental error. 4-HPLA concentration was also relatively stable over time at 20 °C, with changes primarily within the method error (±1 mg/kg of the starting measurement). Changes in concentration of 3-PLA had more variation than 2-MBA or 4-HPLA, but samples were generally within 10% of the T=0 measurement (n = 13 increased, n = 15 decreased). The three exceptions are NZ299 (−119 mg/kg), NZ318 (−176 mg/kg) and NZ298 (−274 mg/kg) which had 12.6, 13.2 and 27.1% decreases in 3-PLA over time. This is contrary to MPI reports that this compound is stable over time in mānuka honey, and thus is potentially due to error in preparation of the T=161 or T=0 day sample. As expected, there were no distinct trends over time for any of the mānuka markers and generally, the greatest changes occurred for the samples which had the largest initial concentration.

For both mānuka and non-mānuka honey samples, there appears to be an increase in MSY over time, however MPI[87] states that MSY is not stable, decreasing over time and at higher temperatures. Conversely, Kato *et al.* (2014)[89] showed that MSY decreased over time at 37 °C. It may be that when stored at elevated temperature, an interfering compound (e.g., a Maillard reaction product) increased in concentration over time and co-eluted with MSY on the HPLC-UV chromatogram.

No correlations were observed between the phenolic compounds and diastase activity for the DBSH, indicating that the analysed phenolic compounds appear not to inhibit diastase activity.

5.2.5 Diastase activity

All DBSH samples showed a decrease in diastase activity over time (Figure 5.13), dropping by 0.4 (NZ340, NZ299) to 21 DN (NZ326). The decrease of NZ326 from DN 26.8 to 5.8 is potentially an experimental error, as all other DBSH samples have lost 10 or less DN units. Due to the lack of Phadebas tablets, this sample could not be reanalysed. It is possible that the diastase test of this sample was affected by contamination by either detergent (used for cleaning labware) or bodily fluids (sweat), both of which can inhibit diastase activity.[181] Sixteen of the samples decreased by less than DN 5, while four samples (mānuka; NZ333, NZ319, NZ316, NZ318) lost between DN 5 and 10.2. For NZ318, part of this loss will be related to the difference in DN calculations (Section 2.2.2.1) as after 161 days, the sample had fallen below DN 8. This resulted in a drop from DN 7.4 to DN 5.5 as the required calculation for DN <8 is different to that for DN >8. Even with this difference, the loss is still substantially greater than the other seventeen DBSH samples.

Korkmaz *et al.* (2017)[271] found diastase activity in honey stored at 22 °C decreased by only DN 1.1-1.7 over a period six months, a much smaller decrease compared to most of the DBSH samples which were incubated for slightly shorter (5.5 months) at a lower temperature (20 °C). The variation in diastase activity loss reiterates the influence that the honey matrix has on its dynamic parameters. While temperature and time also play a role, other components do too.[201]
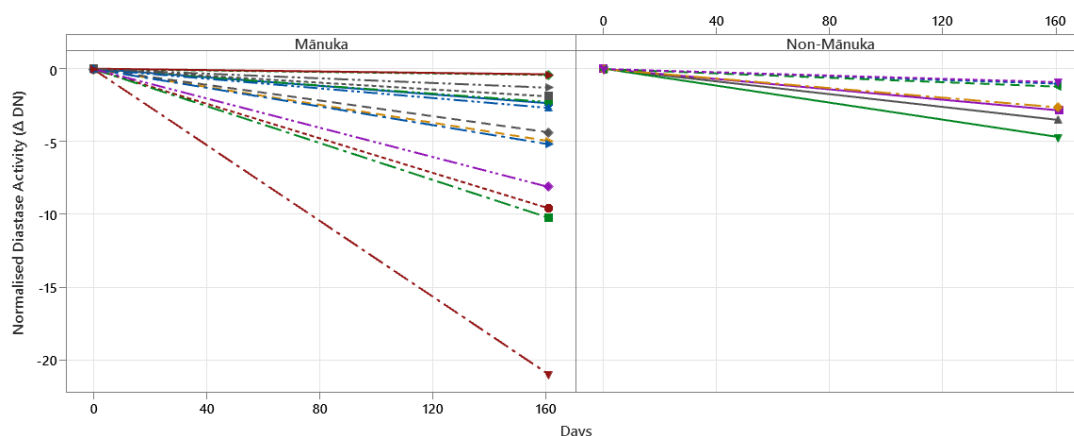
Figure 5.13. Normalised diastase activity vs. time for mānuka and non-mānuka samples stored at 20 °C. Note that sample NZ326 is deemed an outlier.

Samples with larger DN loss (>5) also had a high DHA concentration, and significant MGO gain over the time period; MGO increased by 127.1-199.3 mg/kg, while the majority of other samples had MGO gains of approximately 100 mg/kg or less. While NZ326 also has high DHA and MGO gain, there is no evidence to prove that the extremely large drop in DN is not erroneous. MGO increased by 111.8, 172.7 and 223.1 mg/kg for three samples (NZ340, NZ327 and NZ269 respectively), but only small decreases in diastase activity (<2.5). However, the initial diastase activity of NZ340 and NZ269 was low (DN 0.9 and 3.5 respectively), which prevented a large decrease in diastase activity occurring. NZ327 is an anomaly, with both high initial DHA (2720 mg/kg) and diastase activity (DN 17.8), while gaining 173 mg/kg of MGO (final concentration 585 mg/kg) yet only losing DN 2.3 (net 15.5) after 161 days.

Regression analysis was carried out on a subset of samples (those which had initial (T=0) diastase activity of DN<8 were removed), plotting diastase loss against MGO gain at T=161 days (normalised data, Figure 5.14). This gave a moderate negative correlation (R^2 = 0.635). It appears that diastase activity is negatively affected by increasing MGO concentration in honey as the magnitude of loss in diastase activity is correlated to the extent of gain in MGO. NZ327 does not fit this trend, either due to erroneous measurement of diastase activity, or due to other unknown factors. As honey is such a complex matrix, effectiveness of any inhibition may not rely only on the constituents (MGO and diastase) but other factors such as pH or auxiliary compounds. These may influence the stability of the diastase enzyme or affect the reactivity of MGO to the diastase amino acid residues, preventing or enhancing inhibition. Removal of the two

apparent outliers (NZ326 and NZ327) from the regression analysis improved correlation ($R^2 = 0.841$). There is the possibility that this relationship may be co-incidental due to relationship of both MGO and DN with time and temperature, however MGO is known to react with amino groups on proteins,[204] crosslinking and potentially deactivating them and thus may affect the diastase enzyme (further discussed in Section 6.2.3.3).

For the entire DBSH group ($n = 21$, 14 mānuka, 7 non-mānuka), there was no significant difference ($\alpha = 0.5$) in diastase activity decrease between the mānuka and non-mānuka samples ($p = 0.082$), however, significant difference ($p = 0.023$) was found between mānuka and non-mānuka for a subset containing an initial DN > 8 ($n = 14$, 8 mānuka, 6 non-mānuka). A much larger sample set of samples with initial diastase activity greater than 8 would be required to validate this relationship.
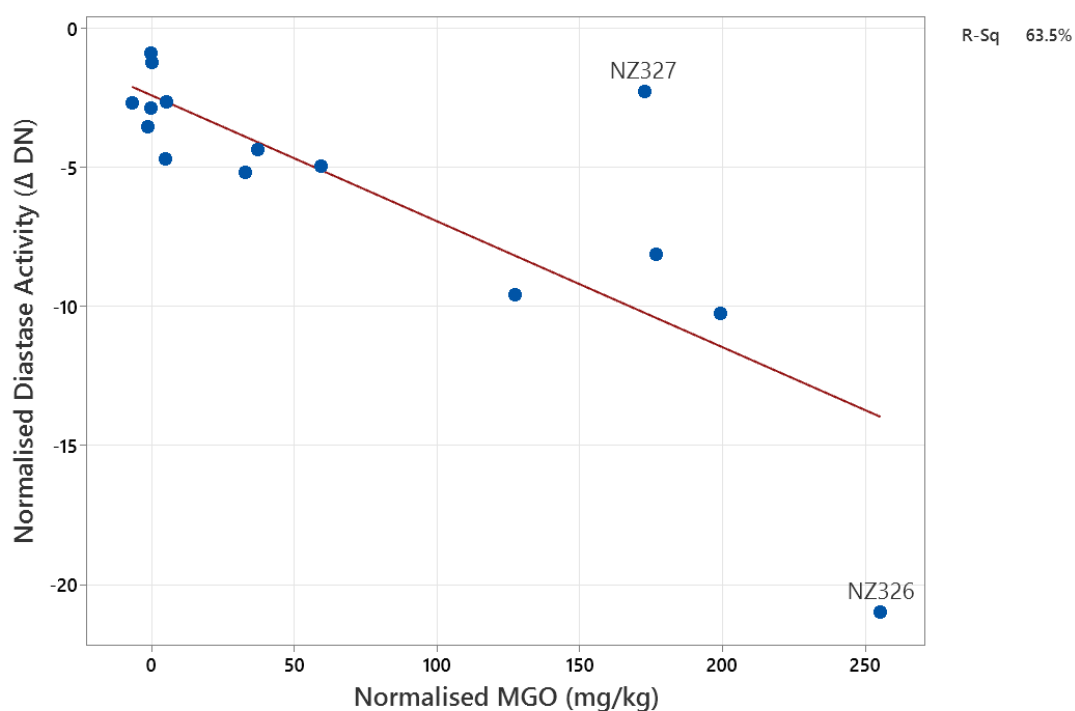
Figure 5.14. Normalised diastase activity (DN) vs. normalised MGO (mg/kg) for mānuka honey after 161 days at 20 °C for DBSH subset with DN>8 at T=0. A moderate negative correlation is observed between diastase activity loss and MGO gain over time ($R^2 = 0.635$).

5.2.6 DBSH summary

DHA and MGO behaved as expected (decreased and increased respectively), while the phenolic compounds were generally stable over time, DN decreased and HMF was

variable. For the majority of analysed compounds, no correlation with diastase activity was observed, however a subset of the data (samples with initial DN < 8) showed strong correlation between MGO formation and decreased diastase activity. The behaviour of diastase may be reliant on intrinsic honey characteristics, not just temperature and time.

5.3   Database trials (DBT) experiment

Eight mānuka and two other honeys (NZ323 tawari and NZ335 kānuka) were chosen for the DBT based on their initial results, to cover a range of values in each parameter (Table 5.7). Samples were stored in duplicate at 27 °C and analysed at 0, 69, 117 and 198 days, using the DB results for the sample as the initial timepoint (Appendix B). The aim of this experiment was to gain a more detailed view of how diastase and other parameters change over time, using a slightly elevated temperature to hasten any reactions compared to the DBSH samples. Analysis in this chapter is carried out on normalised data of the average of the duplicate results. An example of the variation between duplicates is shown in Figure 5.15; the error between measurements is very small, and hence error bars are not visible for some samples. Therefore, error bars have been excluded from the plots in the following section and in the next chapter (Chapter 6).

Table 5.7. DBT honey samples, their floral designation and initial levels (L-low, M-moderate, H-high*) of analysed parameters.

Sample	Floral Designation	DN	HMF	DHA+MGO	Phenolics
NZ269	Mānuka	L	M	H	H
NZ308	Mānuka	L	M	M	H
NZ316	Mānuka	H	L	H	M
NZ319	Mānuka	H	L	H	H
NZ322	Mānuka	M	L	M	M
NZ323	Tawari	L	L	L	L
NZ327	Mānuka	H	L	H	H
NZ333	Mānuka	M	L	M	L
NZ335	Kānuka	M	L	L	H
NZ337	Mānuka	L	M	M	M

*L = DN<8, HMF<5 mg/kg, DHA+MGO<250 mg/kg, Phenolics<600 mg/kg
M = 8≤DN<15, 5≤HMF<10 mg/kg, 250≤DHA+MGO<1000 mg/kg, Phenolics600-1000 mg/kg
H = DN≥15, HMF≥10 mg/kg, DHA+MGO≥1000 mg/kg, Phenolics≥1000 mg/kg
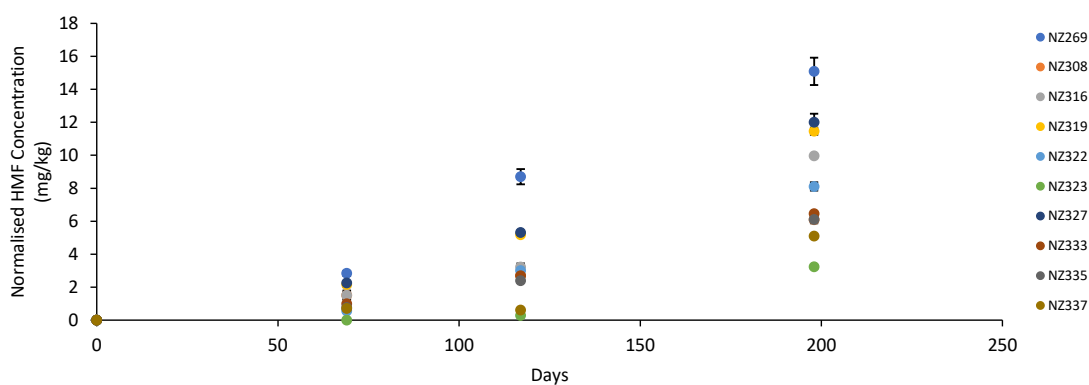
Figure 5.15. Normalised HMF vs. time of the DBT samples at 27 °C. The error bars at set to the standard deviation of the duplicates at each timepoint showing no or little variation between duplicates.

5.3.1   HMF

All DBT samples showed an increase in HMF concentration by the final timepoint (Figure 5.16; Δ HMF 3.2 to 15.7 mg/kg, 198 days, 27 °C), unlike with the DBSH samples (Δ HMF −1.2 to 4.4 mg/kg, 161 days, 20 °C). Even after 117 days, the increase in HMF concentration for the DBT samples was approximately twice (1.7-2.4x) that of the DBSH samples at 161 days demonstrating that temperature is the main driver of HMF formation. As with the DBSH samples, there is variation between the rate of increase, probably due to other honey parameters such as pH.[201] While there is a positive relationship between the HMF gain over 198 days and the initial HMF concentration at T=0, there is no correlation between these two parameters (R^2 = 0.01), indicating the initial concentration of HMF does not have an effect on the rate of HMF formation.

The HMF increase was more prominent in the mānuka honey samples. There was a strong correlation between the gain in HMF and the gain in MGO after 198 days at 27 °C (R^2 = 0.906), which is also observed for the same samples in the DBSH experiment (R^2 = 0.752). Although the storage conditions were different for DBSH (20 °C, 161 days) and DBT (27 °C, 198 days), there was no significant difference (α = 0.05) between the ΔHMF vs. ΔMGO slopes for both experiments.
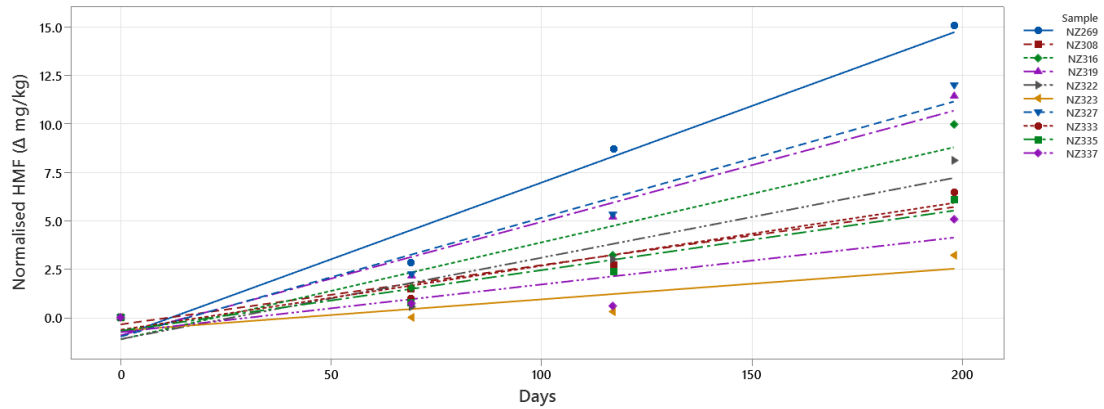
Figure 5.16. Normalised HMF concentration vs time for DBT samples at 27 °C.

5.3.2 DHA and MGO

As with the DBSH samples, DHA concentration decreased and MGO concentration increased over time (Figure 5.17) for all DBT samples with larger changes observed in the samples with high initial DHA concentration. The rate of change for both DHA and MGO is related to temperature as after 117 days at 27 °C there were larger changes compared to 161 days at 20 °C (DBSH) as expected.[28] Correlation to diastase activity is discussed in Section 5.3.4.
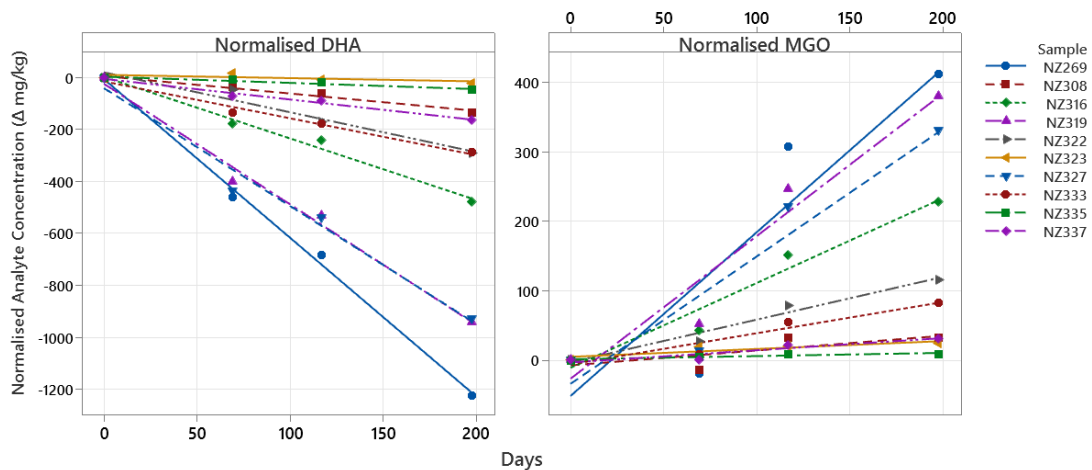
Figure 5.17. Normalised DHA and MGO concentration vs. time for DBT samples at 27 °C.

5.3.3 Phenolic compounds

Similar to the DBSH samples, for the DBT samples, the mānuka marker compounds did not vary by much (Figure 5.18), with the exception of 3-PLA concentration in the NZ269

and NZ327 samples and all MSY concentrations. This is in line with the observation of Stevens *et al.* (2010)[79] who observed that 3-PLA concentration was higher in fresh honey samples than in matured honey. However, Kato *et al*. (2021)[204] have demonstrated that 3-PLA and 2-MBA are stable over time, even at temperatures up to 90 °C, while MSY and 4-HPLA slightly decreased over time when held at the same temperature. Even at lower temperatures (37 and 50 °C), MSY decreased.[89] This is contrary to the results showed for both DBSH and DBT, where MSY increased over time, potentially due to formation of a Maillard reaction product which co-eluted with the MSY peaks. Except for MSY, the literature matches the DBT results where 2-MBA, 3-PLA and 4-HPLA remained relatively constant when tracked over time. Correlation of the phenolic compounds to diastase activity is discussed in the next section.
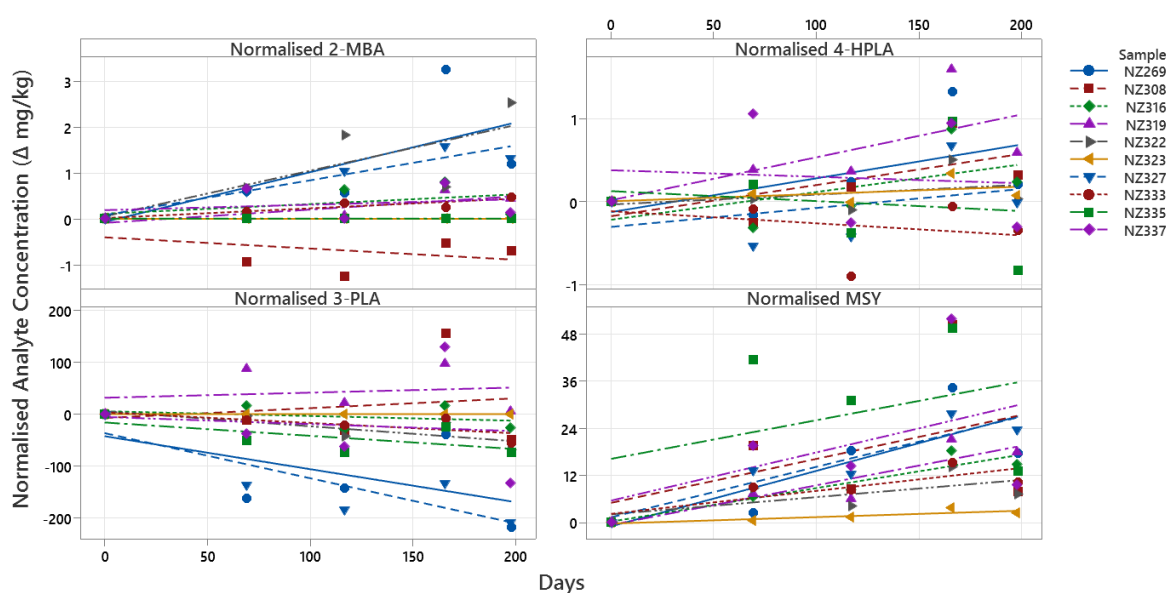
Figure 5.18. Normalised phenolic compounds vs. time for DBT samples at 27 °C.

5.3.4 Diastase activity

All samples showed a decrease in diastase activity over time, however, with a variety of results. There appear to be four clusters in trends (Figure 5.19): little change (<2) in DN (tawari - NZ323), decrease of 2-5 DN (NZ269, NZ308, NZ337), decrease of 5-10 DN (NZ322, NZ327, NZ333, NZ335) and decrease of >10 DN (~15 DN; NZ316, NZ319). Samples with higher initial diastase activity had larger decreases in diastase activity over the storage period. This same trend was observed by White *et al.* (1964);[272] storage of

three honeys with high (DN 41.7), medium (DN 28.2) and low diastase activity (DN 13.4) at 27 °C for 248 days resulted in 73, 51 and 28% losses in diastase activity respectively.

Plotting diastase activity over time, the tawari sample (NZ323) had poor correlation (R^2 = 0.058) due to the slight negative slope producing no real downward trend, with method variation causing results to sit either side of the equation change point of DN 8. All other samples showed moderate to strong negative linear correlation of diastase activity to time at 27 °C, as expected (R^2 = 0.742-0.976, Figure 5.19). However, comparing the trends, the initial diastase activity measured for NZ327 (DN 17.8) may be an error as the following three timepoints followed the trend set by the other samples. The T=0 and T=69 days diastase activity for NZ327 were approximately equal (not shown); instead, T=69 was expected to have a lower diastase activity. NZ327 is also the outlier in the DBSH samples (Section 5.2.2) and is likely lower than expected due to contamination of the sample with detergent or bodily fluids (sweat) during analysis. A higher initial value (T=0) for both the DBSH and DBT would fit the trend. However, there was no way of re-analysing the diastase activity for this sample due to lack of Phadebas tablets. Instead, the NZ327 T=0 timepoint was removed and the diastase data reanalysed by normalising the data by the second timepoint (new T=0). This was deemed plausible because the previous temperature history of all samples is unknown, therefore the heating during time period (0-69 days) should not affect the use of day 69 as day 0. The removal of the initial T=0 timepoint improved the linear regression (R^2 = 0.9987), matching the regressions for the other eight DBT samples. The loss of diastase activity for the DBT samples (27 °C, 198 days) was 1.3-2.2 times greater than the same samples in DBSH (20 °C, 161 days), with the exception of NZ323 which had very little change over time (variation was within the method error); higher temperature increased the loss of diastase activity over time.

Each of the samples which had a decrease of less than DN 5 started with DN<8. Ten samples were included in this study to keep to a manageable scope, however only six samples were usable (initial DN>8) for comparison of diastase activity decrease (not limited by low starting DN) against other components. Two non-mānuka honeys and two mānuka honeys with low starting diastase were initially chosen to include in the experiment to observe what changes occurred at low DN, however the results are not informative. In hindsight, more samples should have been included to gain a better

understanding of correlations, and all chosen samples should have been of higher starting diastase activity. This would have given more usable data when looking at correlation between DN and other compounds. A mānuka with high DN and low DHA/HMF should also have been included in the DBT samples to cover all options.

As with DBSH, regression analysis was carried out on the DBT samples, plotting loss in diastase activity against the changes in analytes, however no clear relationships were observed due to the small usable dataset (six out of ten samples) and wide spread of results. Although there were no clear relationships, the data for the samples with initial diastase activity greater than 8 matched what was described by the DBSH samples; DBT samples with a high MGO gain generally had a greater loss of diastase activity.
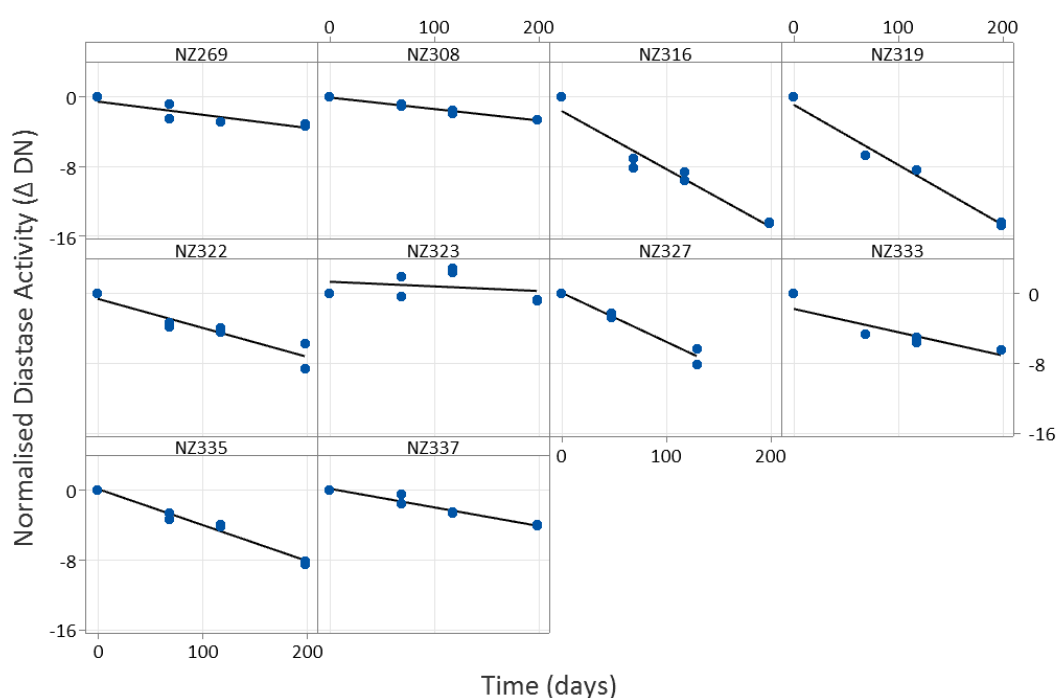
Figure 5.19. Normalised diastase activity vs. time for DBT samples stored at 27 °C for 198 days. NZ327 was adjusted to the second timepoint due to an erroneous initial diastase analysis. Eight samples are mānuka; the non-mānuka samples are NZ323 – tawari and NZ335 – kānuka.

5.3.5 DBT summary

The primary causes of diastase activity loss appeared to be temperature and time (DBT compared to DBSH), as no correlation to other parameters was observed. However, the rate at which it decreased is related to other factors, potentially including concentration of MGO, DHA and 3-PLA. Other unknown factors likely also play a role and may be dependent on the honey type.

5.4   Commercial dataset

A company from the apiculture industry supplied their own mānuka honey dataset with results for the parameters analysed in this study, excluding MSY, but also including 2'-MAP. Due to commercial sensitivity, this company will remain anonymous and all qualitative information of the samples is confidential, therefore only general trends are presented here.

The provided dataset (n = 241 mānuka honeys) was analysed in its entirety and also as a subset of fresh samples (HMF < 10 mg/kg). The commercial dataset showed some moderate correlations (R^2 > 0.25, Figure 5.20a, c-d) between diastase activity with DHA (0.276), 3-PLA (0.612) and 4-HPLA (0.536). Higher concentrations of these compounds were related to lower diastase activity in honey, however this is not necessarily causation. Unless there is reversible inhibition occurring which releases the inhibitor allowing for the same concentration of analyte to be measured over time, a decrease in analyte would be observed, yet 3-PLA and 4-HPLA are known to be stable over time in honey (shown with DBSH and DBT).[88] If it was reversible inhibition, then it would be possible to have occurrences of datapoints outside of the observed trends, high DN and high analyte concentration or low DN and low analyte concentration. However, this is not the case with the commercial dataset, indicating that reversible inhibition may not be the mechanism. If 3-PLA or 4-HPLA are irreversibly inhibiting diastase, it may be that the decrease in these compounds is so small that it is not significant. The concentration of alpha-amylase in honey has not been mentioned in literature, only indirect measurement of its activity with no information on the sensitivity of the diastase assay. If the assay is sensitive, then a small reduction in the concentration of active (non-inhibited nor denatured) diastase may result in a larger decrease of diastase activity. A 1:1 irreversible inhibition stoichiometry would therefore result in a non-significant decrease of the inhibitor.

Alternatively, irreversible inhibition of diastase by these compounds may occur rapidly during honey formation when bees process the nectar, but slow to an insignificant rate due to the viscosity of mature honey preventing interactions between the diastase enzyme and inhibitors.[273] It could also be possible that the concentration of 3-PLA or 4-HPLA are proportional to the concentration of another compound in honey that is the

actual inhibitor, such as how 4-HPLA shows a strong positive correlation to 3-PLA in mānuka honey (Figure 5.21, R^2 = 84.3).

While a moderate relationship is seen with DHA, only a weak relationship is observed with MGO (R^2 = 0.160) although it has a negative trend, with no samples occurring of high MGO and high diastase activity (Figure 5.20b). If MGO is an inhibitor of the diastase enzyme, the weak correlation observed in this dataset could be due to variation in the initial diastase activity of fresh honey or the change in MGO over time both of which could cause the larger scatter of results observed.

As with the DBSH and DBT samples of this research, there was no correlation observed between HMF and diastase. These samples had also been analysed for 2'-MAP (unable to be tested using the method in this research), however little correlation was found between the concentration of 2'-MAP and diastase activity in the commercial honey samples. Results from DB samples analysed for this research were compared to the commercial samples; these roughly fitted the trend that described the commercial results with only minimal outliers.

Table 5.8. Co-efficients of determination for DN vs. other parameters for the commercial dataset, with fresh (HMF<10 mg/kg) subsets. R^2 > 0.25 are bolded.

	Co-efficient of Determination (R^2) and Relationship trend (+/−)	
Parameter	All	Fresh
DHA	**0.276** (−)	**0.286** (−)
MGO	0.160 (−)	0.110 (−)
HMF	0.002 (−)	0.030 (+)
Moisture	0.007 (−)	0.044 (−)
4-HPLA	**0.563** (−)	**0.578** (−)
2-MBA	0.237 (−)	**0.299** (−)
2-MAP	0.216 (−)	0.183 (−)
3-PLA	**0.612** (−)	**0.602** (−)
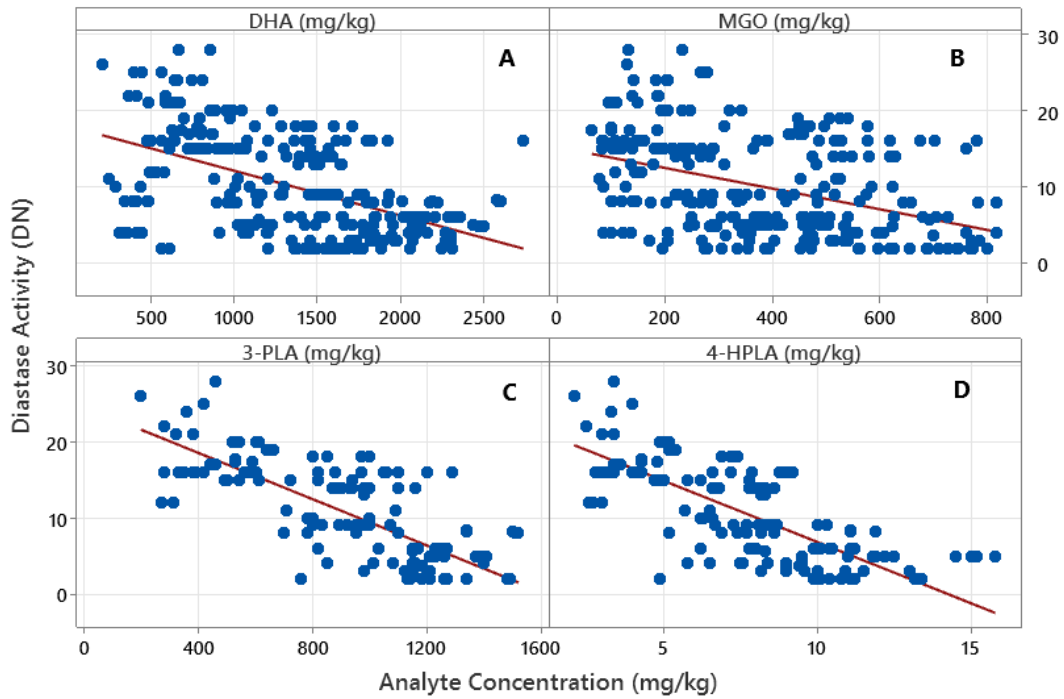
Figure 5.20. Diastase activity plotted against DHA (A), MGO (B), 3-PLA (C) and 4-HPLA (D) for the commercial dataset demonstrating negative relationships.
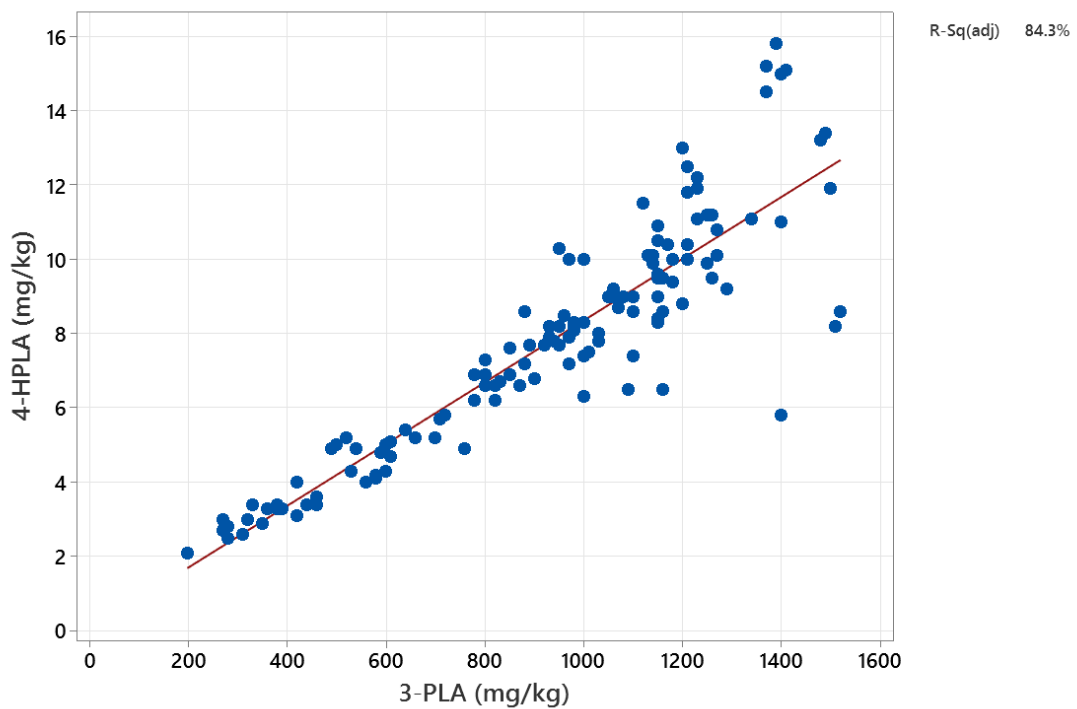
Figure 5.21. Correlation of 3-PLA and 4-HPLA for the commercial dataset samples.

5.5 Summary of the honey database experiments

From the various experiments carried out (DB, DBSH, DBT), and the commercial data, several observations can be made:

- Fresh honey within a floral type shows variability in the tested compounds parameters
- Diastase varies in fresh honey, either due to inhibiting compounds, naturally low diastase activity, or both
    - Only some mānuka honeys have low diastase activity when fresh
- Differences are observed in changes of parameters (e.g., diastase, HMF) over time at the same temperature between honeys, even within floral types. This indicates that individual honey physico-chemical properties and constituents influence these parameters differently.
- Increasing time and temperature are major influences in the rate of change for DHA, MGO, HMF and diastase activity
- Diastase activity in honey may be inhibited by MGO, DHA, 3-PLA and 4-HPLA. However, results are not conclusive and therefore further investigation is required as relationships may only be correlation not causation.

6 Clover Spiked Results and Discussion

Experiments were carried out to investigate the effect of individual compounds on diastase activity. These spiked experiments were carried out in a clover matrix, investigating eight different compounds compared to the control sample. Duplicates of each perturbant and control were stored at three temperatures (20, 27, 34 °C) and periodically analysed over time (up to 185 days). The supplied clover honey for this experiment only had an initial diastase activity of DN 11.6. Although this is sufficient, the honey chosen should have had a higher initial diastase activity to give a greater buffer zone before reaching DN<8 (at which point a different equation is used to calculate results), potentially allowing for any differences to be observed more clearly.

To retain a manageable scope on this project, the control sample was compared to eight perturbants: MGO and DHA, the mānuka chemical markers (2'-MAP, 2-MBA, 3-PLA, 4-HPLA), MSY and tannic acid (TA). MGO and DHA change over time and are also known to be reactive. The marker compounds are known to be stable over time, which seems contrary to testing for inhibitory properties against diastase activity. However, the possible mechanism of inhibition is not known and it may be that these compounds reversibly inhibit the enzyme, making quantification of the inhibitor compound possible so there would be no decrease in the analyte, but observation of reduced diastase activity. Unfortunately, due to the schedule of this thesis, the examination of mānuka honey for compounds that were unique or correlated to reduced diastase in mānuka was not carried out as originally planned and is something to consider for future work.

6.1 Initial reactions of perturbants

The first analysis of samples was carried out at T=0, aliquots were taken on the day the samples were spiked and subsequently stored in the freezer until analysis. As the samples had yet to be subject to storage conditions, not all six samples were analysed; only duplicate or triplicate analysis was taken for each perturbant set to determine the average starting concentration and any initial reactions for each perturbant. A comparison of theoretical spiked concentration and measured concentration is shown in Table 6.1. For the four analysed phenolic compounds, it is important to remember that their recovery in the method validation was below optimal. Except for MSY, the

measured concentration for the other three analysed phenolic compounds (not including 2'-MAP) met the added concentration once adjusted by their validation recoveries (Section 3.3.2.3). Measured MSY concentration was higher than the amount added even prior to recovery adjustment, although the reason for this is unknown. MGO had the most dramatic change as the MGO concentration decreased immediately by 1440 mg/kg (20.1 mmol/kg) after addition. MGO is extremely reactive, binding to proteins, amino acids and phenolic compounds and it is likely these reactions took place as soon as the compounds interacted.[82, 274-275] Rückriemen *et al*. (2017)[276] attributed a loss of MGO inhibitory action when MGO was added to a non-mānuka honey to trapping reactions with phenolic compounds.

Table 6.1. Variation in theoretical spiked vs. measured concentration of spiked analytes in the initial analysis. The measured concentration had been adjusted by subtracting the low endogenous concentrations of analytes in NZ324.

Compound	Theoretical Spiked concentration (mg/kg)	Measured concentration (mg/kg)
MGO	2270 ± 26	820 ± 13
DHA	980 ± 3	990 ± 10
2'-MAP	32 ± 0.3	NA*
2-MBA	30 ± 0.0	24 ± 0.0
3-PLA	1010 ± 4	720 ± 4
4-HPLA	30 ± 0.1	22 ± 1.2
MSY	50 ± 0.5	54 ± 1.6
TA	30 ± 0.2	NA*

*2'MAP and TA were unable to be analysed by the analytical method

6.2 Storage of clover honey perturbed with bioactive compounds

The three storage temperatures were chosen to emulate room temperature storage (20 °C) and two slightly elevated temperatures (27, 34 °C). The elevated temperatures were chosen for two reasons. Firstly, during processing, honey is generally heated to about 40 °C for up to a week to reduce viscosity for pumping and straining (the temperature and heating period vary between processing companies);[19] secondly, mānuka honey may be stored by some companies at slightly elevated temperatures (for an extended period of time) to quicken the conversion of DHA to MGO for faster retail and revenue. As this research only has a short time span (185 days), the higher temperatures will hasten any reactions and indicate what changes in diastase activity, or analyte concentration, might occur in honey over the longer term.

As with the database experiments (Chapter 5), data is this chapter has been normalised by taking the average of the replicates from T = 0 (treatment had yet to begin) and subtracting it from subsequent timepoint results for ease of comparisons between treatments.

6.2.1   Influence of compounds on HMF concentration

The HMF content of the clover honey used for these experiments (NZ324) was extremely high (116 mg/kg), well above the regulated limit of 40 mg/kg. While it would have been ideal to have a honey with low or no initial HMF content to simultaneously observe how HMF changes over time in conjunction with diastase activity, it was not a requirement. Even though the HMF concentration was high, diastase activity was still above DN 8.

As the processing history of this honey in unknown, the cause of the excessively high HMF concentration cannot be determined. However, factors such as low pH, adulteration with high fructose syrup, and storage in metallic containers promote HMF formation, any of which in conjunction with extended storage time and elevated temperature could be the cause of the elevated HMF concentration in NZ324.[94, 200-201]

HMF concentration in honey is expected to increase over time due to its formation in the Maillard reaction;[201, 262, 264, 270, 277] only one article in the literature has described HMF significantly decreasing over time, but this was in samples perturbed with excess HMF.[265] The HMF of the perturbed clover samples during the storage trials showed a variety of responses (both increasing and decreasing) depending on the perturbant and the temperature, contrary to expectations. At 20 °C (Figure 6.1a), HMF concentration in most of the samples decreased by approximately 60 mg/kg over 185 days, with the rate of decrease plateauing over time. However, HMF in the DHA perturbed samples at the same temperature only decreased by 42 mg/kg HMF. The HMF in the MGO perturbed samples reacted differently with little change from the initial concentration over time at 20 °C. For most samples at 27 °C (Figure 6.1b) HMF decreased by a similar amount as at 20 °C after 185 days (approximately 60 mg/kg decrease in HMF), although the HMF decrease over the initial 97 days (51 mg/kg) occurred more rapidly than at 20 °C (44 mg/kg) then plateaued. The DHA perturbed samples had the same HMF trend at both 20 and 27 °C, while in the MGO perturbed samples, HMF increased by approximately 8 mg/kg at 34 days and plateaued the remainder of the timepoints.

At 34 °C, HMF behaved differently than at the lower temperatures (Figure 6.1c). Except for the DHA, MGO and 3-PLA perturbed samples, HMF concentration in the other samples decreased by approximately 30 mg/kg at 97 days before increasing again by approximately 15 mg/kg (approx. 95 mg/kg total at 185 days). DHA and 3-PLA perturbed samples also had a decrease in HMF (approx. 15 mg/kg at 64 days), before HMF concentration increased past the initial value to 138 and 128 mg/kg respectively. HMF concentration substantially increased in the MGO perturbed sample by 69 mg/kg (180 mg/kg total) over 185 days.

Similar HMF behaviour (to the non-MGO samples) was observed by Fallico *et al*. (2008)[265] in three floral honey types fortified with varying levels of HMF (120-250 mg/kg). At lower temperatures, HMF decreased in each floral type, while at higher temperatures (for the multifloral honey only) it initially decreased before increasing.[265] The decrease of HMF could be related to the induction period observed by Zhang *et al*. (2012)[189] and Grainger (2014),[192] where a build-up of intermediaries may be required to achieve a steady-state reaction. It is uncertain how the HMF concentration got so high originally.

The cause of different behaviour of HMF in the MGO, DHA and 3-PLA perturbed samples is unknown. However, MGO is a very reactive compound and could be catalysing formation or inhibiting degradation of HMF by an unknown mechanism (similar effects would be observed in the DHA samples since MGO is formed over time). 3-PLA may be acting as a proton donor for the formation of HMF.[192]

Temperature has a significant effect on HMF formation, as seen at 34 °C, however it has less of an effect at lower temperatures, which is also observed in literature. Fallico *et al*. (2004)[201] found that temperature was the primary driver at higher temperatures (>50 °C), with all honey samples forming HMF. At lower temperatures, factors such as pH and free acidity have a larger role; in some floral types, HMF was not formed even after 96 hours at 50 °C.[201]
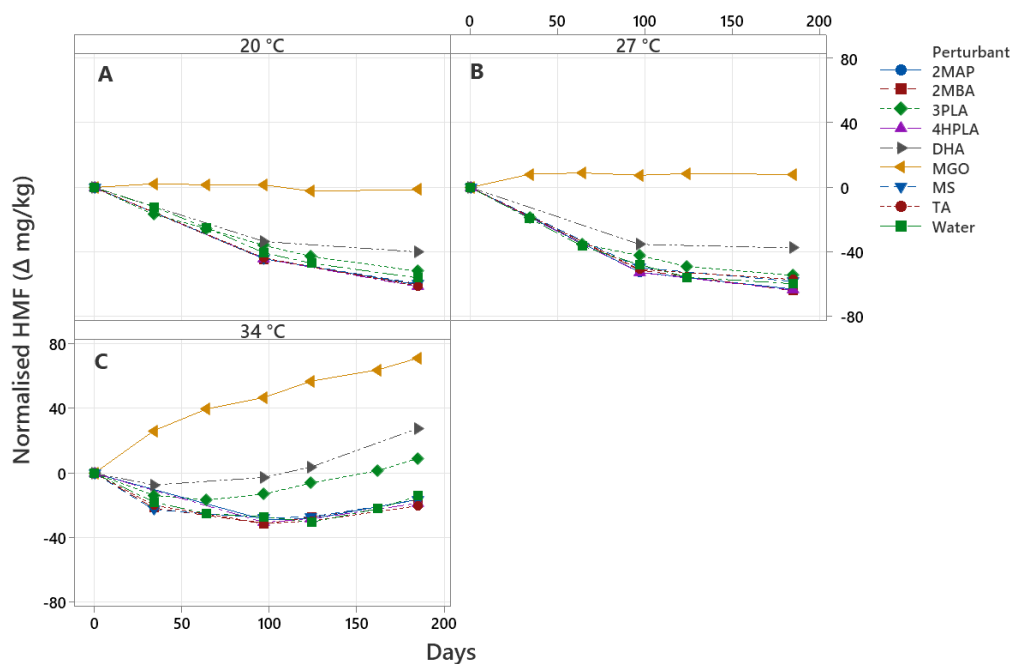
Figure 6.1. Change in HMF with time, normalised to T=0, at different temperatures (a: 20 °C, b: 27 °C, c: 34 °C) for each of the perturbed samples.

6.2.2   Analysis of naturally occurring phenolics in clover matrix

The perturbant was analysed and quantified at time intervals corresponding to diastase testing during the experiment. Not all timepoints were analysed for all samples due to prioritisation of samples which showed greater effect on diastase activity, fewer timepoints were selected for samples which did not appear to have any effect over time.

Analysis of compounds naturally found in the clover honey (e.g., 2-MBA and 4-HPLA in the MSY perturbed sample) showed little to no change in analyte concentration for most of the phenolic compounds (excluding 3-PLA) at all temperatures. Non-perturbed (naturally occurring) 3-PLA increased by approximately 6 mg/kg in all samples over 185 days, regardless of temperature, possibly due to method variation. These results align with the expected stability of these compounds over time.[88] MGO concentration also remained below the LOD over this period in all samples except those perturbed by DHA and MGO. Due to the control and MGO perturbed samples initially starting with 40 mg/kg DHA, there were larger decreases in DHA concentration (15-30 mg/kg decrease in DHA; temperature dependant) for these samples compared to the other non-DHA perturbed samples (7-10 mg/kg decrease in DHA). For the most part, the perturbants did not influence the concentration of other analytes analysed (excluding HMF).

6.2.3 Effect of perturbant on diastase activity

As previously discussed, 2'-MAP and TA could not be quantified using the SULLE method so only diastase activity and 3in1 were analysed for samples perturbed with 2'-MAP and TA. For all samples, the perturbants and diastase activity decreased over time, however to varying extents. The change in perturbant over time at each experimental temperature is shown in Table 6.2, expressed as mmol/kg, and will be briefly discussed in this section alongside the diastase activity results. The molality is used here so direct comparisons can be made between the perturbants. The observed decrease in concentration for 2-MBA, 4-HPLA and MSY is within the method error of the test, hence there is no change in concentration for these perturbants.

Table 6.2. Change in perturbant concentration (mmol/kg) after 185 days of storage at 20, 27 and 34 °C.

Perturbant	Storage Temperature	Change in perturbant after 185 days storage (Δ mmol/kg)		
		20 °C	27 °C	34 °C
DHA		-1.71	-3.24	-5.83
MGO		-0.75	-2.12	-5.98
2-MBA		-0.032	-0.037	-0.041
4-HPLA		-0.020	-0.027	-0.043
3-PLA		-0.83	-0.87	-0.91
MSY		-0.022	-0.035	-0.019

Statistical analysis (Students t-test) was used to determine whether diastase activity in perturbed samples was significantly different than that of the control. This was carried out on each individual timepoint. For the individual timepoints, carrying out a t-test assumes that the data is normally distributed, so using $n = 2$ is not entirely reliable, however it can help confirm visual assumptions. The data from this test was backed up by comparing the least squares regression (LSR) for diastase activity versus days of perturbed samples to the control. LSR was only fitted to datapoints from 0 to 97 days because after 97 days the diastase activity started to plateau (observed for MGO and 3-PLA samples at 27 °C and all samples at 34 °C) and the reaction rates declined, it also gave more indication of what occurs initially in fresh honey. When all datapoints were included for LSR, no significant difference in slope was found for any perturbant. Absorbance units (data obtained before conversion to diastase activity - DN) were used for the t-test calculations instead of DN due to the resultant jump in value that occurred at DN<8 (due to the change in equation used), as this would influence the regression.

Figure 6.2 demonstrates how the diastase activity calculation change causes data to be more spread due to separation of values above and below the DN = 8 threshold.
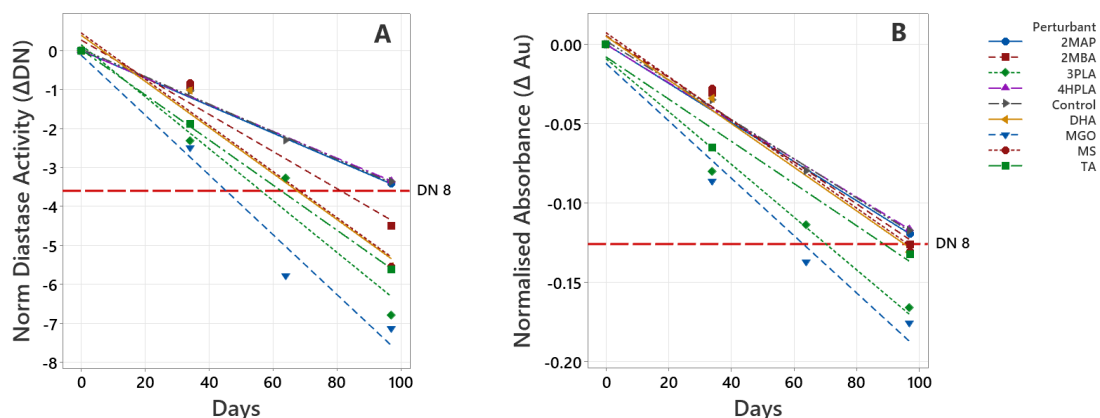
Figure 6.2. Comparison of normalised data for A) DN and B) absorbance versus time plots for the 34 °C samples with the DN = 8 threshold labelled.

6.2.3.1 Analytes with no effect on diastase activity – DHA, 2'-MAP, 2-MBA, 4-HPLA, MSY and tannic acid

Results for the regression slope t-test are shown in Table 6.3, while statistically significant individual data points have been circled on the diastase absorbance versus day plots (Figure 6.3a-c). Comparing individual timepoints, most samples did not have diastase activity loss that was significantly different than the control samples at any of the three temperatures. The exceptions were MGO and 3-PLA (discussed in Section 6.2.3.2), a single timepoint at 34 °C for tannic acid (34 days) and DHA (185 days), and the single data point at 27 °C, day 185 for all samples (Figure 6.3b). The 27 °C, day 185 occurrence is due to the drawback of $n = 2$ as there was no deviation between the duplicates of the control sample. As the t-test works off normal distribution, a standard deviation of 0 reduces the calculated distribution range for the control making it more unlikely that there will be overlap with the other sample distributions. As it is only a singular point, there is not sufficient evidence to claim that all these compounds inhibit diastase activity. The samples that do not have sufficient evidence for diastase inhibition are DHA, 2'-MAP, 2-MBA, 4-HPLA, MSY and tannic acid; further commentary on each compound is provided below.

Table 6.3. T-test results (*p*-values) comparing the diastase absorbance (Au) versus time (days) regression slopes of the perturbed samples to the control samples. Regression was carried out between 0 and 97 days. Statistically significant results are in bold (*p* < 0.05).

Perturbant	Regression slope *p*-values		
	20 °C	27 °C	34 °C
DHA	0.42	0.77	0.34
MGO	0.75	**0.05**	**0.01**
2'-MAP	0.55	0.98	0.97
2-MBA	0.43	0.62	0.57
3-PLA	0.54	0.07	**0.02**
4-HPLA	0.55	0.76	0.76
MSY	0.30	0.86	0.43
TA	0.24	0.41	0.60

4-HPLA

The commercial database samples (Section 5.4) indicate that higher concentration of 4-HPLA (up to 15 mg/kg) is related to lowered diastase activity in mānuka honey. A higher concentration of 4-HPLA (30 mg/kg, 0.16 mmol/kg) was added to the experimental samples. However, there is no evidence that 4-HPLA affected DN. Either the mānuka honey matrix contains other constituents that 4-HPLA interacts with to inhibit the diastase enzyme, or 4-HPLA does not affect diastase but rather is strongly correlated to another compound that does (which is absent in the clover matrix), such as 3-PLA (Section 5.4).

Tannic acid

Tannic acid is an alpha-amylase inhibitor for porcine pancreatic amylase (PPA).[3, 253, 255] However, no effect was observed in the honey amylase due to several possible reasons. Although tannins have been found in honey,[118] there is no literature specifically identifying tannic acid in honey (let alone mānuka[*]), hence an estimate was made for the concentration to add. This was chosen as 30 mg/kg to align with the other minor phenolic compounds (MSY, 2-MBA, 2'-MAP), however molecular weight was neglected in this calculation. Tannic acid is a big molecule (1701 g/mol) and the added amount equated to 0.018 mmol/kg while other compounds were at least 0.16 mmol/kg. Tannic acid has an IC_{50} of approximately 0.14 mmol/L against PPA,[3, 253, 255] and although this cannot be directly compared due to the difference of density and viscosity, the amount added in this experiment is likely not enough to observe any inhibition that may occur.

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[*] Tannic acid was added as it was of interest to the project sponsor

Tannic acid can crosslink with proteins and inhibit enzyme activity; honey contains various proteins and other constituents that could interact with tannic acid before it interacts and inhibits diastase, with viscosity potentially also preventing interactions. The low concentration of tannic acid added to the honey matrix may be altogether irrelevant as tannic acid has not been reported in honey. Tannins have been found in propolis (international)[278] and small quantities of propolis are found in honey, therefore it is plausible that low concentrations of tannic acid may be in mānuka honey.

DHA

DHA is very reactive with amino acids (lysine, glycine and histidine), as such it is used in sunless tanning products, binding to amino acids to cause colour changes in proteins.[279] However, it does not appear to inhibit diastase in this research. It has been well documented by Grainger *et al.* (2016)[28, 280] that in honey, DHA is used up in a myriad of reactions, including degradation into MGO. The added concentration (10.9 mmol/kg) should be sufficient to see any effects DHA might have on diastase, however no effects were observed through monitoring diastase activity. It is possible that DHA binds to alpha-amylase, but not to any sites that impairs diastase activity. Additionally, DHA is a small molecule (MW 90 g/mol) that steric inhibition would be less likely.

2-MBA, 2'-MAP and MSY

There is no literature that explores alpha-amylase inhibition by 2-MBA, 2'-MAP and MSY. Based off the results in this research, these three compounds are unlikely to be the cause of low diastase activity in fresh mānuka honey.

Other considerations

The viscosity of honey must also be considered, as it may prevent interactions between diastase and the perturbants from readily occurring in the honey. Phenolic compounds in honey originate from nectar, therefore they are present during the entire conversion / dehydration process of nectar to honey. The primary source of alpha-amylase is bee hypopharyngeal gland secretion mixed into the nectar. The diastase would immediately be exposed to potential inhibitors and any interactions between the compounds and diastase would not be hindered by honey viscosity, thus reducing diastase activity before maturation, at which time, increased viscosity may protect the diastase from further significant inhibition. Honey viscosity would be more likely to impair inhibitors of low concentrations in honey. Additionally, the higher water

content in nectar will also readily provide H$^+$ compared to honey, which have been shown to help facilitate reactions.[28] This suggests that post maturation addition may not accurately identify all diastase inhibitors that act on diastase.
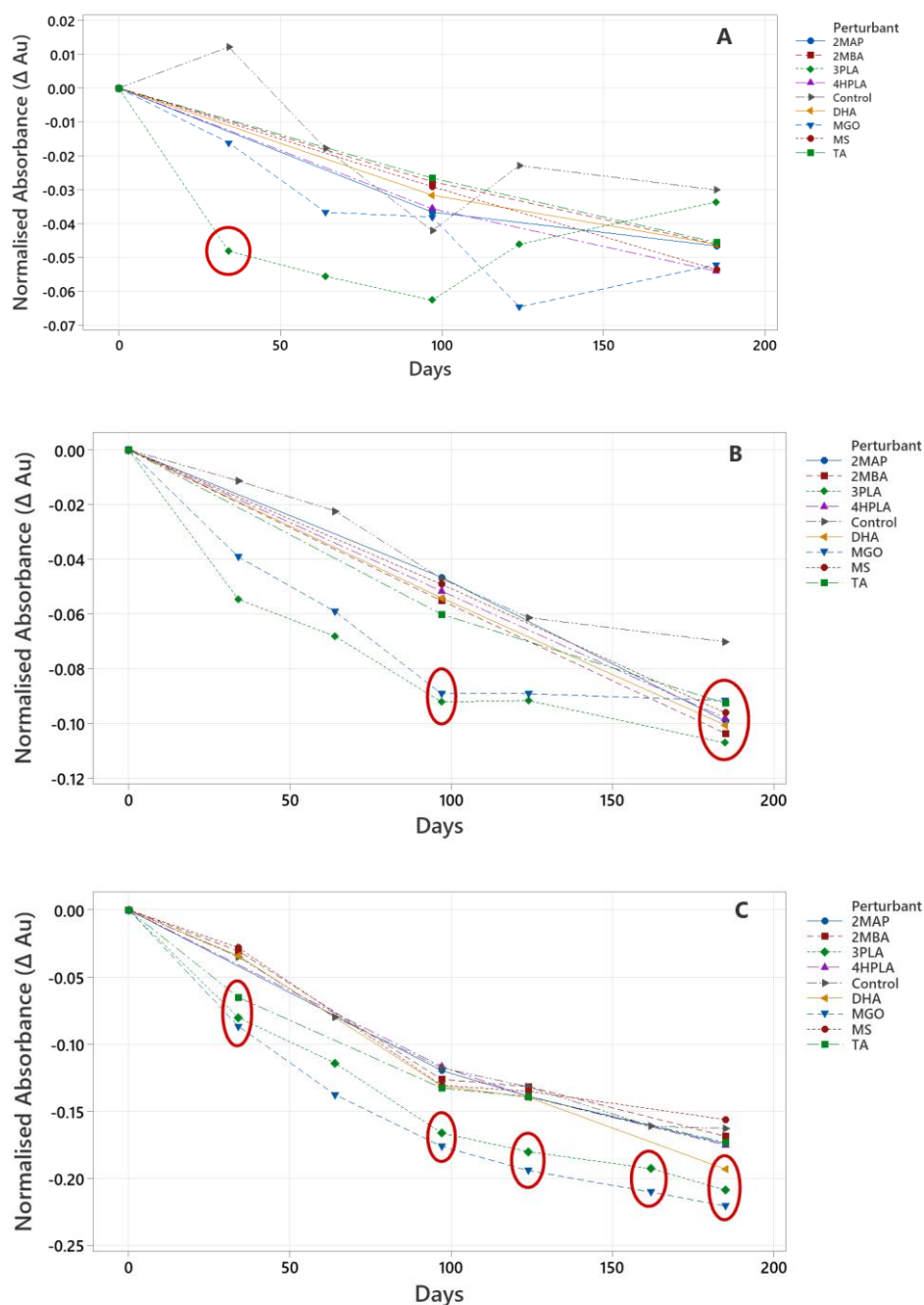
Figure 6.3. Normalised absorbance (as a function of diastase activity) versus storage time for the perturbed samples at; a) 20 °C, b) 27 °C and c) 34 °C. Results with statistically significant difference to the control sample at each timepoint are circled (red). As the t-test was carried out comparing duplicates, all samples are statistically significantly different from the control at 27 °C, 185 days due to zero deviation between the control sample duplicates at this timepoint - this single timepoint across the experiment is not sufficient evidence to suggest diastase inhibition.

6.2.3.2   Analytes with effect on diastase activity – MGO and 3-PLA

Both MGO and 3-PLA perturbed samples show significantly different diastase activity than the control samples for both the 27 and 34 °C datasets, suggesting that MGO and 3-PLA inhibit diastase activity by some unknown mechanism.

The concentration of MGO and 3PLA both decreased over time, with larger decreases at higher temperatures. MGO showed a relatively linear decrease at all three temperatures (Figure 6.4), which is expected as there was no excess of DHA to feed into MGO formation and MGO is very reactive.[280] MGO decreased by 55, 150 and 430 mg/kg (0.75, 2.1 and 6.0 mmol/kg) over 185 days at 20, 27 and 34 °C respectively. Initially, 2270 mg/kg (31.5 mmol/kg) of MGO was spiked into the honey, however 1450 mg/kg (20.1 mmol/kg) was consumed before the first analysis, leaving 820 mg/kg (11.4 mmol/kg) unreacted. This was more than sufficient to observe any inhibitory actions by MGO. There is no initial quick loss of DN that corresponds to this loss, so MGO is either binding to other compounds or reacting further. Over time, there was a positive relationship between diastase activity and MGO as both decreased. Although MGO decrease appears linear, diastase activity decrease is not and therefore the relationship is non-linear (exponential – not shown).
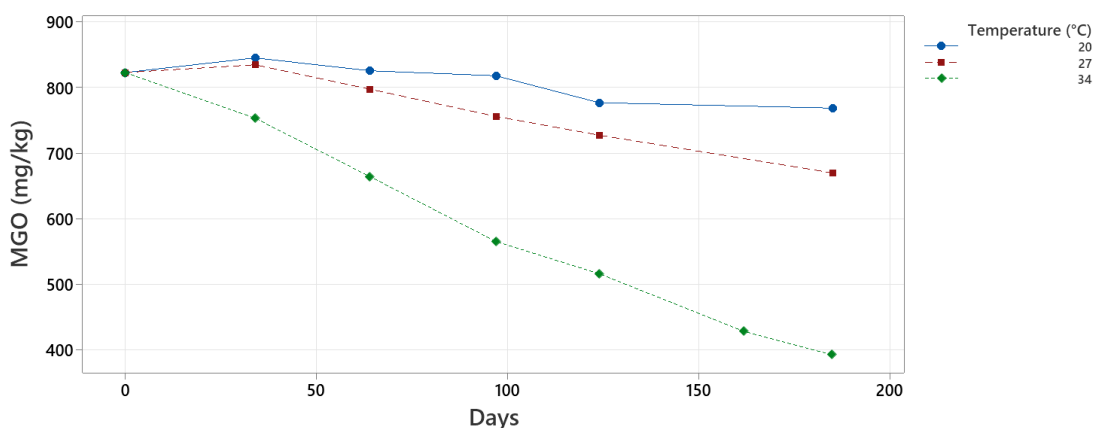
Figure 6.4. MGO concentration versus time for the MGO perturbed sample at 20 °C (blue circle), 27 °C (red square) and 34 °C (green diamond).

3-PLA did not follow the same trend as MGO. After measurement at timepoint zero (724 mg/kg, 4.36 mmol/kg) there was a rapid decrease in concentration over the first 34 days for all three temperatures (concentration lost: 0.53 mmol/kg (20 °C); 0.67 mmol/kg (27 °C); 0.87 mmol/kg (34 °C)) before the concentration stabilised over the remaining storage period (Figure 6.5). However, no correlation was observed between diastase

activity and 3-PLA concentration. 3-PLA is stable over time in honey,[87] hence this initial decrease in concentration is likely to be reaction with some other constituent in the clover matrix due to 3-PLA being introduced into the matrix rather than degradation of the compound. In comparison, in mānuka honey, 3-PLA is present in the nectar and these initial reactions would have already occurred before the honey is analysed. The reaction/binding of 3-PLA appears to rapidly deplete the other compounds/s because 3-PLA concentration stabilises after the first timepoint (the sample at 34 °C, day 97 appears to be an outlier).
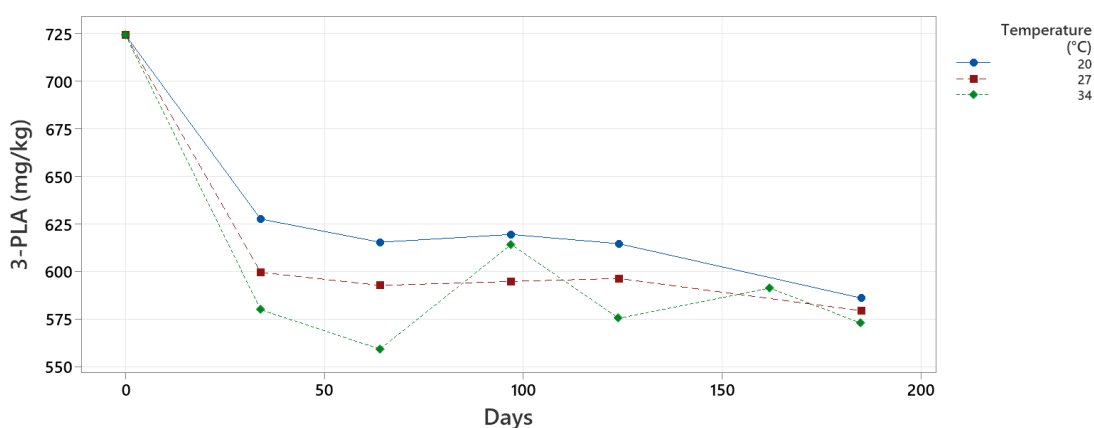

Figure 6.5. 3-PLA concentration versus time for the 3-PLA perturbed sample at 20 °C (blue circle), 27 °C (red square) and 34 °C (green diamond).

At 20 °C, the diastase activity in the MGO perturbed samples were not significantly different than the control samples over the course of the experiment, with an average (*n* = 2) decrease of DN 1.5. For 3-PLA, a similar loss in DN was observed (DN 1.8) after 97 days before a slight increase at 185 days (DN 1.0), which is within the limits of the method variation (DN ± 0.6). Most of the individual timepoints for the MGO and 3-PLA perturbed samples at 27 °C were not significantly different from the control (*n* = 2), although there was a visual difference (Figure 6.3b). At lower temperatures, reactions occur slower and therefore a significant difference from the control may not be observed until the reaction has progressed further. it may take longer to observe a noticeable difference Comparison of the LSR slope over the initial 97 days (27 °C) showed that diastase activity for samples perturbed with MGO was significantly different (*p* = 0.05) while the 3-PLA diastase activity was not (*p* = 0.07), with a 95% confidence interval. For both MGO and 3-PLA at 34 °C, diastase activity for all timepoints (except at 64 days) was significantly different than the control sample; additionally,

there were significant differences between the slopes (p = 0.01 and 0.02 respectively). The profiles of diastase activity loss at 27 °C and 34 °C were similar for both the MGO and 3-PLA perturbed samples but did not describe the same significant difference due to the lower temperature and thus slower reactions. From these results, it could be expected that the DHA perturbed sample would show inhibition properties due to the increase in MGO overtime, however this was not observed. This is likely due to the low gain of MGO in the DHA perturbed samples over the sampling time (70, 100 and 140 mg/kg at 20, 27 and 34 °C respectively) which does not appear to be high enough to inhibit diastase activity over 185 days.

The greater effect of MGO and 3-PLA on diastase activity observed at higher temperatures is significant for honey processing. Mānuka honey is thixotropic which will also affect the rate of diastase inhibition as the honey can solidify at room temperature, preventing molecular interactions. This property also causes processing difficulties at low temperatures (<30 °C), requiring temperatures greater than 30 °C to decrease viscosity.[27] The decreased viscosity will facilitate more interactions between honey components, while the higher temperatures also provide more energy to molecules making it easier to overcome the activation energy. During processing, inhibition rates of alpha-amylase will increase, decreasing diastase activity faster than non-inhibited samples.

MGO is an extremely reactive compound, well known for causing non-specific damage to cells through the formation of advanced glycation end products (AGEs).[86, 281-282] MGO readily reacts with the most susceptible amino acid residues on proteins (arginine - Arg, lysine - Lys and cysteine - Cys) causing irreversible damage through protein conformation alteration or enzyme deactivation by crosslinking proteins, forming AGEs.[86, 204, 276, 281, 283-284] The *A. mellifera* alpha-amylase sequence is made up of 5.25%, 5.65% and 2.10% of Lys, Arg and Cys respectively. Arg195, Lys300 and Arg337 make up the chloride binding site, so it is possible that MGO is binding here, preventing Cl$^-$ binding and decreasing the rate of activity. Alternatively, MGO could be binding to the enzyme via non-catalytic or auxiliary sites, crosslinking it to other compounds or proteins. Lo *et al.* (2011)[274] showed that MGO is also reactive to certain phenolic compounds, with similar results observed by Shao *et al.* (2008).[275] Hypothetically, MGO could crosslink phenolic acids to diastase (a synergistic effect), causing inhibition.

There have been several investigations on the reactivity of MGO in mānuka honey towards proteins.[204, 276, 283] Rückriemen *et al*. (2017)[276] found MGO to be the primary inhibitor of jack bean urease when carrying out an inhibition assay using mānuka honey solution as the treatment. The authors found that a 16.4% (w/v) concentration of mānuka honey (595 mg/kg MGO, 1550 mg/kg DHA) resulted in approximately 90% urease inhibition, while at 0.4 and 5.5% (w/v), urease inhibition was 20 and 45% respectively, suggesting that the MGO could be binding to Cys592 at the active site, or to other Lys or Arg residues. As this is diluted honey, reactions will occur more readily (less viscous, more H^+). The authors repeated the experiment with non-mānuka honey (adding an equivalent concentration of MGO) and did not observe the same level of urease inhibition (approximately 18% inhibition compared to approximately 56% inhibition in mānuka). They attributed this to reactions of MGO with other available compounds in the honey matrix, thus exhausting MGO; in comparison, MGO was at equilibrium in the mānuka matrix, with greater excess free to inhibit urease.[276] MGO has also shown inhibitory activity against catalase, peroxidase and superoxide dismutase.[285] And while MGO was found to carbonylate pancreatin and pepsin, it did not reduce the activity of the enzymes.[284]

Analysis of the hydrolysed high molecular weight protein fraction in both mānuka and non-mānuka honey samples showed higher concentrations of two Maillard reaction products formed from MGO reacting with lysine and arginine in mānuka honey.[283] Another experiment carried out to observe the interaction of MGO with amino acids found that addition of proline, lysine and arginine to a honey matrix reduced MGO concentration at a greater rate than the control sample.[204] Rückriemen *et al*. (2017)[286] found mānuka honey contained a higher concentration of high molecular weight protein fraction than non-mānuka honeys, concluding that MGO covalent crosslinking between proteins was the cause.

Glucose oxidase, which produces hydrogen peroxide in most floral types, is not active in mānuka honey containing high MGO.[287] Matjan *et al*. (2014)[80] found addition of MGO to non-mānuka honey decreased its hydrogen peroxide activity, while incubation of MGO with glucose oxidase induced formation of AGEs through enzyme crosslinking. Similar results of MGO protein modification were found with the major royal jelly protein 1 (MRJP1) and the bee defensin 1 peptide in honey.[82] MGO has shown a broad

enzyme inhibition range, it is entirely likely that the mechanisms of MGO observed in literature also affect diastase activity in honey.

3-PLA is a common phenolic compound found in honey of different botanical origins.[125, 288] While prominent in mānuka honey (82.2-1760 mg/kg, this research), it is also found in high concentrations in thistle (122-760 mg/kg),[125] heather (378-1060 mg/kg),[288] and kānuka (905-1640 mg/kg, this research). The heather honey origin was not given by the authors.[288] No information could be found on the diastase activity of thistle honey, however heather (both *Erica* or *Calluna*) honey diastase activity ranges from DN 10.1 to 57.3, with most values greater than DN 15.[48, 289-291] This implies that 3-PLA does not affect diastase activity in heather honey, however as none of these studies also investigate phenolic compounds, it is not definitive as the 3-PLA concentration in these honeys is unknown. Also, the specific floral origin of the heather samples with diastase activity (unknown) may be different than the samples with 3-PLA information (*Erica* and *Calluna*). Other factors will also play a role in diastase activity, such as nectar moisture concentration (higher moisture will require more manipulation by bees, theoretically adding more diastase to the honey) or honey matrix effects. The diastase activity of the three kānuka samples in this research were low (DN 8.2-13.1), which could indicate that their high 3-PLA concentrations (905-1640 mg/kg) may be having an effect on the diastase activity.

Unlike MGO, the mechanism by which 3-PLA might inhibit diastase activity is unclear. Comparison of the structure of 3-PLA to the phenolic compounds which inhibit PPA (Chapter 4)indicates that the inhibitory action of 3-PLA is different than the other phenolic acids. Greater inhibitory action against PPA by phenolic acids is known to be attributed to more hydroxyl groups on the phenol ring, as well as free chain restriction of the acid group.[2, 4, 252, 254-255] However, 3-PLA does not have either of these properties; it is 2-hydroxypropanoic acid with a phenyl group substituted at carbon 3. The reactivity of 3-PLA with diastase will be through hydrogen bonding of the acid and alcohol groups to amino acid residues, potentially in the active site, however further investigation is required to deduce the mechanism. The phenyl group would also be able to interact with amino acids through hydrophobic interactions, and also π-π stacking with tyrosine, tryptophan or phenylalanine residues.[292]

Although there is no literature on 3-PLA inhibition of enzymes, it is a broad spectrum inhibitory compound towards bacteria, yeast and moulds.[293-297] Sorrentino *et al.* (2018)[297] investigated the inhibitory effect of 3-PLA and three other phenolic acids (gallic, caffeic, and ferulic acids) against *Listeria innocua*, finding that the strongest inhibition occurred with 3-PLA or gallic acid. It is possible that 3-PLA is interacting with proteins within these organisms and causing inhibitory action. The mechanism of action of 3-PLA against gram negative bacterium *Pseudomonas aeruginosa* was investigated by Chatterjee *et al*. (2017)[292] to determine how the molecule inhibited the formation of biofilm. It was determined that 3-PLA could bind to three bacterium receptors (proteins) at the active sites through possible hydrogen bonding with serine, tryptophan, tyrosine or cysteine residues and π-π stacking interaction with phenylalanine.[292] It indicates that similar interactions could occur between 3-PLA and *A. mellifera* alpha-amylase, however this would have to be confirmed.

The main issue to be addressed by this research is investigating the cause of low diastase activity in *fresh* mānuka honey. In fresh honey, MGO concentration is very low and while MGO inhibits diastase, it may not be the cause of low diastase activity in fresh honey unless it is reacting with diastase as it forms during honey ripening. Although it will contribute to a greater loss in diastase activity over time, faster than in a non-mānuka honey, especially if heating is applied to make the honey mature faster. The results from the spiking experiments indicate that 3-PLA is a contributor to low diastase activity in fresh mānuka honey.

6.2.3.3 *Diastase half-life in MGO and 3-PLA perturbed clover samples*

For the MGO and 3-PLA perturbed samples, as well as the controls, the half-life of diastase was calculated (Table 6.4). To determine the reaction order of diastase activity loss, zero-order ([Abs] vs. time), first-order (ln[Abs] vs. time) and second-order (1/[Abs] vs. time) plots were created, with absorbance (Abs) used as the unit for diastase activity[*]. The reaction order is determined by the plot with the best linear fit; an example is shown with the 3-PLA perturbed sample incubated at 34 °C (Figure 6.6). A zero-order reaction rate is constant over time and independent of reactant concentration, however the half life depends on the initial concentration as it is the time required for the

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[*] Due to the issue with diastase activity calculation discrepancy for DN < 8.

concentration to decrease by half. For a first-order reaction, the reaction rate linearly depends on the concentration of one reactant, however the half-life is constant throughout the reaction. In second-order reactions, the rate depends on the concentration of the reactants, with the half-life being inversely proportional to these concentrations. In plotting concentration vs. time, first- and second-order plots appear similar (exponential decays), however the concentration in a second-order reaction approaches zero more slowly than a first-order reaction. The loss of diastase activity was determined as a second-order reaction, as the 1/[Abs] vs. time plot was linear, with the following differential (Equation 11) and integrated (Equation 12) rate laws:

$$\frac{\Delta[Abs]}{\Delta t} = k[Abs]^2$$
<div align="right">**Equation 11**</div>

$$\frac{1}{[Abs]} = kt + \frac{1}{[Abs]_0}$$
<div align="right">**Equation 12**</div>

The second order plot (1/[Abs] vs. time) slope gave the second-order rate constant (k) for the loss of diastase activity, with the intercept equal to $1/[Abs]_0$. The half-life in second-order reactions is dependent on the initial concentration, which can make the concept less useful, however as each of the perturbed samples started with the same diastase activity (same honey), they are comparable. The half-life of diastase in the control sample is much longer than the two perturbed samples, with the diastase half-life for MGO and 3-PLA being similar (except for 3-PLA, 20 °C due to experimental variation in diastase results). White *et al*. (1964)[272] found that diastase had a half-life of 1,480, 540, 200 and 78 days at 20, 25, 30 and 35 °C respectively. While there are some similarities between those values and values obtained in this experiment at each temperature (20 °C control samples, 27 and 34 °C MGO and 3-PLA samples), it is not consistent across any sample. Part of this can be attributed to White *et al*. (1964)[272] determining the diastase degradation as first-order, as well as determination of the half-life using Equation 13. As shown in Table 6.4, diastase activity half-life is dependent on the honey constituents, and therefore another reason for the difference between literature and this research.

$$log\ t_{\frac{1}{2}} = \frac{\frac{1}{T} - 0.003000}{0.000130}$$
<div align="right">**Equation 13**</div>

These half-life values for diastase highlight a potential issue for mānuka honey producers. Mānuka honey with diastase activity less than DN 15 and high 3-PLA concentrations are potentially more at risk for falling under the threshold of DN 8 if they are stored at slightly elevated temperatures in order to speed up the DHA to MGO conversion. Processing and straining the honey can require heating the honey to approximately 40 °C for several day up to two weeks,[19, 27] which could dramatically decrease diastase activity. Further storage at the slightly elevated temperatures may drop the diastase activity below DN 8 after only a couple of months (temperature dependant).
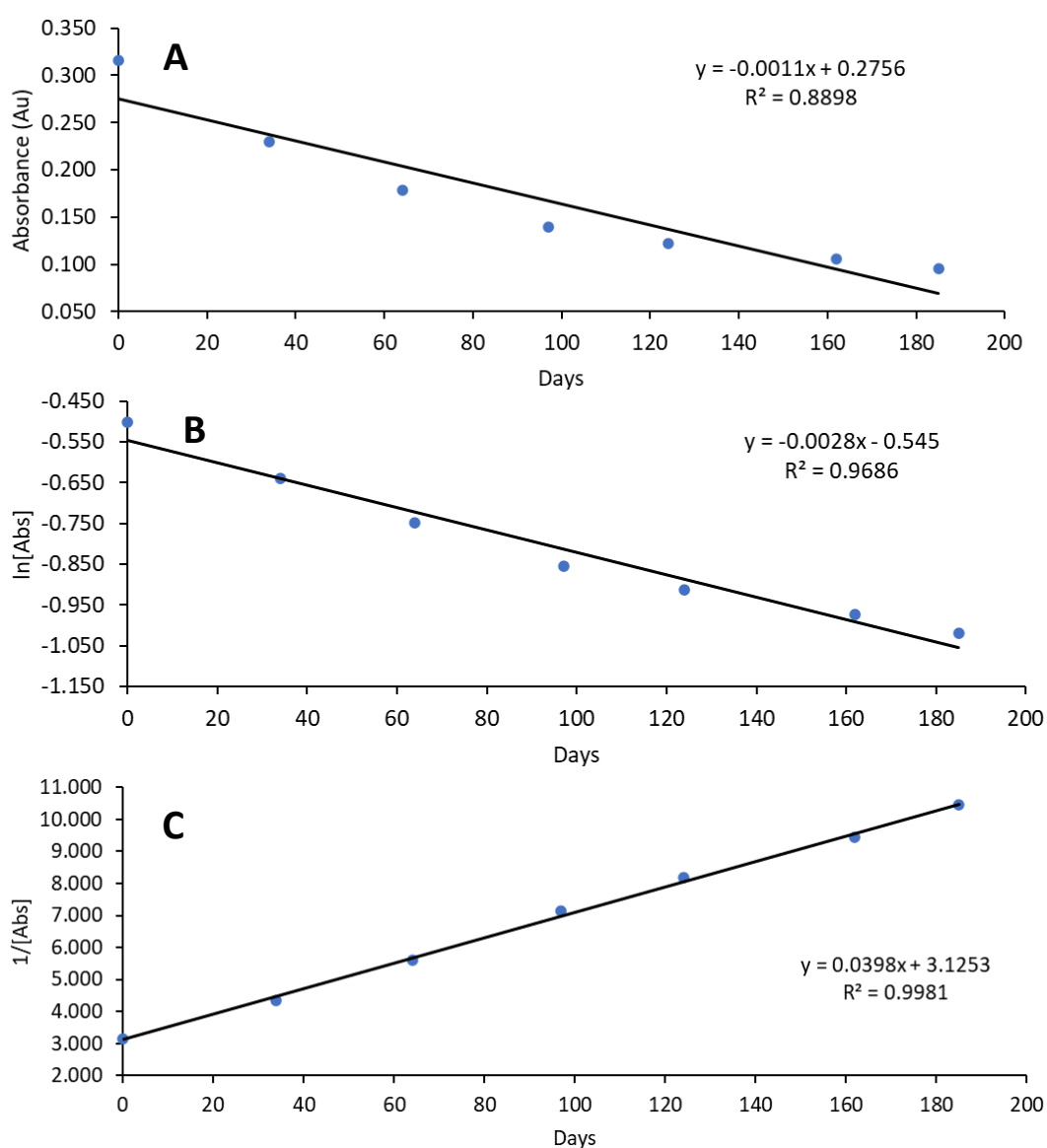

Figure 6.6. Reaction order plots for clover honey perturbed with 3-PLA and stored at 34 °C. The zero-order (A. [Abs] vs. time) and first-order (B. ln[Abs] vs. time) plots deviate from linearity compared to the linear second-order (C. 1/[Abs] vs. time) plot.

Table 6.4. Calculated half-life of diastase for the control, MGO and 3-PLA perturbed samples at each storage temperature.

	Diastase Half-life (days)		
Perturbant	20 °C	27 °C	34 °C
Control	1340	590	160
MGO	820	440	80
3-PLA	340	380	97

6.3 Comparison of diastase activity in clover and mānuka matrices

Clover honey perturbed individually with bioactive compounds provided information on which compounds (MGO and 3-PLA) cause a greater loss in diastase activity over time. However, these are just singular compounds and do not fully represent what is occurring in mānuka honey, where multiple compounds may be interacting with alpha-amylase. Comparison of three clover perturbed samples (control, MGO and 3-PLA) to three DBT honey samples (two mānuka – NZ322/333, kānuka – NZ335) with similar initial diastase activity (to partially negate the second order reaction concentration issue) showed that the diastase activity loss for NZ322 and NZ335 was significantly lower ($p < 0.05$, slope comparison) than clover control sample at 27 °C, but not the MGO and 3-PLA perturbed samples (Figure 6.7). The initial values for diastase activity (DN and absorbance), MGO and 3-PLA in each of the samples are shown in Table 6.1. The three DBT samples have lower concentrations of MGO than the MGO perturbed clover sample, similarly, the two mānuka samples have lower concentrations of 3-PLA than the 3-PLA perturbed sample. Excluding NZ333, the rate of loss for the DBT samples was greater than any of the perturbed samples. It indicates that there may be several compounds in mānuka honey (and kānuka) simultaneously interacting with diastase to cause a larger loss of diastase activity than observed in the clover, reiterating that while diastase activity is dependent on time and temperature, other mānuka honey constituents contribute to accelerating diastase activity loss.
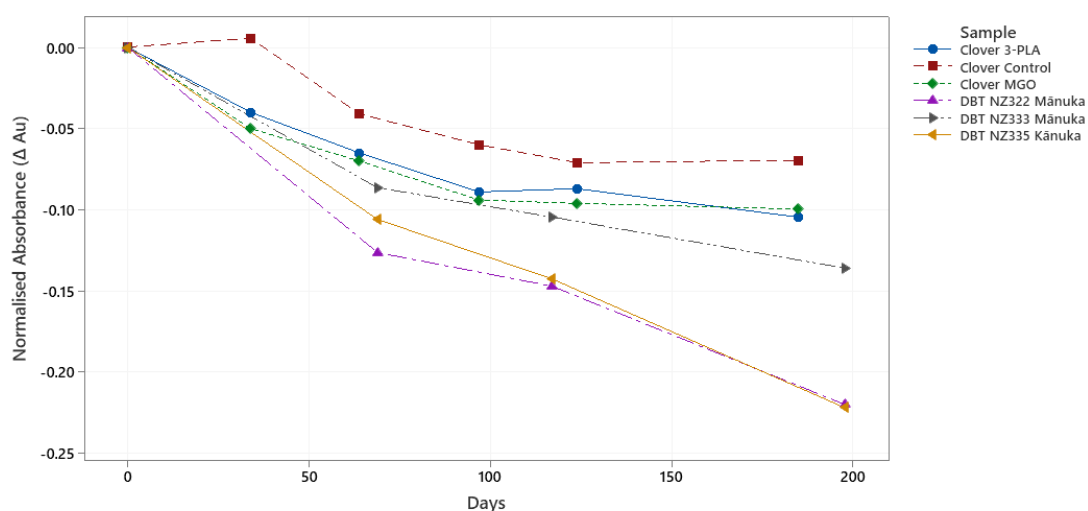
Figure 6.7. Comparison of diastase activity loss between the clover perturbed samples (control, MGO and 3-PLA) and several DBT honey samples (NZ322 mānuka, NZ333 mānuka and NZ335 kānuka) when stored at 27 °C. The DBT honey samples show a greater loss over time compared to the clover perturbed samples.

Table 6.5. Comparison of starting concentrations for 3-PLA and MGO in the clover and DBT samples.

Sample	Diastase Activity		MGO Concentration		3-PLA Concentration	
	(DN)	(Abs)	(mg/kg)	(mmol/kg)	(mg/kg)	(mmol/kg)
Clover Control	11.6	0.316	<LOD	<LOD	<LOD	<LOD
Clover MGO	11.6	0.316	823	11.4	<LOD	<LOD
Clover 3-PLA	11.6	0.316	<LOD	<LOD	724	4.36
NZ322 – Mānuka	14.2	0.409	171	2.37	550	3.31
NZ333 – Mānuka	8.5	0.209	117	1.62	472	2.84
NZ335 – Kānuka	13.1	0.372	38.2	0.53	904	5.44

6.4   Summary of the spiked clover experiments

Of the compounds investigated, 3-PLA and MGO were the only ones that showed significantly lowered diastase activity compared to the control, although only at higher temperatures. As MGO concentration is low in fresh honey, 3-PLA is likely a contributing factor to low diastase activity in fresh mānuka honey. MGO potentially has an effect on the rate of diastase activity loss over time. As MGO is formed in mānuka honey, some of it may bind to alpha-amylase, possibly decreasing the diastase activity at a faster rate than that attributed to temperature alone. The mechanism of action by 3-PLA is not yet understood and requires further investigation. Temperature and time are also significant drivers in diastase activity loss. Mānuka and kānuka samples showed a greater loss of diastase activity compared to the clover perturbed samples.

7 Conclusion and Future work

7.1 Conclusion

The failure of mānuka honey to meet the diastase activity export regulation is detrimental to New Zealand's multi-million dollar honey export industry. The cause of this failure was hypothesised to be due to a contribution from an interaction of a compound found in mānuka honey, opposed to high temperature treatment of honey or prolonged storage. This research aimed to identify the effect of several bioactive compounds (MGO, DHA (MGO precursor), mānuka chemical markers (2'-MAP, 2-MBA, 3-PLA, 4-HPLA), tannic acid and MSY) on measured diastase activity in mānuka honey to determine if any of these compounds had inhibitory activity on alpha-amylase (diastase).

Validation of the methods (Chapter 3) used in this research (diastase, 3in1, SULLE) was carried out to ensure that reliable results were produced. Most validation parameters of these methods were comparable to those in the literature and suitable for the quantification of analytes in this research. Although the recoveries for phenolic compounds using the SULLE extraction method produced results that were lower than optimal (<80%), they were adequate for use in this research.

Due to the lack of literature on *A. mellifera* alpha-amylase, the mechanism of porcine pancreatic alpha-amylase (PPA) catalysis was investigated (Chapter 4), and important amino acid residues identified. Comparison of PPA amino acid sequence to *A. mellifera* alpha-amylase showed 51% conservation, including a conserved active site, indicating that the catalytic mechanism is the same, however enzyme properties and susceptibility to inhibition by phenolic compounds may not be. Investigation of PPA phenolic compound inhibitors included several that were found in honey, however they were not found to be unique or significantly high in mānuka honey.

Analysis of a database (DB) of honeys ($n = 65$) highlighted the large variation of the analysed parameters, even within the mānuka honey subset (Chapter 5). Diastase activity varied from DN 1.1-28.9 in fresh (HMF < 10 mg/kg) mānuka honey with no correlation observed to other measured parameters. This variation indicated that mānuka honey may have naturally low diastase activity (unknown reasons) and / or

other parameters (chemical or physico-chemical) are influencing the diastase activity. Two storage experiments (20 °C, 161 days; 27° C, 198 days) of a subset of DB honeys showed that temperature and time are significant drivers of diastase activity loss. However, differences between the rates of diastase loss indicated that intrinsic honey parameters also influence diastase activity loss. These experiments alongside analysis of a provided commercial dataset showed that loss of diastase activity is correlated to the concentration of MGO, DHA, 3-PLA and 4-HPLA, however there was no strong evidence of causation.

Further investigation into the effect of the individual compounds was carried out by perturbing a clover matrix and storing samples at three temperatures (20, 27, 34 °C) for 185 days (Chapter 6). Both MGO and 3-PLA perturbed samples showed significantly different diastase activity loss (and diastase half-life) than the control and other perturbed samples, suggesting that interactions of these compounds with alpha-amylase in mānuka honey decrease diastase activity. The mechanism is unknown. MGO concentration is low in fresh honey, therefore low diastase activity in fresh mānuka honey is more likely to be related high 3-PLA concentration. As with the database experiments, temperature and storage duration also had an impact on diastase activity.

It is evident throughout this research that diastase activity in honey is not only affected by heating or prolonged storage but accelerated by chemical and possibly physico-chemical properties of the individual honeys. Specifically, two compounds (MGO and 3-PLA) which are found at high concentrations in high grade mānuka honey, appear to cause a greater loss of diastase activity compared to control samples. The Codex states that honey must not have diastase activity less than DN 8, except honey with low natural enzyme content, which should not have diastase activity less than DN 3 while HMF concentration should not exceed 15 mg/kg. However, this experiment has also highlighted that HMF formation varies between individual honeys, and mānuka honey with low diastase activity may also have a faster rate of formation of HMF. The increase in HMF is further exacerbated as mānuka honey is stored to accelerate MGO formation before retail.

The regulatory values for the quality parameters (diastase activity and HMF concentration) for indicating heat treatment and prolonged storage may not be suitable

for mānuka honey (and potentially other New Zealand honeys). The rate variability of both diastase activity loss and HMF formation between floral honey types due to intrinsic properties (not just temperature and storage) implies that the regulations could be too strict for mānuka honey. Further investigation is needed to determine the main intrinsic honey chemical and physico-chemical properties which affect diastase activity and HMF formation in mānuka honey, as currently there is an incomplete understanding of what may influence these parameters. Significant evidence that show that the current regulations are too strict on mānuka honey compared to other floral types is needed, and may be grounds for an exemption of mānuka honey to the diastase activity regulation.

7.2   Recommendations for future research

Throughout this research, several points of improvement or ideas for future research were identified. Points of improvement included alterations to the experimental methods as well as the experimental designs. These recommendations are provided below.

For the experimental methods:

- The sugaring out liquid-liquid extraction (SULLE) method with analysis by HPLC-PDA was suitable for this research, however there were issues with the recovery of analytes and also their selectivity. Most of the recoveries were below 80% and may have been resolved by adding salt during the extraction. For future research, it is recommended that the method is altered to include salt to investigate if this improves recoveries.
- Neither the extraction method nor the detection method were very selective, making integration difficult due to the number of compounds visible in the chromatogram. The instrument method run time (48 minutes) could have been extended to get better resolution of analytes (and adjustment of the gradient), however this is not suitable for high sample throughput. A different column could also be tried. A more selective (and sensitive) detector, such as MS, could be utilised either by itself or in conjunction with the PDA.

For the experimental design:

- Pre-screening the honey matrix used for storage trials is recommended to select a honey with the ideal components (high diastase activity, low HMF and other analyte concentrations).

- Carry out perturbed experiments in mānuka honey as well as clover; perturbing a high diastase activity mānuka honey with further excess of MGO and 3-PLA could avoid any 'mopping up' reactions that may occur in a non-mānuka matrix.

- Add potential inhibitors to the honey matrix individually and collectively to determine if inhibition is additive. Additionally, add potential inhibitors at a range of concentrations to investigate if inhibition is dose dependent.

This research focused on five bioactive compounds (and one precursor) that are unique / elevated in mānuka honey as well as two other compounds. However, honey is a complex matrix with other compounds that may have the potential to affect diastase activity. More avenues to investigate with regards to diastase activity are:

- Pre-screening of honey samples for a wide range of compounds should be carried out to identify specific compounds (that may not be in literature) that are correlated to lower levels of diastase activity in mānuka honey.

- Isolate and purify alpha-amylase from honey and carry out inhibition assays with phenolic compounds in an artificial honey matrix and compare to inhibition in a real honey matrix. This would allow kinetic experiments to be carried out without uncontrolled interferences from multiple compounds in the real honey matrix.

- For MGO and 3-PLA, investigate the mechanism for which they decrease diastase activity. For example, competitive inhibition or crosslinking.

- Investigate other chemical and physico-chemical properties, such as pH, viscosity, other phenolic compounds, water activity / moisture and elemental analysis, and their influence or correlation to diastase activity as it would lead to better understanding of diastase in honey. From this, a model which accurately predicts diastase activity loss in honey could be built. Alongside this, the effect of these parameters on HMF formation could be investigated.

- Proline concentration in honey may be directly correlated to diastase activity as both are secreted into the honey during maturation, analysis of both should be carried out on fresh honey.

- Honey pH could be adjusted by varying levels to determine the effect of pH change on diastase activity and HMF formation in fresh, high diastase activity honeys.

- This research found several other New Zealand floral honey types which had low diastase activity. As there were not enough samples to be conclusive, a more thorough study should be carried out to determine the behaviour of diastase activity in all New Zealand honey varieties, and how this might also be linked to seasonal or regional variation. Honey should be collected straight from the hive to determine diastase activity in truly fresh honey, including multiple samples from the same apiary and of different floral types (determined from pollen analysis) to determine the variability.

- The phenolic compound extraction method provides chromatogram profiles for each honey. Using these profiles, 'bucket analysis' could be trialled to investigate if floral type can be determined based on the phenolic profile.

- Investigate how the honey bee may affect diastase activity in honey as diastase is secreted from the hypopharyngeal glands: do genetics and honey bee age have a role in diastase secretion?

- An investigation could be carried out to determine if moisture content in nectar affects diastase activity. A nectar with high moisture content may require more processing by the honey bee and therefore result in a higher level of alpha-amylase in the mature honey. This could account for some of the natural variation in diastase activity in fresh honey.

- Future work could include investigating diastase inhibition in nectar by diluting a honey with high diastase activity to a moisture content similar to nectar, adding the perturbant, then dehydrating it back to 20% moisture before measuring diastase activity.

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Appendix A.   Database Honey Index

Table A.1. Sample number, floral type (as classified in this research), and harvest region for the database of honeys used in this research. The experiments the honeys were used in are described: DB – Database, DBSH – Database Snapshot, DBT – Database Trials, CD – Clover Spiked.

Sample Number	Floral Honey Type	Harvest Region	Experiments
NZ269	Mānuka	NA	DB, DBSH, DBT
NZ298	Mānuka	NA	DB, DBSH
NZ299	Mānuka	NA	DB, DBSH
NZ300	Mānuka	NA	DB
NZ301	Mānuka	NA	DB
NZ306	Mānuka	Central	DB, DBSH
NZ307	Mānuka	Wairarapa	DB
NZ308	Mānuka	Wairarapa	DB, DBSH, DBT
NZ309	Mānuka	Central	DB
NZ310	Mānuka	Wairarapa	DB, DBSH
NZ311	Mānuka	Central	DB
NZ312	Mānuka	Central	DB
NZ313	Mānuka	Central	DB
NZ314	Mānuka	Northland	DB, DBSH
NZ315	Mānuka	Wairarapa	DB
NZ316	Mānuka	Taumaranui	DB, DBSH
NZ317	Mānuka	Kaiteke	DB
NZ318	Mānuka	Whanganui	DB, DBSH
NZ319	Mānuka	Whanganui	DB, DBSH, DBT
NZ320	Mānuka	Taumaranui	DB
NZ321	Southern Rata	Westland	DB, DBSH
NZ322	Mānuka	East Coast	DB, DBSH, DBT
NZ323	Tawari	Gisborne	DB, DBSH, DBT
NZ324	Clover	South Canterbury	DB, CD
NZ325	Mānuka	Wanganui	DB
NZ326	Mānuka	Wanganui	DB, DBSH
NZ327	Mānuka	Wanganui	DB, DBSH, DBT
NZ328	Mānuka	New Plymouth	DB
NZ329	Clover	New Plymouth	DB, DBSH
NZ330	Bush blend	New Plymouth	DB
NZ331	Mānuka	Far North	DB, DBSH
NZ332	Mānuka	Far North	DB, DBSH
NZ333	Mānuka (Mānuka/Rewarewa)*	North West Waitakene Ranges	DB, DBSH, DBT
NZ334	Kānuka	Far North	DB, DBSH
NZ335	Kānuka	North West Waitakene Ranges	DB, DBSH, DBT
NZ336	Kānuka	Far North	DB, DBSH
NZ337	Mānuka (Pohutukawa)*	Bay of Islands	DB, DBSH, DBT
NZ338	Towai	Northland	DB, DBSH
NZ339	Mānuka	NA	DB, DBSH
NZ340	Mānuka	NA	DB, DBSH
NZ341	Thyme	NA	DB, DBSH
NZ342	Kāmahi	NA	DB, DBSH
NZ343	Rewarewa	Rotorua	DB
NZ344	Multi flora blend	Port Ohope	DB

Sample Number	Floral Honey Type	Harvest Region	Experiments
NZ345	Mānuka	Napier	DB
NZ346	Mānuka	NA	DB
NZ347	Mānuka	NA	DB
NZ348	Mānuka	NA	DB
NZ349	Mānuka	NA	DB
NZ350	Mānuka	NA	DB
NZ351	Mānuka	NA	DB
NZ352	Mānuka	NA	DB
NZ353	Mānuka	NA	DB
NZ354	Mānuka	NA	DB
NZ355	Mānuka	NA	DB
NZ356	Mānuka	NA	DB
NZ357	Mānuka	NA	DB
NZ358	Other (Mānuka)*	NA	DB
NZ359	Other (Mānuka)*	NA	DB
NZ360	Honeydew	North Canterbury	DB
NZ361	Honeydew	North Canterbury	DB
NZ362	Kāmahi	NA	DB
NZ363	Citrus	Gisborne	DB
NZ364	Tawari	BOP	DB
NZ365	Rewarewa	BOP	DB

* Floral type in brackets describes the original classification by the beekeeper.

Appendix B.   Database Honey Results

Table B.1. Analysis results for database honey. These results were also used as T = 0 for the DBSH (*) and DBT (†). Fourteen samples were not tested (NT) for diastase activity due to a shortage of Phadebas tablets.

Sample Number	Moisture (%)	Diastase Number (DN)	Diastase Absorbance (Au)	DHA (mg/kg)	HMF (mg/kg)	MGO (mg/kg)	2-MBA (mg/kg)	4-HPLA (mg/kg)	3-PLA (mg/kg)	MSY (mg/kg)
NZ269*†	20.0	3.5	0.113	3960	5.42	995	19.2	10.4	1770	122
NZ298*	20.6	1.6	0.058	1780	4.57	391	12.5	12.6	1290	91.4
NZ299*	20.6	1.1	0.044	1980	4.88	435	12.4	13.5	1070	90.9
NZ300	20.4	1.5	0.057	1900	5.40	426	12.0	12.6	972	92.4
NZ301	20.4	1.1	0.045	1960	6.10	437	13.0	12.7	936	88.8
NZ306*	19.8	8.6	0.213	899	2.29	297	6.97	4.74	784	73.6
NZ307	19.2	9.3	0.237	404	7.69	163	4.68	5.86	748	146
NZ308*†	20.2	6.1	0.186	445	6.38	153	4.30	5.64	831	177
NZ309	20.0	8.3	0.200	417	7.17	134	4.21	3.90	483	120
NZ310*	19.8	10.0	0.262	174	7.67	72.5	1.77	2.21	215	47.0
NZ311	20.7	16.1	0.477	201	9.44	67.8	3.27	2.39	272	49.0
NZ312	19.7	14.7	0.427	192	12.2	69.6	3.08	2.48	321	68.6
NZ313	19.0	13.1	0.372	243	10.0	90.8	3.71	2.69	299	55.9
NZ314*	21.1	2.1	0.074	1600	11.9	572	2.23	7.96	1150	110
NZ315	20.1	13.6	0.390	401	4.17	117	5.13	4.17	545	68.4
NZ316*	20.2	28.9	0.931	1750	<LOD	185	5.29	5.58	592	47.2
NZ317	21.7	18.4	0.558	2260	1.78	234	7.96	7.71	950	72.8
NZ318*	23.1	15.7	0.464	2830	<LOD	258	8.78	9.78	1510	71.9
NZ319*†	22.1	18.2	0.551	2680	<LOD	341	6.99	9.38	1210	87.6
NZ320	20.3	26.7	0.854	1550	1.92	177	4.32	5.61	550	53.1
NZ321*†	20.3	8.4	0.204	57.5	<LOD	<LOD	<LOD	0.87	<LOD	<LOD
NZ322*†	19.0	14.2	0.410	830	<LOD	171	4.77	4.24	550	49.3

Sample Number	Moisture (%)	Diastase Number (DN)	Diastase Absorbance (Au)	DHA (mg/kg)	HMF (mg/kg)	MGO (mg/kg)	2-MBA (mg/kg)	4-HPLA (mg/kg)	3-PLA (mg/kg)	MSY (mg/kg)
NZ323*	18.9	6.0	0.185	80.9	<LOD	11.0	<LOD	0.97	<LOD	4.94
NZ324	20.3	11.6	0.316	41.6	117	<LOD	<LOD	<LOD	43.7	3.42
NZ325	18.9	25.9	0.827	2600	2.26	344	7.30	8.32	1320	76.0
NZ326*	20.3	26.8	0.858	3850	1.83	625	6.13	9.51	1750	98.8
NZ327*†	20.2	17.8	0.537	2720	<LOD	412	6.33	8.08	1380	83.5
NZ328	20.8	10.3	0.272	548	9.24	163	6.17	4.55	606	44.6
NZ329*	19.4	16.8	0.502	7.51	19.5	<LOD	<LOD	<LOD	12.9	2.42
NZ330	19.3	15.7	0.464	102	23.8	37.3	<LOD	1.80	79.8	6.63
NZ331*	19.4	10.2	0.267	800	24.5	407	5.17	6.20	662	135
NZ332*	20.7	10.7	0.288	91.3	27.1	55.8	5.25	5.37	1250	305
NZ333*†	17.3	8.5	0.210	742	<LOD	117	3.11	3.87	472	48.7
NZ334*	20.6	8.2	0.196	36.8	24.5	14.2	<LOD	8.01	1640	354
NZ335*†	20.2	13.1	0.373	179	2.63	38.2	<LOD	5.76	905	261
NZ336*	19.5	10.2	0.268	26.7	8.46	8.02	<LOD	8.34	1030	284
NZ337*†	20.3	6.1	0.186	406	5.80	83.7	3.32	4.24	849	109
NZ338*	20.1	16.8	0.502	191	4.32	48.9	<LOD	2.17	194	45.9
NZ339*	19.4	8.6	0.212	179	15.9	112	3.78	3.14	313	93.7
NZ340*	20.5	0.9	0.038	2420	14.3	939	8.68	7.64	1070	72.2
NZ341*	20.1	19.4	0.593	16.7	4.12	<LOD	<LOD	<LOD	<LOD	<LOD
NZ342*	20.3	12.7	0.357	12.6	1.98	<LOD	<LOD	<LOD	<LOD	<LOD
NZ343	18.5	5.6	0.173	23.0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
NZ344	20.3	NT	NT	235	9.91	40.9	<LOD	<LOD	77.7	11.8
NZ345	17.6	NT	NT	67.9	4.16	16.8	6.23	1.12	82.2	6.81
NZ346	18.7	NT	NT	160	<LOD	85.7	3.88	2.73	185	56.4
NZ347	18.9	NT	NT	244	2.90	115	3.81	2.86	200	50.2
NZ348	20.2	5.3	0.163	1410	5.34	487	8.12	6.31	886	63.3
NZ349	19.3	NT	NT	1470	6.47	522	6.80	6.59	871	56.9
NZ350	19.1	NT	NT	1500	6.73	529	6.60	6.35	865	56.5

Sample Number	Moisture (%)	Diastase Number (DN)	Diastase Absorbance (Au)	DHA (mg/kg)	HMF (mg/kg)	MGO (mg/kg)	2-MBA (mg/kg)	4-HPLA (mg/kg)	3-PLA (mg/kg)	MSY (mg/kg)
NZ351	19.2	8.1	0.194	1440	5.19	519	6.40	6.10	860	54.7
NZ352	19.0	NT	NT	150	6.56	522	7.74	6.34	892	60.9
NZ353	20.1	NT	NT	1460	8.21	447	7.17	6.29	711	54.7
NZ354	20.1	5.6	0.173	1430	7.78	446	7.06	6.67	705	53.9
NZ355	19.5	NT	NT	1490	7.82	453	7.13	6.72	723	53.8
NZ356	19.3	5.1	0.159	1430	6.56	448	6.87	6.77	739	52.9
NZ357	19.8	NT	NT	111	9.26	393	6.71	5.41	649	45.3
NZ358	18.7	NT	NT	83.2	11.4	28.8	<LOD	1.23	82.0	17.0
NZ359	18.9	NT	NT	87.5	12.4	30.5	<LOD	1.54	78.3	16.1
NZ360	15.8	14.4	0.417	13.4	<LOD	<LOD	3.16	2.49	38.9	16.9
NZ361	15.1	27.1	0.868	29.3	<LOD	<LOD	2.65	1.47	37.5	10.0
NZ362	20.1	12.1	0.335	167	<LOD	92.9	4.10	1.73	111	23.8
NZ363	18.8	8.5	0.208	117	47.1	38.2	<LOD	2.25	155	44.7
NZ364	20.4	NT	NT	14.8	34.8	9.52	<LOD	1.00	<LOD	2.66
NZ365	20.3	NT	NT	22.7	32.9	16.1	<LOD	1.56	72.9	39.8