Kinetics of Conversion of Dihydroxyacetone to Methylglyoxal in Honey

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Abstract

Mānuka honey contains a unique non-peroxide antibacterial activity which is highly sought after due to the perceived health benefits. Methylglyoxal (MGO) is the compound predominately responsible for this non-peroxide activity (NPA). Varying levels of dihydroxyacetone (DHA) are found in mānuka nectar which converts to MGO once honey is harvested; however, this is not a 1:1 conversion. The price of mānuka honey increases as the MGO concentration increases; hence some beekeepers store their honey for extended periods of time in an attempt to increase the concentration of MGO. The level of 5-hydroxymethylfurfural (HMF, a potentially toxic compound) must stay below 40 mg/kg if the honey is to be exported. Formation of HMF is predominately dependent on time and temperature. Therefore there is a trade off between storing honey to maximise the MGO concentration and retaining a low concentration of HMF.

Currently there is not a lot of information on the conversion of DHA to MGO in a honey matrix and many beekeepers are unable to predict the maximum MGO concentration from an immature honey. The principal aim of this thesis was to learn more about the conversion of DHA to MGO in a honey matrix in order to create a tool that can predict the concentration of DHA and MGO over time when held at certain temperatures.

Four high performance liquid chromatography methods were compared to find a suitable method for the analysis of the three compounds of interest (DHA, MGO and HMF). The chosen method allowed all three compounds to be detected (using *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine, PFBHA, derivatisation) in a single 30 minute analysis.

A set of mānuka honeys were analysed for various chemical and physical properties (including moisture content, pH, acidity, amino acids, selected phenolics and trace elements) to identify potential factors that may contribute to the conversion of DHA to MGO. Amino acids and phenolic acids were identified as potential compounds that affected the conversion of DHA to MGO.

Model systems (sugar and water) were used to control the reactions occurring during storage; DHA and individual perturbants were added to the artificial honey to isolate the effect of the perturbant on the conversion. Amino acids were the main focus of these systems; alanine enhanced the conversion of DHA to MGO, whereas DHA was lost to side reactions when proline was present. Real honey samples were also analysed; correlations between the rate constant for DHA disappearance and certain compounds were observed. Temperature influenced the rates of DHA conversion to MGO and also the efficiency of the reaction.

The information from the storage trials was used as a starting point to build a predictive model. This included equations for reactions involving DHA and perturbants (either enhancement of conversion to MGO or removal to a side product) and removal of MGO. The model used the initial concentrations of DHA, MGO, amino acids and some phenolic compounds to accurately predict the change in DHA and MGO over time when stored between 20 and 37 °C.

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List of Abbreviations

~	Approximately
°C	Degrees Celsius
σ	Standard deviation
λ_{max}	Wavelength of maximum absorption
AB	Airborne clover honey
ACN	Acetonitrile
ARP	Amadori rearrangement product
ATHP	6-Acetyl-1,2,3,4-tetrahydropyridine
AGE	Advanced glycation end-products
Ala	Alanine
CoA	Certificate of analysis
Conc	Concentration
DAD	Diode array detection
DHA	Dihydroxyacetone
DMSO	Dimethyl sulfoxide
E_a	Activation energy
EDL	Estimated detection limit
et al.	et alia
FMOC	Fluroenylmethyloxychloroformate
GC	Gas chromatography
HA	Hydroxyacetone
HB	Happy Bee clover honey
HMF	5-Hydroxymethylfurfural
Hol	Hollands honey
HPLC	High performance liquid chromatography
HS	Head space
ICP-MS	Inductively coupled plasma mass spectrometry
IHC	International Honey Commission
ILCP	Inter-laboratory comparison programme
K	Equilibrium constant
k	Rate constant for DHA disappearance
k'	Rate constant for MGO appearance
k_{HMF}	Rate constant for HMF formation

Kat	Katikati clover honey
LOD	Limit of detection
log	logarithm to the base 10
LOQ	Limit of quantification
ln	logarithm to the base e
Ltd.	Limited
Lys	Lysine
MDL	Minimum detection limit
MeOH	Methanol
MGO	Methylglyoxal
MP	Maillard product
MPI	Ministry of Primary Industries
MS/MS	Tandem Mass Spectrometry
NPA	Non-peroxide activity
NPA	Nitrogen phosphorous detector
o	Ortho
OPA	*o*-Phthaldialdehyde
OPD	*o*-Phenylenediamine
PAA	Primary amino acids
PDA	Photodiode array
PFBHA	*O*-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine
Pro	Proline
R^2	Coefficient of determination
Rcf	Relative centrifugal force
RI	Refractive index
RP	Reversed phase
RSD	Relative standard deviation
Ser	Serine
SMF	5-sulfoxymethylfurfural
SD	Standard deviation
Temp	Temperature
UMF	Unique mānuka factor
UMFHA	Unique Mānuka Factor Honey Association
UoW	University of Waikato
UPLC	Ultra performance liquid chromatography

USDA	United States Department of Agriculture
UV	Ultra violet
vs.	Versus
v/v	Volume/volume
WDA	Well diffusion assay
w/v	Weight/volume
w/w	Weight/weight

Chapter One

1 Introduction

Chapter one introduces the reader to mānuka honey and gives background information on why this research is important and relevant to the New Zealand mānuka honey industry. This chapter covers exports of honey, the *Leptospermum genus*, antibacterial properties of honey and details the non-peroxide activity that is unique to mānuka honey. The intentions of this research are described and the aims are identified.

1.1 Beekeeping in New Zealand

The first documented introduction of the honey bee (*Apis mellifera*) into New Zealand was in 1839 when hives from England were introduced into Hokianga. Many bees were imported until the Apiaries Act of 1924 prohibited further importation of bees.[1] The honey bee is found throughout New Zealand and is used for the collection of honey.[2]

Honey production has grown in recent years. In 2005, there were 2,911 beekeepers, 19,281 apiaries and 294,886 hives in New Zealand. This rose to 3,806 beekeepers, 25,253 apiaries and 422,728 hives in 2012. In 2004, 8,888 tonnes of honey were produced compared to 10,385 tonnes in 2012. A national record honey crop for New Zealand was created in 2013; this was up 72% on the previous year (17,825 tonnes produced), due to better weather conditions and an increased number of hives. Values for New Zealand honey production and exportation for the years 2008-2013 are shown in Table 1.1. However, New Zealand Ministry of Primary Industries (MPI) do not keep records on what proportion of this honey is mānuka. A report by MPI[3] in December 2013 breaks down the New Zealand data into regions; the reader is directed to this report for revenue, exports, numbers of bees and operating costs. The domestic consumption of honey in New Zealand is around 5,000 tonnes annually. The remaining honey is either stored or exported. In comparison, around 75,000 tonnes/year are exported from China and 60,000 tonnes/year are exported from Argentina. New Zealand focuses on speciality honeys, such as mānuka honey.[4]

Due to its perceived health benefits, mānuka honey retails at a much higher price than any other honey. The wholesale value of mānuka honey which possesses non-peroxide antibacterial activity (NPA, see section 1.5.4 for a discussion on NPA) has risen 10-fold in the last 20 years.[5] Table 1.2 summarises the price per kg of clover and mānuka honeys (with and without NPA) for the years 2008-2013; mānuka honey is worth more than clover honey, with active mānuka honey worth more depending on the level of NPA. A report from December 2010 from Statistics New Zealand[6] reported NZ$31,700 of honey was exported to the United Kingdom and NZ$9,141 was exported to Singapore. However, there is no mention of the export value of mānuka honey.

Table 1.1 Production and export of honey in New Zealand for years 2008 to 2013 (extracted from MPI).[3]

Year	Honey production (tonnes)	Honey exports (tonnes)	Export value ($ millions)
2008	12375	5366	62.6
2009	12565	7384	81.0
2010	12553	7147	97.6
2011	9450	6721	101.6
2012	10385	7658	120.8
2013	17825	8054	145.0

Table 1.2 Price of clover and mānuka (with and without NPA) honeys for years 2008 to 2013 (extracted from MPI).[3]

Year	Light (clover type)	Mānuka (without NPA*)	Mānuka (with NPA*)
		Price ($/kg)	
2008	2.80-3.75	8.50-13.25	12.10-45.00
2009	3.80-5.60	7.00-12.00	12.50-56.28
2010	4.00-6.00	7.00-13.00	11.00-37.50
2011	4.10-6.80	8.00-12.00	12.50-80.50
2012	4.40-7.30	9.00-15.00	14.75-50.00
2013	5.00-7.30	10.45-16.50	13.00-60.00

* NPA = non-peroxide activity

1.2 Honey

The Codex Alimentarius is the World organisation responsible for generation of standardised codes for food worldwide. They define honey as:

> *"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."*[7]

The New Zealand/Australian Food Standard's definition is based on this definition.

Bees collect nectar and hold it in their internal honey sac. A pair of hypopharyngeal glands produce enzymes which are added to the nectar. The foraging bees pass the nectar to the house bees for ripening into honey. The moisture in the nectar can range from 4 to 60% or more when it arrives at the hive.

House bees expose the nectar as a thin film on their proboscis, which evaporates the moisture into the warm dry air of the hive. This process takes about 20 minutes. Small droplets of half ripened honey are deposited by bees onto a thick film on the wax cells to complete its ripening. When the moisture level is reduced to approximately 19%, the cells are sealed with wax caps.[8-10]

The major constituents in honey collected by *A. mellifera* are similar for honeys of different floral origin (e.g. sugars). Minor constituents (e.g. trace elements) may be different due to environmental influence. The composition of honey is important to this research as any one of the compounds present may act as either a catalyst or inhibitor in the conversion of dihydroxyacetone (DHA) to methylglyoxal (MGO). A review of components in mānuka honey has been undertaken and can be found in chapter 4.

1.2.1 Mānuka honey

Mānuka honey derived from the *Leptospermum scoparium* (mānuka) tree in New Zealand has been observed to have a high level of non-peroxide antibacterial (NPA, see section 1.5.4) property that is not found in other honey.[11] Methylglyoxal (MGO) is responsible for the majority of the NPA.[12-13] MGO is formed from the conversion of dihydroxyacetone (DHA) over time when honey is extracted from the hive and stored. Dihydroxyacetone is present in the nectar of mānuka flowers.[14] The level of DHA in the nectar may vary due to a number of factors, including genetics and environment,[15] as a result the NPA level of mānuka honey will also vary between batches. Furthermore, if bees visit non-mānuka trees the NPA will be diluted.

Mānuka honey is a dark colour, with an average Pfund* of 84 ± 11.8 mm. It has a conductivity which is about 4 times higher than normal floral honeys (5.8 ± 1.54). Conductivity is reported to be an indirect measurement of the mineral content of a honey.[16] The conductivity of mānuka honey is closer to honey dew honey, but the

* Honey colour is measured on a Pfund grader; the Pfund scale is measured in millimetres, and goes from 0 mm (colourless) to 140 mm (black).

reason for this is unknown. Mānuka honey is classified as an over-represented* pollen type and the pollen is indistinguishable from that of kānuka honey. Mānuka honey is often heated more than other honeys due to the difficulty in straining and pumping it at the usual temperatures due to its thixotropic nature in liquid form. In addition, it may be heated to try and increase its NPA rating.

1.3 Leptospermum genus

The *Leptospermum* genus belongs to the Myrtaceae family, which are a family of dicotyledon plants. The *Leptospermum* genus contains 79 species in the world; they are found in South East Asia, New Guinea, Australia and New Zealand. 77 of the 79 species live in Australia; 75 of which are endemic. New Zealand only has one species of *Leptospermum* – this is *Leptospermum scoparium*.[18]

1.3.1 Mānuka (*Leptospermum scoparium*)

Leptospermum scoparium is the only *Leptospermum* species that lives in New Zealand. It also grows in Australia in Victoria and Tasmania. The tree is commonly referred to by the Maori name mānuka; which will be used to describe it throughout this thesis. In addition, it is also known as tea-tree in both New Zealand and Australia because in the colonial days the aromatic leaves were used as a substitute for tea.[19]

In New Zealand, *L. scoparium* is one of the most widely spread native plants. Mānuka trees can grow on rocky cliffs, in thermal areas and in muddy swamps.[19] The size of the tree depends on its location; it grows as a shrub up to 4 metres, but is also found as a slender tree up to 15 meters tall.[19-20] The foliage is grey-green to bronze-green and the small close-set leaves have sharp pointed tips.[20] They have woody seed capsules (5-6 mm in diameter) that are retained on the plant for at least a year after flowering.[21]

* Honey that has 20,000-100,000 pollen grains in a 10 g sample is considered to be normal. If there are more pollen grains, the honey is said to be over-represented. This occurs because mānuka trees are andromonoecious - male and hermaphrodite flowers are produced on the same plant. Nectar is produced in small quantities in both types of flower. Therefore pollen is over-represented in the honey due to the availability of pollen when the bees are collecting nectar.[17]

Mānuka is highly polymorphic; the environment plays a part in some variation, but most is genetically determined and maintained in cultivation. Generally in the wild, mānuka flowers have white petals with a reddish central disk. However, occasionally pink and red flowers are seen. A map detailing the origin of wild *L. scoparium* that have been brought into cultivation is shown in Figure 1.1. Around 150 cultivars have been named; over 70 are still cultivated.[22] The variety *incanum*, which occurs naturally in the far north of the North Island, has contributed to several ornamental cultivars; this includes *L. 'Keatleyi'*. Various wild mutations exist, such as red-flowered plants (e.g. *L. 'Nichollsii'*, in the South Island) and double flowered plants (e.g. *L. 'Leonard Wilson'*), and have been brought into cultivation. Figure 1.2 shows examples of single and double flowers with white or pink petals.

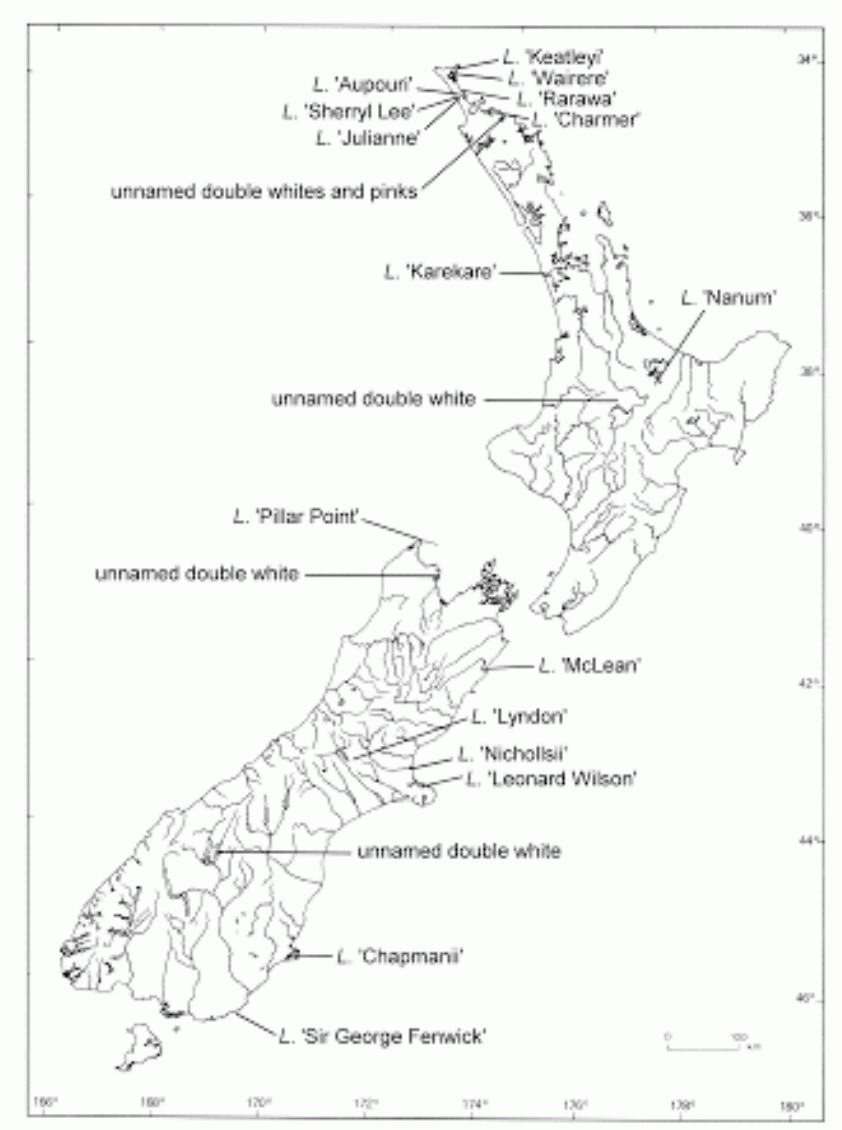


Figure 1.1 Map of cultivar origins of *L. scoparium*. ©Murray Dawson.[22]

Figure 1.2 Different varieties of mānuka flowers: Nanum Tui – single white flower (top left); Red Ensign – single pink flower (top right); Snow Flurry – double white flower (bottom left); Wiri Kerry – double pink flower (bottom right). ©Megan Grainger.

Mānuka trees have a relatively short flowering period of approximately four weeks. Due to New Zealand being a long narrow country oriented North-South, the time of flowering depends on the tree's location; flowering occurs from November through to January, North to South. The trees may also flower in irregular bursts at other times of the year.[21]

Several mānuka varieties have been identified by botanists in New Zealand that are broadly associated with certain regions. Stephens[23] studied the regional variability of the non-peroxide antibacterial activity in mānuka honey for his PhD thesis. The NPA of 461 mānuka honey samples were analysed and adjusted for dilution of the nectar by bees foraging non-mānuka trees. Stephens reported that different regions had significantly different NPA ratings; in addition, samples from the same region also differed. Stephens analysed mānuka from different populations in New Zealand and identified four varieties in two major divisions. The Northern division contains core populations in Northland and Coromandel (*L.*

scoparium variety *incanum*) and the Waikato (*L. scoparium* variety *linifolium*). These samples were found to contain high non-peroxide activity, equivalent to 14-16 NPA units. Studies have suggested that an unnamed variety of mānuka on the West Coast is closely related to the *incanum* variety. This honey has medium to high non-peroxide activity.[24] Both varieties have lanceolate-linear* leaves; the incanum variety has rose-tinted petals that are about 8 mm long.[25]

The Southern division contains core populations in the Central North Island (*L. Scoparium* variety *myrtifolium),* which has a low non-peroxide antibacterial activity as reported by Stephens.[23] The *myrifolium* variety has smaller ovate† recurved leaves.[25] Another unnamed variety, which is associated with *L. Scoparium* variety *myrtifolium,* grows predominantly on the East Coast and is unique because it contains triketones in the essential oil which gives it antibacterial properties. According to Stephens,[23] this honey has moderate antibacterial activity.[24]

The mānuka tree is host to a sap-sucking scale insect (*Eriococcus orariensis*), which was accidentally introduced from Australia around 1937. In the following 10 years farmers used the insect to clear mānuka from their land and it became wide spread around New Zealand. The scale insect sucks sap from the tree and causes a loss of sap nutrients. Over 2-5 years this can debilitate and kill mānuka trees. The insect exudes excess sugars as honey dew. Shaggy sooty mould grows on the honey dew, giving the trees a fire-burned appearance.[19, 27] In the mid 1950s a parasitic fungus (*Myriangium thwaitesii)* which destroys the scale insect appeared in the country and allowed the recovery of mānuka trees in most areas.[28]

A study[29] of antibacterial activity of Australia honeys reported that Australian honeys (n = 11) originating from *L. scoparium* in Tasmania did not contain any detectable non-peroxide activity. The NPA was detected indirectly using well diffusion assay (WDA, see section 1.7), not by directly measuring MGO. The results suggest that New Zealand and Australian *L. scoparium* are different. This may arise from environmental differences or sub-species. The study analysed

* Lancelolate leaves are lanced shaped with the leaf being several times longer than wide; the greatest width is about one-third from the base. It gradually tapers to the apex and tapers more rapidly to the base.
† Ovate leaves are elliptical or reverse egg shaped; they are widest near the apex.

other *Leptospermum* species from Australia and they were found to display NPA (see section 1.3.2). Another study[30] reported low levels of MGO in mānuka honey from Tasmania (n = 7). However, the concentration of DHA ranged from 23-2,313 mg/kg. Further studies should be carried out on other *L. scoparium* in Australia to discover whether or not this is a consistent result for *L. scoparium* in other areas of Australia.

1.3.1.1 Nectar analysis of mānuka flowers

In 1988, Tan *et al.*[31] compared ether extracts of mānuka flowers to honey; they found little similarities between the two. The organic extractable components of mānuka honey were not present in mānuka flowers. The extractable components of the honey were suggested to arise from honeydew, which coats the mānuka tree. Triterpenoids were reported as the main compounds in the flower; however, they were not found in the honey. Triterpenoids have also been found in the leaves.[32] In comparison, phenolic compounds, including phenyllactic acid, found in the nectar were reported to be the same as those found in the honey, but are in much higher concentration in the nectar.[5] The difference may arise from bees visiting multiple trees that have varying levels of the compounds, thus diluting its presence in the honey.

When MGO was discovered as the NPA component of mānuka honey, a search for its origin began. MGO levels in plants are reported to increase under stressful conditions such as salinity, drought and cold stress.[33] There is very little reported work on MGO in the nectar of mānuka flowers. Adams *et al.*[14] found no MGO in the nectar of the mānuka flowers. The absence of MGO may be due to the time of collection (not mentioned), the method of extraction, the detection limits of the analysis method or because DHA does not convert to MGO until it is in the honey matrix. One study[5] reported 31.9 mg/kg MGO in the nectar of mānuka flowers collected during autumn using OPD derivatisation with solid phase micro extraction and GC-NPD detection, which is more sensitive than HPLC-UV detection. They repeated the study in spring, and reported trace amounts of MGO were detected in the nectar of trees. Detection of MGO in the nectar may arise from:

- conversion of DHA in the nectar before collection by bees

- trees that are under stress may form MGO.
- the way samples were stored before analysis
- some DHA converting to MGO during the derivatisation process.

The last two reasons are the most plausible. Further study at Waikato University showed no MGO in the nectar of a study of *L. scoparium*.[34-35]

In 2009, DHA was found as the precursor to MGO. Adams *et al.*[14] reported the presence of DHA in nectar samples from mānuka flowers in varying amounts (a value was not given).Williams (2012)[34] and King (2013)[35] studied DHA in *L. scoparium* from various regions in New Zealand and limited samples from Australia. They both reported varying levels of DHA in the nectar. Inter-regional trends were not observed, but the sampling year had an influence, which is likely to be related to environmental conditions. Personal communication with Revell[15] suggests that genotypes of *L. scoparium* may play a role in the varied amount of DHA in the nectar of mānuka flowers.

1.3.2 Jelly Bush (*Leptospermum polygalifolium*)

Leptospermum polygalifolium is found in Australia from the South coast of New South Wales to Cape York in far North Queensland. It grows either as a shrub (0.5 to 3 m) or as a slender tree (7 m or higher). It is commonly known as jelly bush.

L. scoparium and *L. polygalifolium* are closely related and share a number of physical characteristics. Jelly bush honey displays non-peroxide antibacterial properties, which has been labelled the Unique Leptospermum Factor (ULF). Blair *et al.*[36] reported that Australian *Leptospermum* honey is effective against bacterial and non-bacterial pathogens. In 2011, Irish *et al.*[29] studied 477 honey samples from Australia by well diffusion assay. They found *L. polygalifolium* honeys had high levels of NPA. They also noted that honey from *L. liversidgei* and *L. laevigatum*, plus some unspecified species had NPA. The NPA increased with storage of the honey; however, MGO content was not tested in this study.

Windsor *et al.*[37] studied Australian *Leptospermum* honeys and reported the highest MGO concentrations in *L. polygalifolium* honeys; values up to 1,723

mg/kg were reported. A press release from The University of Queensland reported very high levels of MGO in honeys from *L. polygalfolium* (higher than 1,750 mg/kg).[38] Another study[30] reported 1,166 mg/kg MGO in jelly bush honey.

1.4 Kānuka (*Kunzea ericoides*)

Kānuka *(Kunzea ericoides)* is found in New Zealand and Australia as a shrub or tree (up to 30 m). It was commonly called mānuka until the 1930s and was classified as part of the *Leptospermum* genus (*L. ericoides*) until 1983.

Kānuka is very similar to mānuka superficially, but the two can be differentiated by the leaves and flowers; kānuka leaves are softer and the smaller white flowers grow in clusters (Figure 1.3), not singly like mānuka flowers. In addition, kānuka does not have the 'sooty' appearance because it is not affected by mānuka blight.[39] This is due to kānuka shedding its bark, which gives it a smooth surface upon which the blight cannot grow.

Figure 1.3 Cluster of kānuka flowers. ©Paul Ashford, 2013, www.NZplantpics.com.[41]

The seed capsule is less woody compared to the mānuka seed capsule and is smaller (2-3 mm); in addition, the capsules are not usually seen on the tree as they fall off after a month or two.[21] Kānuka is genetically very different from mānuka.

In 2010, Stephens *et al.*[5] reported MGO was only found in kānuka/mānuka blend honeys, not pure kānuka honeys. This is in line with Adams *et al.*[14] who reported

that there was no DHA in the nectar of kānuka flowers. In contrast, Holt *et al*. (2012)[42] reported that samples of raw and medical-grade kānuka honey had high levels of MGO (1,024 mg/kg and 1,154 mg/kg respectively). The levels found were higher than what is commonly found in mānuka honey. The samples were tested by *o*-phenylenediamine (OPD) derivatisation with RP-HPLC and UV detection. This is the method used for commercial testing of MGO in mānuka honey. No details were reported on the location from which this honey was sourced. This is the only report of MGO in kānuka honey. Mānuka and kānuka trees are often found in the same areas and as a result blended honeys can be produced. The pollen grains of the two species are very hard to distinguish[5] and currently there is no reliable method to distinguish the two. The reported level of MGO is, however, extremely high for a blend honey. Subsequent analysis of this honey and other kānuka honeys at University of Waikato did not find the presence of MGO.

1.5 Antibacterial properties of honey

Honey has been known since ancient times for its wound-healing properties. It has a range of putative nutritional and health effects. Antibacterial, antioxidant, anti-inflammatory, prebiotic, antitumor, antimutagenic and antiviral properties have all been attributed to honey.[43]

In 1882, van Ketel was the first person to attempt the identification of antibacterial compounds in honey.[44] In 1919, Sackett discovered that dilution of honey increased its activity (see section 1.5.2).[45] Many studies have been dedicated to the identification of components in honey that are responsible for its antibacterial nature.

The frequency of antibiotic resistant bacteria is increasing worldwide and causing a significant risk to public health. However, the rate of development of new antibiotics is very slow, due to the fact that many large pharmaceutical companies are no longer investing time or money into this area.[46] Therefore a different defence against bacteria is required. The use of honey for wound dressings is advantageous for multiple reasons; it has the ability to keep the wound moist, it is non-toxic, has anti-inflammatory actions, debriding activity (cleaning the wounds

by releasing pus or dead tissue), reduces scarring, stimulates re-epithelisation and bacteria will not become resistant to it.[36] However, its use in modern medicine has been prevented from becoming a mainstream application due to extensive differences in the diverse range of honeys available.[46]

Medical-grade honey was first licensed for treating wounds in Australia in 1999. Its use has now been extended to New Zealand, Canada, Europe, the United States of America and Hong Kong.[47] There are several commercially available honey items, approved by various regulatory authorities, which are produced specifically for wound care. This includes a variety of dressing fibres impregnated with honey, ointments and gels. These designs have overcome the problem of honey being difficult to apply and keep in place.[48]

All honeys have three main antibacterial properties; pH, hydrogen peroxide (H_2O_2) and osmolarity. Mānuka honey has an extra antibacterial property (of non-peroxide origin) that is not seen in other honeys. These antibacterial properties are discussed below.

1.5.1 pH

The pH of honey ranges from 3.4 to 6.1 with an average of 3.9.[31] The low pH is mostly due to the conversion of glucose into H_2O_2 and gluconic acid by glucose oxidase, which originates from the hypopharyngeal glands of the bees (equation 1-1).[49]

$$Glucose + H_2O \xrightarrow{Glucose\ oxidase, O_2} Gluconic\ acid + H_2O_2 \qquad (1\text{-}1)$$

The low pH slows or prevents growth of many species of bacteria. However, if the honey is diluted with buffer solutions (e.g. body fluids), this acidity will be neutralised.[50] Early explanation of the antibacterial activity of honey considered that the low pH of honey was important. However, other studies have found no correlation between antibacterial activity and pH.[51]

1.5.2 Hydrogen peroxide

In full density honey, glucose oxidase is almost fully inactive. This is due to the product, gluconic acid, lowering the pH of honey to a point where it inhibits the glucose oxidase enzyme.[52] A maximum of 0.3 ppm H_2O_2 has been found in full density honey.[53] Once honey is diluted the enzyme becomes active again and produces H_2O_2 and gluconic acid from glucose.[31, 54] In many honeys hydrogen peroxide is the main contributing component to the antibacterial nature of honey. Hydrogen peroxide in honey has more of an antibacterial effect than that of an equivalent hydrogen peroxide solution,[55] suggesting that there are other factors which contribute to its activity.

Glucose oxidase is inactivated by heating honey and is also sensitive to light. In addition, some honeys contain catalase which destroys H_2O_2.[50] Rate of production of H_2O_2 varies disproportionately when honey is diluted and dilution varies in wounds depending on the exudates. Maximum levels of H_2O_2 vary depending on honey type; it has been reported that maximum levels are generated in honey that has been diluted 2-3 fold.[56] Thus H_2O_2 may become ineffective in serum and tissues, due to catalase decomposing it.[48] Most mānuka honey that has a high MGO content does not exhibit peroxide activity due to deactivation of glucose oxidase by MGO.[57]

1.5.3 Osmolarity and water activity

Honey is a saturated or super-saturated solution of sugars; the water content of honey is about 15-21% by weight. Eighty four percent of the solids in honey are a mixture of the monosaccharides fructose and glucose which have a strong interaction with water molecules. Free water (water which does not interact with the sugars) is measured as water activity, a_w, and is too low in ripened honey to allow growth of any microbial species.[51] The water activity of honey is reported as 0.56-0.62.[55] However, most organisms grow best in a_w values between 0.995-0.980. Therefore microbes are unable to grow in honey until it is diluted to about 2-12%.[55, 58] This would occur when honey is digested or from exudates if it is used as a wound dressing. It is important to note that honey will ferment if the water content is higher than 17% and contains a sufficient number of yeast spores.[59] *Staphylococcus aureus* are very osmotolerant and can survive in high

sugar concentrations.[45] Most microbes stop growing below a_w 0.90; but at this water activity, *S. aureus* will grow at approximately 10% of the maximum rate it grows when the water activity is 0.98.[60] Thus honey with only osmolarity as an antibacterial property is not effective against *S. aureus* after it is diluted three or four times its volume by exudates. However, honey that is produced for wound care, which has other antibacterial activity (for example hydrogen peroxide), is still effective after dilution to 45-60 times its volume.[48]

1.5.4 Non-peroxide antibacterial activity (methylglyoxal)

A unique non-peroxide antibacterial activity of mānuka honey was discovered in 1982. This research was first published in 1983 in an MSc thesis,[61] and subsequently in 1988.[11] This activity is still present when H_2O_2 effects are removed by catalase and also in undiluted honey, unlike the antibacterial effect of H_2O_2. The compound responsible for this activity has been heavily studied and was originally believed to originate from the floral source.[23] The cause of the variation in activity levels was also unknown and it was conjectured that it may occur from nectar harvest of different varieties of *L. scoparium*, environmental factors or another species interacting with *L. scoparium.* The term 'unique mānuka factor' (UMF) was coined by Molan in 1997 as a way to identify the non-peroxide activity (NPA) in mānuka honey. [62 6262] UMF has since been registered as a trade mark; hence the term non-peroxide activity (NPA) will be used throughout this thesis.

Since the discovery of NPA in mānuka honey, many compounds have been examined in order to find the one associated with NPA. Russell *et al*. (1990)[64] isolated aromatic acid derivatives from mānuka honey; syringic acid and phenyllactic acid were the main compounds found. They reported that syringic acid, 3,4,5-trimethoxybenzoic acid and their methyl esters exhibited antibacterial activity against *S. aureus.* However, methyl syringate and phenyllactic acid are the major acids in several European honeys so therefore could not be responsible for the unique property in mānuka honey.[49] Russell *et al*.[64] also considered that the total phenolic extract might be responsible for the antibacterial honey; however, whilst the extracts showed some activity, they were not solely responsible for the antibacterial activity of mānuka honey. In 1997, Bogdanov[65]

separated ten different honeys into four fractions and found that the antibacterial activity was acids > bases = non-polar, non-volatiles > volatiles. However, mānuka was an exception, where 90% of the activity was in the acidic fraction compared to only 44% in other honeys. However, the alkaline conditions used in separation would have destroyed the NPA, hence Bogdanov's conclusions are not valid. In 1999, phenolic derivatives of benzoic acids, cinnamic acids and flavonoids were ruled out as potential compounds because they also occur in other honeys which do not exhibit NPA.[49]

In 2000, Weston[66] published a review on various components in honey which contribute to the antibacterial activity. He believed that NPA was due to residual hydrogen peroxide activity, and not a certain compound in mānuka honey. He believed that the catalase used in the well diffusion assay was not sufficient to remove all hydrogen peroxide activity. In 2004, Snow and Manley-Harris[67] reviewed previous literature about the nature of the non-peroxide activity. They observed no statistical difference between the normal amount of catalase and 10-fold excess added for the well diffusion assay; they concluded that the non-peroxide activity did not occur from residual hydrogen peroxide.

In 2004, Weigel *et al.*[68] reported the occurrence of 1,2-dicarbonyl compounds (3-deoxyglucosulose, MGO and glyoxal) in multifloral honey. Mavric *et al.*(2008)[44] focused on 1,2-dicarbonyl compounds in mānuka honey to investigate if they were related to the non-peroxide activity. They discovered that MGO is directly responsible for the pronounced antibacterial effect of mānuka honey. They confirmed this result by comparing mānuka and 'inactive' forest honey to MGO in a well diffusion assay. The inactive honey showed no inhibition zone, while the mānuka honey had an inhibition zone the same size as the equivalent amount of MGO (1.9 mM). In addition, they reported that out of the samples analysed by HPLC (n = 50) only New Zealand mānuka honey contained MGO (n = 6). These samples contained 38-761 mg/kg MGO; this is up to 100-fold higher than found in other honey samples. They also reported that mānuka honey diluted to 15-30% (MGO concentration of 1.1 to 1.8 mM) showed antibacterial activity.

In the same year, Adams *et al.*[12] independently reported that MGO was the compound which accounted for the non-peroxide activity of honey. They isolated

the active fraction using HPLC and characterised it by NMR. They noted that treatment of active mānuka honey with alkali and titration back to the original pH resulted in loss of non-peroxide antibacterial activity and loss of the MGO peak in the HPLC chromatogram. This concurs with Snow and Manley-Harris[67] who reported that the compound responsible for NPA was unstable in alkali conditions and at pH 11 there was immediate loss of activity which was irreversible. MGO is alkali labile. Bowden and Fabian (2001)[69] reported three reactions of dicarbonyls in basic solution; benzyl-benzilic acid (more commonly known as the benzillic acid rearrangement), fission of carbon-carbonyl carbon bond and fission of carbonyl-carbonyl carbon bond. This may be the reason why the loss of activity was immediate. Adams *et al.*[12] also reported that there was no change in peak area after prolonged evaporation (high vacuum, ca. 0.02 mbar) of an aqueous solution of active honey, which is to be expected since MGO can hydrogen bond to proton donors and hence would not be volatile in honey

The presence of MGO in mānuka honey influences other compounds, such as other antibacterial compounds and enzymes. Defensin1, a peptide in honey which has antibacterial activity against Gram-positive bacteria, has been reported in Revamil® honey[70*] and was recently reported as a regular component of honey, including mānuka honey, in varying amounts.[71] However, Kwakman *et al.*[70] noted that both defensin1 and H_2O_2 were not active in mānuka honey, due to influence from MGO. Furthermore Majtan *et al.*[71] assessed *in vitro* the effect of MGO (0.7 mg/g) on defensin1 and reported a loss of defensin1 which was time-dependent. After 51 hours of incubation at 37 °C there was no remaining activity from defensin1 in samples that contained MGO. The ability of MGO to modify defensin1 was confirmed by immunoblot analysis which showed a decrease in the amount of the native 5.5 kDa form of defensin1. MRJP1,[†] a dominant protein (55 kDa) in honey, was also modified in mānuka honey, shown by a higher molecular weight on the immunoblot, which increased with increasing MGO added to honey. In addition, the authors suggested that α-glucosidase, which converts sucrose into glucose and fructose, may also be modified. This was identified by the loss of the 70 kDa band in the immunoblot. The study did not examine if there was an

[*] Revamil honey® is medical grade honey which has been produced under controlled conditions in a greenhouse.[70]

[†] MRJP1 is a major 55 kDa protein found in honey and royal jelly, produced in the cephalic glands of honey bees.

associated change in function on α-glucosidase or MRJP1, thus further research is required.

MGO has also been reported to modify and inhibit glucose oxidase, thus preventing it from producing hydrogen peroxide.[57] Daglia *et al.*[72] studied the effect of MGO on digestive enzymes, *in vitro*, and reported that there was no significant change on the physiological activity of pepsin and pancreatin from carbonylation. A recent study[73] examined the use of cyclodextrins to complex mānuka honey for oral use to overcome the problem of MGO reacting with other food components. This may allow for enhanced delivery and slow release of MGO in the gastrointestinal tract. There are many reviews on reactions of MGO in food and in biological systems; including the ability of MGO to modify proteins, mechanisms for detoxification and effects *in vivo*; however, this information lies outside the scope of the current research. The reader is directed to a selected number of reviews and the references within if more information is required.[74-78]

1.6 Mānuka honey as an antibacterial agent

Mānuka honey has a large potential for curing bacterial infections because it has been shown to be effective against multi-drug-resistant organisms.[79-80] Furthermore, there have been no reports of antimicrobial resistance to honey; hence it is a very promising substance for medical application. Blair *et al*. (2009)[36] reported that bacteria were unable to form a resistance to mānuka honey. Cooper *et al*. (2010)[47] extended this study by examining the possibility that bacteria might become resistant to low concentrations of mānuka honey if they are continually exposed. They also examined whether or not resistant strains were selected. They reported that the test organisms had reduced susceptibilities to mānuka honey during long-term stepwise resistance training; however, this was not permanent. In addition, honey-resistant mutants were not detected.

Mānuka honey has been shown to have an antibacterial effect *in vitro* against *Helicobacter pylori*, the main cause of stomach ulcers.[81] It is also effective *in vivo*[82] and *in vitro*[47, 83-84] against *Staphylococcus aureus* as well as active *in vitro* against vancomycin resistant *Enterococci*.[79] and *in vitro* on gram-negative

bacterium *Pseudomonas aeruginosa*.[80] Active mānuka honey has also been reported to have activity against non-bacterial pathogens, for example yeast and filamentous fungi.[36] Recently mānuka honey has been reported as a possible way to control dental biofilm deposit after *in vitro* studies,[85] and has been shown to be effective *in vitro* against *Clostridium difficile.*[86] There is a vast array of literature on the use of mānuka honey as an antibacterial agent. However, this is outside the scope of this research and will not be covered here. The reader is directed to a recent review by Patel and Cichelllo[87] (and the references within) which summaries research carried out on the use of mānuka honey as an anti-bacterial agent both *in vitro* and *in vivo* as well as in clinical trials.

MGO reacts readily with thiol groups of proteins and with guanine bases of DNA. This can lead to inhibition of some enzyme activity and cause arrest of cell division. Fidaleo *et al.*[83] suggested that the ability of the antibacterial effects of MGO may be due to the overall cellular damage caused by random multiple detrimental effects on cytoplasmic constituents, rather than interaction with specific target sites. They examined the antibacterial nature of MGO in liquid and gel formulations using the agar well diffusion test. The preliminary results showed that MGO has a high bactericidal activity against *Staphylococcus spp.*

Although MGO is toxic to microbial cells, they also produce it; this may be to relieve cells from stress caused by high sugar phosphate concentrations. Therefore they are equipped with MGO detoxification systems. There are several detoxification mechanisms – glutathione-dependent GlcI/GlxII (glyoxylase II) pathway, aldo-keto reductase and the glyoxylase (GlxIII) pathway. Blair *et al.* report no change in the gene expression of these pathways when bacteria were exposed to mānuka honey.[36]

The glyoxalase system in the body is used to detoxify MGO. This enzyme system has been found in mammals and the simplest life form, indicating that MGO detoxification is universally important to most life forms.[16] It is important to note, that in a honey wound dressing there is a slight chance that MGO will enter cells, but poses no risk.

1.7 Non-peroxide activity and MGO agreement

Before MGO was discovered as the compound responsible for NPA in mānuka honey, the activity was measured indirectly. A well diffusion assay (WDA) was used to quantify the level of activity, related to a phenol standard and this method is still used commercially to measure the NPA of honey. The WDA is carried out on *Staphylococcus aureus* inoculated agar plates. Diluted honey, which has had catalyse added (to remove the effect of H_2O_2) is placed into cut wells, along with phenol standards and the plate is incubated over night before the diameters of inhibition zones* are measured (Figure 1.4). These zones are compared to the phenol standards; a honey that has a zone of inhibition with a diameter equivalent to a 10% phenol solution is said to have a NPA 10 rating. This principal is applied up to a 30% phenol solution.[12] In 2008, Molan[88] released an article with improvements for the well diffusion assay including reanalysing honey with an NPA of 20 or higher by diluting the honey 12.5% and using mānuka honeys with known NPA ratings for standards.

With the discovery that MGO is the compound responsible for NPA, direct analysis of MGO is now possible. However, well diffusion assay is still the analysis of choice in some laboratories. The relationship between NPA and MGO is disputed. In 2009, Atrott and Henle[13] reported a perfect linear correlation between MGO (analysed by HPLC with *o*-phenylenediamine (OPD) derivatisation) and the corresponding antibacterial activity of honey samples for samples between 12.4 and 30.9% equivalent phenol concentration. They stated that for honey with levels higher than 150 mg/kg MGO, the MGO is directly responsible for the antibacterial properties of mānuka honey. Therefore they suggested that MGO is a sufficient tool for labelling the bioactivity of mānuka honey. Furthermore they noted that their data was in perfect agreement with the study by Adams *et al*..[12] However, Adams *et al.* subsequently issued a corrigendum which stated that they failed to apply a dilution factor of 1.87.[89] Therefore the two independent studies do not completely agree.

* The zone of inhibition is the area around the well where no bacteria have grown due to the non-peroxide activity of the honey in the well. The higher the NPA, the larger the zone of inhibition.

Figure 1.4 Well diffusion assay plate after incubation. Various sized zones of inhibition can be seen around wells. ©Ben Deadman.

Adams *et al.*[12] analysed mānuka honey (n = 47) for NPA and MGO content (Figure 1.5). They showed that a MGO concentration of 500 mg/kg is equivalent to a NPA rating of 15, compared to Atrott and Henle[13] (Figure 1.6) who assign this to a rating of 20. Wallace *et al.* stated that a NPA 20 honey contains approximately 800 mg/kg MGO.[43] A commercial mānuka honey with a NPA rating of 15 analysed by Donarski *et al.* was reported to contain 817 mg/kg of MGO.[90] The unique mānuka factor honey association (UMFHA) uses a correlation curve verified by independent laboratories, which matches that of Adams *et al.*[12, 89] (Figure 1.5). Table 1.3 shows the conversion of NPA to MGO (both mg/kg and mmol/kg) for the main NPA points using the correlation provided by UMFHA.[91] Appendix A summarises the conversion of NPA to MGO (both mg/kg and mmol/kg) for NPA 0 to 30.

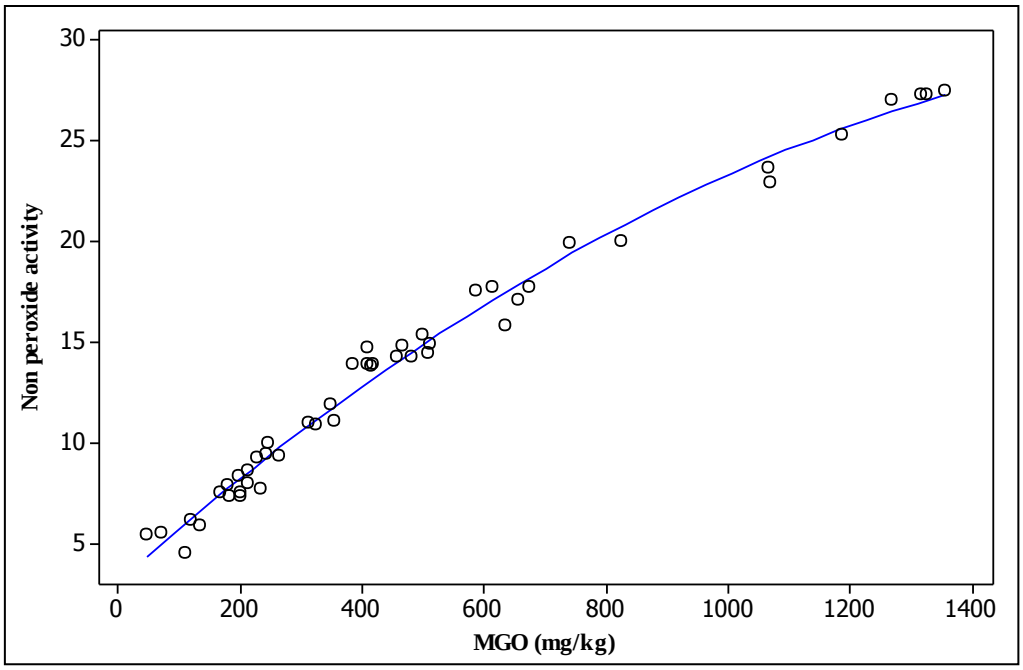


Figure 1.5 Relationship of NPA vs. MGO for honey samples (n = 47) analysed at the University of Waikato.[12, 89]

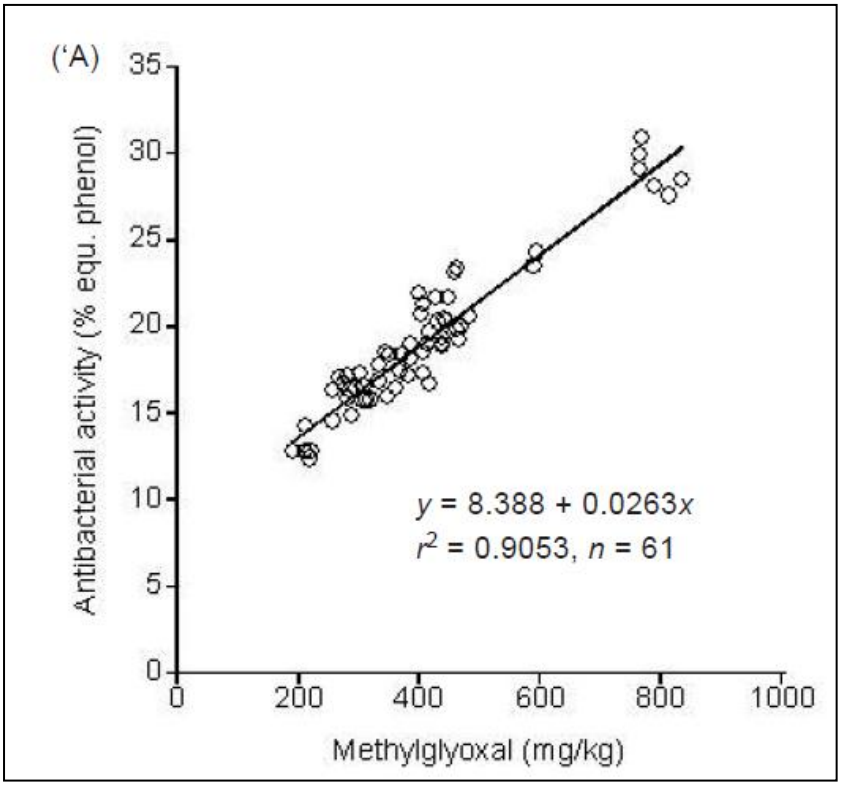


Figure 1.6 Relationship of NPA vs. MGO for honey samples analysed by Atrott *et al.*.[13]

Table 1.3 Conversion of NPA to MGO (mg/kg and mmol/kg) for NPA points that are used to rate commercial products using the correlation curve from UMFHA.[91]

NPA	MGO (mg/kg)	MGO (mmol/kg)
5	134	2
10	263	4
15	478	7
20	779	11
25	1167	16
30	1640	23

The NPA rating of honey and the MGO level may not completely agree as MGO may not be the sole non-peroxide component contributing to the antibacterial activity of mānuka honey. There is divided opinion on whether or not MGO accounts for all of the non-peroxide antibacterial activity of mānuka honey. Atrott and Henle[13] suggest that at levels below 10% equivalent phenol polyphenolics or organic acids may also contribute to the activity. Chan *et al.*[92] compared the flavonoid profiles of mānuka honeys to their NPA and did not find a correlation with most flavonoids. Unknown compound 01 had a possible relationship (R^2 = 0.364) and luteolin has an even weaker correlation (R^2 = 0.227). Rosendale[93] reported that the *in vitro* antimicrobial activity is from both MGO and osmotically active sugars and also pH and aeration; at high honey doses, MGO was the main contributing factor, but at low levels the osmotically active sugars were the dominant growth-limiting factors of *E. coli*.[93]

Molan (2008)[94] stated that there is a synergistic occurrence between MGO and non-antibacterial compounds in honey; this was based upon observations that the antibacterial activity of MGO in honey was higher than MGO in water. However, earlier work carried out by Farr[95] compared DMSO and distilled water as solvents in the well diffusion assay. The two solvents diffuse differently, thus giving the phenol different zones of clearing in agar. In low concentrations of DMSO (2-3%) the phenol had larger zones of clearings; in comparison, phenol in water gave larger zones of clearing at higher concentrations (5-7%). Therefore the results of the experiment by Molan would not be able to directly compare MGO in water and in honey due to the matrix effect. For the results to be directly comparable, a sugar solution would allow a better comparison with the honey.

Mānuka honey has varying levels of NPA and consumers rely on adequate labelling to ensure they are purchasing a honey with an activity fit for purpose. Over the years a number of ratings have been found on the market, which may lead to confusion for the industry and consumer. UMF® was the first labelling method for active mānuka honey. It is the registered name and trademark of the Active Mānuka Honey Association (AMHA); who have since changed their name to Unique Mānuka Factor Honey Association (UMFHA). Only licensed users who meet a set of criteria, which includes the monitoring and auditing of honey quality, can use the UMF® rating on their honey. In 2011, 29 of the 44 AMHA members held a license to use the UMF® trademark; this included Comvita, SummerGlow Apiaries Limited and Arataki Honey Limited (Rotorua and Hawkes Bay).[96] Additional labels are now being used to quote the level of NPA in mānuka honey. Some companies (such as Mānuka Health New Zealand Ltd.) use MGO™ to rate the NPA of honey. Wedderspoon®, a Canadian based company rate their New Zealand South Island organic mānuka honey using Organic mānuka active™ (OMA) as the standard for activity. This rating measures the NPA against phenol.[97] Additionally, Australian *Leptospermum* that has NPA is rated by registered companies; they rate their honey using Unique Leptospermum Factor (ULF™). Other mānuka honey companies do not appear to have a regulated rating system; they only state a number (e.g. 20+) with no explanation, or have the word 'active' alongside a number. These honeys have not passed strict tests to ensure the honey has a certain level of bioactivity. The various labels have led to confusion within the industry and throughout the market place.

The New Zealand Ministry of Primary Industries (MPI) and representatives from the mānuka honey industry are currently drawing up labelling guidelines for mānuka honey. There is currently a transition in the Australia New Zealand Food Standards Code from 1.1A.2[98] to 1.2.7.[99] After a 3 year transition period, all food businesses must comply with Standard 1.27 by 18 January 2016. Under this new legislation, claims about health benefits of food cannot be made unless human trials have occurred. Therefore a new way to label active mānuka is required because terms such as UMF, NPA, and activity will no longer be able to be used. Guidelines for labels will be put in place so that there is clarity for both the industry (entire supply chain) and consumers.[100] In July 2014, MPI published an

interim labelling guide for mānuka honey,[101] which suggests including "presence of a chemical marker" on labels to identify that the honey contains MGO.

1.8 Rationale of this research project and outline of the thesis

1.8.1 Rationale

New Zealand mānuka honey is a natural product that is sold without any additives. Honey producers sell mānuka honey by its NPA rating; the higher the rating the higher the price per drum. Figure 1.7 shows the price for which one commercial honey company buy their honey; the price increases exponentially with increasing NPA rating. A standard mānuka honey sells for $17.54/kg,* compared to NPA 20 which sells for $62.10/kg. Beekeepers may negotiate a price with honey companies for honey above NPA 25.

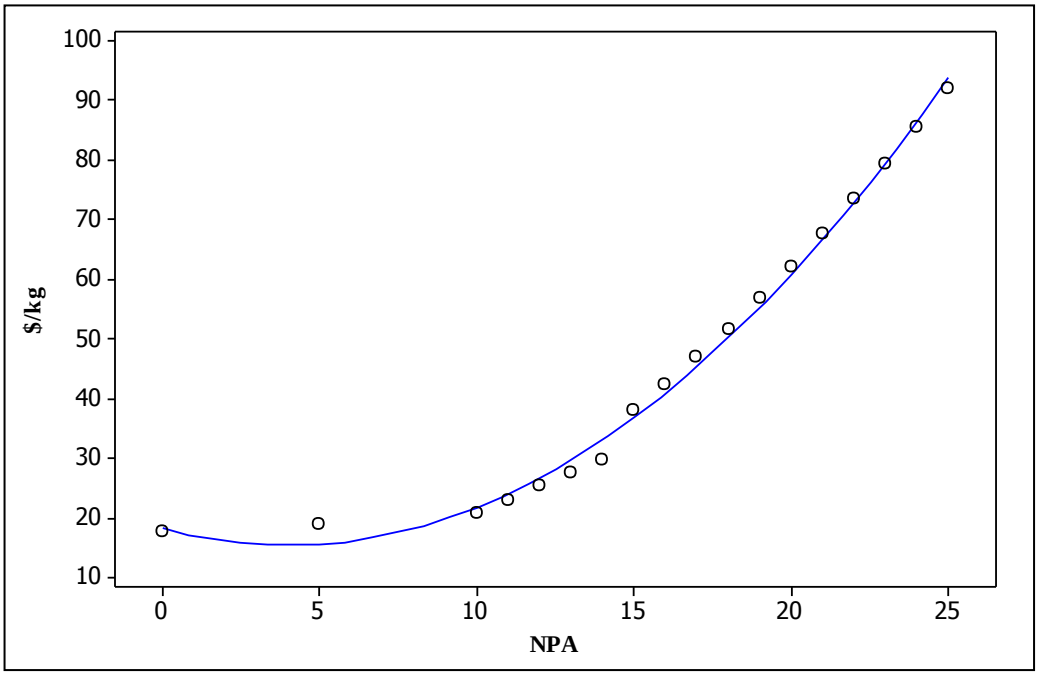


Figure 1.7 Price per kg of mānuka honey vs. NPA.

NPA increases as the honey matures; currently honey producers store barrels of honey for an extended period of time to try and increase its value. Some producers ensure that the barrels are kept at a constant temperature. This costs the producer money in terms of floor space and electricity and they are unable to predict what the final NPA rating will be. Repeated analyses in commercial laboratories to

* These prices are based on August 2012, but are subject to market requirements.

follow the maturation can be expensive and they provide only snapshots of the MGO and DHA concentrations. These tests are usually carried out until the honey reaches a level required by a buyer or until MGO no longer increases.

Length of storage and temperature are the only two variables that honey producers can vary in order to increase the MGO concentration. At present there is not a lot of information on the conversion of DHA to MGO in a honey matrix. For many producers, optimum storage temperature and time are unknown and there is currently no way to predict the final MGO concentration of individual batches of immature honeys. Knowledge of this conversion will allow honey producers to choose optimum storage conditions for their honey so that they can gain the most efficient conversion in the shortest time, which will reduce their expenses and increase their return.

In addition to finding the most efficient conversion of DHA to MGO, the concentration of HMF must also be taken into consideration because honey must contain less than 40 mg/kg HMF if it is to be sold internationally. Therefore the return in maximum MGO concentration must be complemented by a low HMF concentration; the duration of storage must also be reasonable.

1.8.2 Outline of thesis

Chapter 2 details the experimental procedures used throughout this study.

This research first examined four HPLC methods to accurately quantify DHA, MGO and HMF (chapter 3). There is currently no official method for simultaneous detection of DHA, MGO and HMF and at the beginning of this research, commercial laboratories did not have a method for simultaneous detection of DHA, MGO or HMF. The four methods were compared and the advantages and disadvantages of each method were examined to choose the most suitable HPLC method for this research.

Chapter 4 profiles a database of mānuka honeys to quantify some major and minor components (including moisture content, proline, primary amino acids, major and minor elements and selected phenolic compounds) that may affect the

conversion of DHA to MGO. New Zealand clover honeys (n = 4) were also analysed for comparison. Currently there is little information on the profile of mānuka honey; there is no large sample set of mānuka honeys that have been tested for a range of chemical and physical properties already in the literature. Therefore this work will be valuable as it contributes a range of information on mānuka honey for a reasonable size of samples. At the time of printing, the New Zealand Ministry of Primary Industry began work on profiling a large set of mono-floral honeys.

Model systems (using artificial honey doped with DHA and other compounds) were stored at various temperatures (20, 27 and 37 °C) and periodically tested to examine the effect of individual compounds on the conversion of DHA to MGO (chapter 5). The collective effect of perturbants was also examined.

Mānuka and clover honeys doped with DHA stored at various temperatures (4-37 °C) were examined (chapter 6). Various compounds present in these honeys were examined to see if they had an effect upon the conversion of DHA to MGO.

The appearance of HMF in artificial honeys with various perturbants and in real honeys (chapter 7) has also been examined.

Ultimately, a model was constructed (chapter 8) using information from chapters 5 and 6 to simulate the conversion of DHA to MGO. The model was tested against the artificial and real honey matrices that have been stored in controlled environments. The model may allow beekeepers to sell their freshly harvested honey based on a projected outcome; thus removing the requirement for them to store and periodically test the samples. This chapter also includes a model to simulate the formation of HMF in honey, using information from chapter 7. A combination of the two models may allow honeys to be packaged and shipped under controlled conditions so that the honey reaches its optimal potential when it is on the shelf to be sold. Furthermore the model may set preferred storage conditions for the honey.

Each chapter has its own small conclusion, but the main conclusion and recommendations for further research are summarised in chapter 9.

Chapter Two

2 Materials and Methods

2.1 Honey samples

Twenty three mānuka honey samples were kindly provided by Steens Honey Ltd. (Te Puke, New Zealand). These honeys were used in the database of mānuka honeys and storage trials and ranged from harvest years 2003 to 2013, with varying NPA values. NPA measurements analysed by a commercial laboratory were provided by Steens.

Mānuka honey samples were also kindly provided by Gibbs Honeybees, Masterton, NZ. These included honeys for storage trials and samples that were used in the database of mānuka honeys. Mānuka honey 1404 was kindly provided by New Zealand Honey Traders, Northland, NZ. Other mānuka honeys were purchased from commercial outlets. These were Katikati mānuka honey (Katikati, New Zealand, batch 5/12, best before 3/2017) and SummerGlow mānuka honey (Hamilton, New Zealand).

Clover honeys, were purchased from commercial outlets; therefore their origin and date of harvest is not known. There is no known benefit to storing clover honey, therefore it is expected that it was harvested in the same year as purchased. These samples were Happy Bee clover honey (Hamilton, New Zealand), Airborne pure natural New Zealand clover honey (Leeston, Canterbury, New Zealand, batch 113411, best before 29/03/15), Katikati clover honey (Katikati, New Zealand, batch 43/11, best before 11/2016) and Holland clover honey (Timaru, New Zealand).

All honeys were stored in a cold room (3-5 ºC) or freezer (−20 ºC) when not used.

2.2 Standards and derivatising agents

HPLC

Methylglyoxal (MGO, 40% *w/w*), 1,3-dihydroxyacetone (DHA, 97%) and 5-hydroxymethyl-2-furaldehyde (HMF, 99%) were obtained from Sigma-Aldrich. *O*-(2,3,4,5,6-Pentaflurobenzyl)hydroxylamine hydrochloride (PFBHA, 99+%)

was obtained from Alfa Aesar. Hydroxyacetone, (HA, ≥ 90%) was from Aldrich. *o*-Phenylendiamine (OPD, ≥ 98.0%), D-(+)-glucose, sucrose, D-(-)-fructose and D-gluconic acid lactone (99-100%) were obtained from Sigma-Aldrich.

There is a complication with the concentration of purchased MGO. Bottles of MGO purchased from Sigma Aldrich and Fluka have a batch number corresponding to a certificate of analysis which provides the concentration of MGO in the bottle. An older bottle of MGO (Sigma Aldrich, product number M0252, lot number 1375410) shows MGO is 47.0% in water; a different bottle (Fluka, product number 67028, lot number 1343502) reports 43.2%. There is no indication on either bottle (or other bottles) whether this is *w/w*, *w/v* or *v/v*. Correspondence with Sigma has provided responses variously of *w/v*, *w/w* and *v/v*. As the density of purchased MGO is not equal to 1.000 the difference between these is significant.

The method used by Sigma to determine the concentration and molecular weight of MGO describes the final concentration as being in the form of "theoretical molecular weight divided by the apparent molecular weight,"[102] thus the concentration of MGO in this thesis is taken to be *w/w*%.

Proline analysis

Ninhydrin and L-proline were obtained from Sigma-Aldrich.

ICP-MS

ELAN DRC Setup/Stability/Masscal 10 ppb solution was used for X-Y calibration, ion lens voltage, auto lens calibration and daily performance. Stock solutions of elements used for calibration standards, Multi-element standard Merck IV (Ag, Al, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Sr, Tl and Zn) and individual standards S, Si and Hg were obtained from Merck.

2.3 Solvents, compounds and consumables

Solvents

Type I water, also called milli Q, (distilled and deionised) was obtained using a Barnstead E-pure system (18.0 MΩcm).* Acetonitrile was obtained from Honeywell, Burdick and Jackson or by Merck. Methanol was supplied by Scharlau.

Hydrochloric acid, propan-2-ol, and hydrogen peroxide were obtained from Ajax Fine Chem Pty Ltd.. Nitric acid and formic acid (98-100%) were obtained from Merck. Sodium hydroxide pellets were obtained from Univar APS Fine Chem.

Compounds

Phosphate buffer was made up using di-sodium hydrogen orthophosphate dihydrate, obtained from BDH chemicals and sodium dihydrogen phosphate, obtained from M&B.

Compounds used as perturbants in the storage trials were as follows: propylamine, diethylamine, *N*-methylacetamide, L-proline, L-alanine and L-serine and L-lysine obtained from Sigma-Aldrich and iron sulphate and potassium phosphate obtained from Ajax Fine Chem Pty Ltd.

Consumables

Falcon tubes™ were used for ICP-MS analysis. Millipore syringe filters (cellulose acetate, 0.45 µm) were used for filtering honey.

2.4 General Methods

Glassware cleaning

All vials used for HPLC analysis were acid-washed before use; vials were first washed in detergent and rinsed before being submerged into a bath of concentrated nitric acid and soaked overnight. On removal, tap water was run over them for ten minutes before being rinsed at least three times with tap water then distilled water. The vials were oven-dried.

* From this point forward in the thesis 'water' will refer to type I water unless otherwise stated.

All other glassware was washed with warm water and dishwashing liquid, rinsed with distilled water and oven-dried.

Statistical analysis

Statistical analyses were carried out in Microsoft© Excel 2007, Minitab® 16 Statistical Software or Matlab® 2013a Student Version.

2.5 Determination of moisture content

Moisture content of honey samples was determined using a Misco Palm Abbe PA203 digital refractometer. Samples were left at 20 °C for four hours before analysis. The samples were stirred then placed into the machine and left for one minute for the temperature to equilibrate. Readings were taken at 30 second intervals until three consecutive values were constant.

The digital refractometer was calibrated against results from Steens in-house method (n = 35), using an Atago handheld honey moisture meter. Eurofins LTS Ltd. also provided measurements for six of these samples using AOAC 18th edition method 950.46 B, following AOAC 826.08 pretreatment.

2.6 Determination of pH and acidity of honey

The determination of pH and acidity in honey was based on the AOAC Official method 962.19[103] and International Honey Commission (IHC)[104] methods.

Honey (10.0 ± 0.2 g) was dissolved in water (75 ± 0.1 mL). The pH was recorded. The sample was stirred with a magnetic stirrer and titrated with 0.05 M NaOH at a rate of 5.0 mL/min until pH 8.5 was obtained. The titration was completed within two minutes. 0.05 M NaOH (10 ± 0.02 mL) was immediately added and the sample was back-titrated with 0.05 M HCl to pH 8.3.

Free acidity was calculated with the following equation:

$$Free\ acidity\ \left(\frac{milliequivalent}{kg}\right) = \frac{volume\ of\ titrant\ used\ \times\ [titrant]}{mass\ of\ titrand\ honey} \times\ 1000 \quad (2\text{-}1)$$

Lactone was calculated with the following equation:

$$Lactone\ \left(\frac{milliequivalent}{kg}\right) = \left(10 - volume\ of\ titrant\ (ml)\right) \times [titrant]\ \times \frac{1000}{mass\ of\ titrand\ honey} \quad (2\text{-}2)$$

Total acidity was calculated with the following equation:

$$Total\ acidity = free\ acidity + lactone \quad (2\text{-}3)$$

2.7 Analysis and quantitation of DHA, MGO and HMF by high performance liquid chromatography

HPLC was performed using four different methods. One method directly analysed the samples for DHA, MGO and HMF simultaneously so will be known as the Direct method; the other three methods required derivatisation before analysis; *o*-phenylendiamine (OPD) was used to detect MGO and DHA in two different methods, hence they will be known as OPD-MGO and OPD-DHA methods respectively. *O*-(2,3,4,5,6-Pentaflurobenzyl)hydroxylamine hydrochloride (PFBHA) was used as the derivatisation agent for analysis of DHA, MGO and HMF simultaneously; this method will be known as the PFBHA method.

2.7.1 HPLC by the Direct method

2.7.1.1 Instrumentation and method

The Direct method was based on Adams *et al*..[12] HPLC was performed on an HPLC system fitted with a Waters 515 pump and 996 photodiode array (PDA) detector (240-400 nm), refractive index (RI) detector, column oven, Rheodyne 7725i injector fitted with a 20 µm loop and an Alltech Elite degassing system. Two columns were used in series for separation of compounds; these were Shodex KS-801 and Shodex KS-802 and were maintained at 50 ºC. The system was controlled using Waters Empower™ 2 Chromatography software.

The system was operated isocratically with 1 mL/min of water. UV detection at the λ_{max} was used to confirm the presence of MGO and DHA (284 and 270.9 nm respectively). HMF (284 nm) was integrated using UV. MGO and DHA were integrated using the RI chromatogram.

2.7.1.2 Standards for DHA, MGO and HMF

The HPLC instrument was calibrated using six point calibration curves for DHA, MGO and HMF. All standards were made up as *w/w*. Stock solutions of the compounds of interest were used to dope a clover honey matrix to the required concentrations.

Stock solutions of DHA were made up to 10 ± 0.02 g, to give a final concentration in the standard between 100 to 1,500 mg/kg. Stock solutions of MGO and HMF (together) were made up to 10 ± 0.02 g to give a final concentration in the standard between 200 to 2,000 mg/kg for MGO and 0 to 100 mg/kg for HMF.

To make the standards, a 50% (*w/w*) stock of clover honey solution was made. The resulting honey solution was weighed into 6 vials (5.00 ± 0.02 g) and the corresponding DHA stock standard (500 ± 5 mg) and the corresponding MGO and HMF stock standard (500 ± 5 mg) were added to the honey solution. This was filtered through a 0.45 µm syringe filter and injected into the HPLC for analysis. DHA concentrations decreased from standard 1 through to 6, while MGO and HMF concentrations increased.

Large masses were used to minimise weighing errors. For standard 1, which did not have MGO and HMF stock added, and for standard 6 which did not have DHA stock added, water (500 ± 5 mg) was added to keep the matrix consistent. Equations 2-4 to 2-6 show the calculations used for the standards.

$$Concentration\ of\ stock\ solution\ \left(\frac{mg}{kg}\right) = \left(\frac{Weight\ of\ compound\ (mg)}{Weight\ of\ final\ solution\ (kg)}\right) \times\ purity\ of\ compound \tag{2-4}$$

$$Compound\ added\ to\ standard\ (mg) = \left(\frac{Weight\ of\ stock\ added\ (mg)}{10^6\ \frac{mg}{kg}}\right) \times\ concentration\ of\ stock\ solution\ (\frac{mg}{kg}) \quad (2\text{-}5)$$

$$Compound\ in\ honey\ standard\ \left(\frac{mg}{kg}\right) = \frac{Compound\ (mg)}{Total\ weight\ of\ standard\ (kg)} \quad (2\text{-}6)$$

Linearity was evaluated by least-squares regression analysis. A value of 0.995 was deemed to be the lowest acceptable correlation coefficient (R^2) acceptable for analysis.

Elution times and wavelengths at which chromatograms were extracted for integration are summarised in Table 2.1. Stock solutions were stored in the freezer until required. Riddle and Lorenz[105] observed that solutions of DHA in distilled water were stable in the refrigerator for 6 months or longer.

Table 2.1 Elution time and λ_{max} of compounds of interest for the Direct method.

Compound	Elution time for RI (min)	Elution time for UV (min)	λ_{max}
MGO	20.5	20.4	284
DHA	24.0	23.5	270.9
HMF	58.0	52.1	284

2.7.1.3 Sugar standards

All standards were made up as *w/w*. Sugar standards were made up in a water matrix. Glucose was in a standard by itself. Sucrose and fructose were weighed out into the same standard. Equation 2-7 shows the calculation used for the sugar standards.

$$Concentration\ of\ sugar\ \left(\frac{g}{100g}\right) = \left(\frac{Weight\ of\ sugar\ (g)}{Total\ weight\ (g)}\right) \times\ 100 \quad (2\text{-}7)$$

Fructose and glucose standards ranged from 0-50 g/100 g and sucrose standards ranged from 0-2.5 g/100 g. Due to the high proportion of sugar in honey, the standards and samples had to be diluted 40-fold for glucose and fructose to have baseline separation. High concentration standards were made up and then diluted to minimise weighing errors. The concentrations of fructose, glucose and sucrose were calculated by integrating the corresponding peaks in the RI chromatogram. Sucrose eluted at 13.5 minutes; glucose and fructose eluted at 14.5 and 17.5 minutes respectively. Stock standards were stored in the freezer until required.

2.7.1.4 Sample preparation

Honey samples were thawed and thoroughly mixed before weighing into a vial (3 ± 0.2 g). Water (3.0 ± 0.2 g) was added to give a 50% solution (*w/w*). Vortexing and sonication were required to dissolve the honey. Samples were centrifuged at 4.4 x10^3 rpm for 20 minutes. The supernatant was filtered through a 0.45 µm syringe filter before analysis. Samples were injected directly for determination of DHA, MGO and HMF; they were diluted 40-fold for sugar analysis to allow for separation of glucose and fructose peaks.

2.7.2 HPLC by the PFBHA method

2.7.2.1 Instrumentation and method

The PFBHA method is based on Windsor *et al.*.[37] HPLC was performed on an HPLC system fitted with two Waters 515 pump, 996 photodiode array (PDA) detector (240-400 nm), autosampler (set at 20 ºC) and an Alltech Elite degassing system. A Waters SymmetryShield RP18 column (5 µm, 3.0 x 250 mm) was used for separation of compounds; a 20 µL injection was used. The system was controlled using Waters Empower™ 2 Chromatography software. The system was operated with gradient elution (Table 2.2) using 30:70 (*v:v*) acetonitrile (ACN):H_2O as mobile phase A and 100% ACN as mobile phase B. The gradient

elution was run at a constant combined flow rate of 0.8 mL/min. The time of elution and wavelength at which the chromatogram for each compound was extracted at is summarised in Table 2.3. Two isomers of MGO were observed, with the largest (peak 2) accounting for approximately 95% of the MGO. HMF also had two isomers; however only isomer 1 (11.19 min) was quantified, due to an unknown interference with isomer 2.

Table 2.2 Gradient elution program for the PFBHA method.

Time (min)	Solvent A*	Solvent B†
0	90	10
2.5	90	10
10.5	50	50
18	0	100
25	90	10
35	90	10

* 30:70 (*v/v*) ACN:H_2O

† 100% ACN

Table 2.3 Elution time and λ_{max} for compounds of interest.

Compound	Elution time (min)	λ_{max}
DHA	6.52	260
HA	9.89	260
HMF	11.19	279
MGO isomer 1	18.53	244.3
MGO isomer 2	19.00	244.3

2.7.2.2 Standards

Hydroxyacetone (HA, 3.34 mg/ mL) was used as the internal standard solution. The PFBHA derivatising solution (20 mg/mL) was made up in citrate buffer (0.1 M) and adjusted to pH 4 with NaOH (4 M). Both solutions were stored in the fridge.

A six point calibration curve was made up for each compound (DHA, MGO and HMF) using a multi-compound standard. Stock standards of DHA and MGO + HMF were made up in water in varying concentrations. DHA ranged from 0 to 13,000 mg/kg, MGO ranged from 0 to1,900 mg/kg and HMF ranged from 0 to 500 mg/kg. The stock solutions were stored in the freezer until required.

Standards were made up in an artificial honey matrix (0.20 ± 0.02 g). Increasing DHA and decreasing MGO and HMF concentrations were in standards one through to six. Internal standard (250 ± 2.5 µL), DHA stock (100 ± 1 µL) and MGO + HMF stock (40 ± 0.4 µL) were added, mixed and left for one hour. PFBHA derivatisation solution (1,200 ± 30 µL) was added and mixed. Samples were left for one hour to complete derivatisation. ACN (6 ± 0.05 mL) was added and the sample mixed until all crystals dissolved; water (2 ± 0.02 mL) was added and the standards were mixed before analysis.

A linear calibration curve was constructed using the HPLC peak area ratios of DHA:HA, MGO peak 1 + MGO peak 2:HA and HMF peak 1:HA against the mass of the compounds.

2.7.2.3 Sample preparation

Honey samples (0.20 ± 0.02 g) were weighed into test tubes, HA (250 ± 2.5 µL) was added, the sample was mixed and left for one hour, at which time PFBHA derivatising solution (1,200 ± 30 µL) was added, mixed and left for a further hour. ACN (6 ± 0.05 mL) was added and mixed until all crystals were dissolved; water (2 ± 0.02 mL) was added and mixed before analysis.

2.7.3 HPLC by the OPD Method for MGO determination

2.7.3.1 Instrumentation and method

The OPD method was based on Adams *et al.*.[12] HPLC was performed on an HPLC system fitted with two Waters 515 pump, 996 photodiode array (PDA) detector (240-400 nm), Waters 717plus Autosampler (5 µL injection), and an Alltech Elite degassing system. A Waters SymmetryShield RP18 column (5 µm, 3.0 x 250 mm) was used for separation of compounds. The system was controlled using Waters Empower™ 2 Chromatography software. The system was operated with gradient elution (Table 2.4) with 0.075% acetic acid in water as mobile phase A and 80:20 (*v/v*) methanol (MeOH):H_2O as mobile phase B. The gradient elution was run at a constant combined flow rate of 0.3 mL/min. MGO eluted at 31.49 minutes and the chromatogram was extracted at 312 nm.

Table 2.4 Gradient elution program for the MGO-OPD method.

Time (min)	Solvent A[1]	Solvent B[2]
0	90	10
4	90	10
5	58	42
30	45	55
31	0	100
34	0	100
35	90	10
40	90	10

[1] 0.0075% acetic acid in water

[2] 80:20 (*v/v*) MeOH:H_2O

2.7.3.2 Standards

MGO standards were made up in water, ranging from 0 to 0.2 mg/mL. Standard (1.50 ± 0.02 mL) was added to 2% (*w/v*) OPD in 0.5 M phosphate buffer (0.75 ± 0.01 mL, pH 6.5) and reacted for four hours at room temperature with light excluded. Standards were stored in the freezer until required.

2.7.3.3 Sample preparation

Honey (0.60 ± 0.02 g) was dissolved in water (30% (*w/v*)). Honey solution (1.50 ± 0.02 mL) was reacted with 2% (*w/v*) OPD in 0.5 M phosphate buffer (0.75 ± 0.01 mL, pH 6.5) and left to react for four hours. Reactions were performed in the dark at room temperature. Samples were filtered using a 0.45 µm filter before analysis.

2.7.4 HPLC by the OPD Method for DHA determination

2.7.4.1 Instrumentation and method

The OPD method was based on Atrott *et al.*[106] HPLC was performed on an HPLC system fitted with two Waters 515 pump, 996 photodiode array (PDA) detector (240-400 nm), Rheodyne 7725i injector fitted with a 20 µL loop, and an Alltech Elite degassing system. A Waters SymmetryShield RP18 column (5 µm, 3.0 x 250 mm) was used for separation of compounds. The system was controlled using Waters Empower™ 2 Chromatography software. The system was operated with gradient elution (Table 2.5) with 0.075% acetic acid in water as mobile phase A

and 80:20 (*v/v*) MeOH:H_2O as mobile phase B. The gradient elution was run at a constant combined flow rate of 0.3 mL/min. DHA eluted at 13.25 minutes and the chromatogram was extracted at 312 nm for integration.

Table 2.5 Gradient elution program for the DHA-OPD method.

Time (min)	Solvent A*	Solvent B†
0	60	40
1	60	40
21	0	100
25	60	40
32	60	40

* 0.0075% acetic acid in water

† 80:20 (*v/v*) MeOH:H_2O

2.7.4.2 Standards

A 6 point calibration curve was created. DHA stock solutions were made up so that the final DHA standards ranged from 100 to 2,000 mg/kg. Artificial honey (1.00 ± 0.02 g) and the corresponding DHA stock (1.00 ± 0.01 mL) were added to a volumetric flask (10 mL) and made up with acetate buffer (0.5 M, pH 4.29).

2.7.4.3 Sample preparation

Honey (0.50 ± 0.02 g) was dissolved in 0.5 M acetate buffer (10% (*w/v*), pH 4.29) to make a honey solution. Honey solution (3.00 ± 0.02 mL) was reacted with 1% (*w/v*) OPD in acetate buffer (0.90 ± 0.01 mL) and acetate buffer (0.90 ± 0.01 mL) and left to react for 16 hours at 37 °C. Samples were filtered using a 0.45 µm filter before analysis.

2.8 ICP-MS

A Perkin-Elmer SCIEX ELAN DRC II inductively coupled mass spectrometer (ICP-MS) with a quadrupole mass spectrometer (PerkinElmerSciex, Concord, Ontario, Canada) was used for trace element analysis. The ICP-MS was controlled by ELAN software, version 3.3. The front end of the ICP-MS consisted of a Seaspray nebuliser and a baffled cyclonic spray chamber both from Perkin Elmer. A mixing block was used to dilute the sample 1:1 with internal standard.

2.8.1 ICP-MS calibration

A series of liquid calibrations were run in a specific order to ensure optimum performance of the ICP-MS. All calibrations apart from the Dual Detector Cross Calibration were performed using ELAN DRC Setup/Stability/Masscal 10 ppb Solution. The dual detector solution was used for dual detector calibration.

X-Y calibration of the cones

The sampler cone and the skimmer cone were adjusted in the X and Y directions until maximum counts for In were obtained.

Ion lens voltage

The lens voltage was adjusted until maximum counts for In were obtained.

Autolens

An autolens calibration was automatically performed by the instrument. The autolens was steadily increased to find the maximum for Mg, In and Pb. A lens voltage versus mass graph was obtained, which should be linear with a positive slope. The calibration was repeated if the line was not correct or if a point sat too far from the line.

Daily performance

The requirements for a successful daily performance calibration are listed in

Table 2.6. Net intensity mean values were arbitrarily chosen to monitor the sensitivity of the ICP-MS. The values were chosen according to the reported sensitivity from the manufacturer's specifications and by monitoring the ICP-MS on a long term basis. If the machine failed to meet these criteria, the instrument was recalibrated until they were achieved.

Dual detector cross calibration

A dual detector cross calibration was automatically performed by the instrument. This ensured the two detectors overlapped. The calibration was repeated if one or more elements only had one data point.

Table 2.6 Expected values for calibration using liquid standard Element/Parameter.

	Concentration in Solution	Net intensity mean/Threshold
Mg	10 ppb	> 42000
In	10 ppb	> 200000
Pb	10 ppb	> 100000
Background 220	N/A	< 3%
% Ba^{2+}/Ba	Ba at 10 ppb	< 3%
% CeO/Ce	Ce at 10 ppb	< 3%

2.8.2 Sample preparation

A stock of honey (10.0 ± 0.2 g) and water (5.0 ± 0.2 g) was made up to take sub-samples from because honey is not homogenous. The aid of sonication was used to dissolve the honey. Sub-samples (1.80 ± 0.02 g) were taken in triplicate for analysis from the stock and weighed into Falcon® tubes (50 mL). HNO_3:H_2O_2 (2:1, 6.00 ± 0.04 mL) was added and samples were left to digest overnight before being heated in a heating block at 75 ºC for 90 minutes. The tops were only loosely fastened to avoid pressure build up. Samples were cooled before diluting 200 times to bring the acid concentration down to 2% for ICP-MS analysis.

Blank samples were run in triplicate with every batch of samples analysed.

2.8.3 ICP-MS parameters and data analysis

ICP-MS was used to determine ^{10}B, ^{23}Na, ^{24}Mg, ^{27}Al, ^{31}P, ^{34}S, ^{39}K, ^{43}Ca, ^{51}V, ^{52}Cr, ^{54}Fe, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{68}Zn, ^{75}As, ^{82}Se, ^{88}Sr, ^{109}Ag, ^{111}Cd, ^{137}Ba, ^{202}Hg and ^{207}Pb. ^{56}Fe, ^{55}Mn and ^{75}As isotopes were also determined using the dynamic reaction cell (DRC) with NH_3 as the reacting gas. Table 2.7 summarises the ICP-MS parameters.

Table 2.7 Parameters used for ICP-MS analysis.

Parameter	Unit	Value
RF power	W	1350
Dwell time per AMU	ms	50
Plasma gas	mL/min	15
Sweeps/reading	-	5
Reading/replicate	-	3
Replicates	-	3
Scan mode	-	Peak hopping

2.9 Amino acid determination

2.9.1 Primary amino acids

Honey samples were sent to the Nutrition Laboratory at Massey University for primary amino acid analysis. The samples were analysed using AccQ Tag derivatisation, AOAC 994.12 with HPLC separation.[107]

2.9.2 Proline determination

Honey samples (n = 10) were sent to the Nutrition Laboratory at Massey University for proline analysis. Honey samples were analysed using AccQ Tag derivatisation, AOAC 994.12 with HPLC separation.[107]

The proline content of honey was also carried out on site using ninhydrin derivatisation. The proline method was based on the International Honey Commission Method,[108] which is similar to the original method by Ough[109] and also the AOAC Official Method 979.20 Proline in Honey.[110]

2.9.2.1 Standards

Proline Stock – A stock solution of L-Proline (40.0 ± 0.2 mg) was made up to 50.00 ± 0.05 mL using water and stored in the freezer until required.

Proline Standard – Stock solution (2.00 ± 0.02 mL) was transferred and made up to 50.00 ± 0.05 mL with water to make the standard (0.8 mg/25 mL).

Ninhydrin Solution (3%) – Ninhydrin (0.60 ± 0.05 g) was dissolved into monomethyl glycol ether (20.00 ± 0.03 mL). This solution was made fresh daily.

In a vial (15 mL), standard solution (0.50 ± 0.05 mL), formic acid (1.00 ± 0.01 mL) and ninhydrin (1.00 ± 0.01 mL) were added and sonicated for 15 minutes. Samples were placed in a steam bath for 15 minutes then transferred to a 70 ºC water bath (10 minutes). Propan-2-ol: water (1:1) solution (5.00 ± 0.03 mL) was added to each vial and the lid immediately replaced. Samples were analysed by UV within 30 minutes at 510 nm. Standards were repeated in duplicate and the average reading was used for calculation. A duplicate of standards was run with every batch of samples.

2.9.2.2 Samples

Honey (2.50 ± 0.05 g) was weighed into a beaker. Water (20.00 ± 0.02 mL) was added to dissolve the honey. The solution was transferred to a volumetric flask (50.00 ± 0.05 mL) and made up with water.

In a vial (15 mL), honey solution (0.50 ± 0.05 mL), formic acid (1.00 ± 0.01 mL) and ninhydrin (1.00 ± 0.01 mL) were added and sonicated for 15 minutes. Samples were placed in a steam bath for 15 minutes then transferred to a 70 ºC water bath (10 minutes). Propan-2-ol: water (1:1) solution (5.00 ± 0.03 mL) was added to each vial and the lid immediately replaced. Samples were analysed by UV within 30 minutes at 510 nm against a blank of water. Samples were analysed in triplicate.

2.10 Storage trials

2.10.1 Artificial honey

Model systems for the storage trials were made using artificial honey and doping them with either DHA or MGO. Artificial honey was made with fructose (41.60%), glucose (37.30%), sucrose (2.91%) and water (18.20%). The water was used to dissolve the stock solution of DHA or MGO. The pH was adjusted to 3.8-4 using gluconic acid for DHA as the solution was above 4. Sodium citrate was used to adjust the pH of MGO solutions because the solution was too acidic (~pH 2). MGO is not expected to be acidic; however, due to a small amount of pyruvic acid impurity, the solution is acidic. Pyruvic acid is a relatively strong organic

acid (pKa = 2.49). Sareen *et al.*[111] noted that a solution of MGO with a pH of 2 corresponds to 0.07% by mole impurity of pyruvic acid.

40.00 ± 0.02 g of artificial honey was used for the storage trials. Fructose (16.64 ± 0.02 g), glucose (14.92 ± 0.02 g) and sucrose (1.16 ± 0.02 g) were mixed together. 7.28 ± 0.02 g of water was required to give it the correct water content. For control samples, 7.00 ± 0.02 g of the DHA/MGO stock solution were used. In cases where additional compounds were added to the matrix the concentration of the stock solution was adjusted so that only 6.5 g of water was required to give the correct concentration in the honey. The other 0.50 g was used to dissolve the additional compounds; the pH was corrected to 3.5-4 before addition to the honey. DHA stock solutions were made so that the final concentration in the honey was either 10,000 mg/kg or 2,000 mg/kg. MGO stock solutions were made so that the final concentration in the honey was 2,000 mg/kg. Samples were incubated at 20, 27 or 37 °C and sub-samples were periodically removed for analysis by HPLC.

2.10.2 Real honey matrices

Real honeys (40.00 ± 0.02 g) had DHA stock solution (1.00 ± 0.01 mL) added to them. The stock solution of DHA was made to have a final concentration of 2,000 or 10,000 mg/kg when added to the honey. The pH was adjusted to 3.8-4 using gluconic acid before being added to the honey. Honeys were incubated at 4, 20, 27 or 37 °C. Sub-samples were periodically removed for analysis by HPLC.

2.11 High pressure processing (HPP)

Artificial honey doped with 2,000 mg/kg DHA, two store-bought clover honeys doped with 1,200 mg/kg and one store-bought and two freshly harvested mānuka honeys were used for analyses. Doped samples were prepared the same way as described for the storage trials in section 2.10. Analyses were carried out in duplicate. Each sample was divided into ten sub-samples. One sub-sample was the control and was not subjected to HPP treatment. The other nine sub-samples (5.0 ± 0.2 g) were packed in 5 x 5 cm transparent plastic film pouches (Cas-Pak plastic vacuum pouch, New Zealand) and thermosealed under vacuum. Each sub-sample was subjected to a different high pressure processing (HPP) treatment.

HPP was carried out in the Department of Chemical and Materials Engineering at The University of Auckland. A HPP unit Avure 2 L – 700 Laboratory Food Processing System (Avure Technologies, Columbus, OH, USA) was used. The equipment consisted of a 2 L cylindrical shaped pressure treatment chamber, water circulation, a pumping system and a control system operated through a computer with software supplied by the manufacturer. Distilled water was used as the medium in the chamber in which the honey samples were placed.

Samples were subjected to different combinations of pressure (100, 400 and 600 MPa) and time (15, 45 and 90 min) while the temperature was held close to ambient. The treatment time was the holding pressure time and did not include the time taken to reach the desired pressure (approximately 1.5 minutes) or the decompression time (<20 seconds). The adiabatic heating of 100, 400 and 600 MPa gave an average processing temperature of 25.75 ± 0.95, 28.71 ± 0.90 and 30.18 ± 2.14 °C, respectively during the holding pressure phase.

After treatment, the samples were immediately cooled in ice water, then stored at −18 °C until analysis by HPLC.

2.12 UPLC-PDA-MS/MS

Ultra Performance Liquid Chromatography PhotoDiode Array Tandem Mass Spectrometry (UPLC-PDA-MS/MS) data was obtained for some clover and mānuka honey samples. The samples were kindly analysed by Analytica Laboratories.

Sample preparation

Honey (1.00 ± 0.02 g) was weighed into a 50 mL tube and extraction solution (9 mL, methanol:water, 50:50, + 0.1% acetic acid) was added. The sample was thoroughly mixed then sonicated (10 minutes). A 1.5 mL aliquot was centrifuged (15,000 rcf*, 20 min, 20 °C) and the supernatant was used for analysis.

* rcf = relative centrifugal force

UPLC-DAD-MS/MS

Separation was performed on a Thermo Scientific UPLC system with diode array detection (DAD) and Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer detection in tandem. Samples were separated on an Agilent Eclipse Plus column (100 x 2.1 mm, 1.8 µm) at 40 °C. The system was operated with gradient elution with 0.1% acetic acid in water as mobile phase A and 0.1% acetic acid in acetonitrile as mobile phase B. The gradient elution was run at a constant combined flow rate of 0.4 mL/min and the analysis time was 33 minutes; a 5.0 µL injection was used. Due to commercial sensitivity, the gradient program, retention times of compounds of interest and instrument parameters are not given.

Leptosperin, methyl syringate, phenyllactic acid, 2-methoxybenzoic acid, 4-methoxybenzonic acid, syringic acid and luteolin were quantified.

Chapter Three

3 High performance liquid chromatography (HPLC) analysis of DHA, MGO and HMF – comparison of methods

As previously mentioned in chapter 1, MGO was discovered as the compound responsible for the non-peroxide antibacterial activity in mānuka honey in 2008. Since then, commercial laboratories have focused on directly quantifying MGO in mānuka honey instead of using an indirect method (well diffusion assay). Until 2014, commercial laboratories routinely analysed MGO predominately by *o*-phenylenediamine (OPD) derivatisation with HPLC-UV or GC to detect MGO in honey. As this thesis goes to print, commercial laboratories are starting to adopt PFBHA derivatisation as the method of choice. However, when this research began, to the authors knowledge, only one non-commercial laboratory was using this method.

A reliable method for analysing DHA, MGO and HMF was required for this research. A method that simultaneously detects all three analytes would be beneficial. Until part way through this research (2013), there was no method reported in the literature which simultaneously analysed DHA, MGO and HMF.

This section of the thesis describes a comparison of four different HPLC methods for the analysis of DHA, MGO and HMF. One method required no prior derivatisation to quantify all three compounds. The other three methods required pre-column derivatisation; one method used PFBHA as the derivatising agent to quantify all three compounds and two methods, with different sample preparation used OPD as the derivatising agent to quantify MGO or DHA.

3.1 Review of analysis methods for MGO, DHA and HMF

3.1.1 Analysis of methylglyoxal

Literature covering MGO detection is common for food matrices and beverages due to the role of MGO in the Maillard reaction. Foods that are heated, processed or stored for a long period of time and contain carbohydrates have been studied, including coffee, baked foods, beer and wine. Biological samples (including plasma, organs, urine and blood) are also frequently analysed for MGO due to the role of MGO in formation of advanced glycation end products (AGEs) in the body.[78, 112] Nemet[78] provides a detailed review on the derivatisation agents, detection and quantification for analysis of MGO in biological and food samples. Recently detection of MGO has become important in mānuka honey because this is the compound responsible for the non-peroxide antibacterial property. This review will be restricted to the analysis of MGO in honey matrices.

The reactivity, low molecular mass and the lack of a strong UV chromophore make the detection of MGO difficult; therefore it usually has to be derivatised before analysis. HPLC or GC analysis are the main techniques used for quantification of MGO. A further difficulty for MGO analysis is its availability in a matrix; MGO is known to bind to other compounds in the matrix, such as amino acids. Lo *et al.*[113] suggested that over 99% of MGO in physiological systems is involved in reversible or irreversible reactions. There is no information on the percentage of MGO bound in food matrices.

HPLC is a common technique for MGO analysis in honey. Weigel *et al.*[68] first reported MGO in multifloral honeys in 2004, while examining MGO in foods. They used OPD derivatisation with HPLC-UV analysis. MGO reacts with the amine groups on OPD to give a stable quinoxaline, which can be detected by UV (Figure 3.1). This method has been reported a number of times for MGO analysis in mānuka honey[12, 68, 114] and was originally the method of choice for MGO analysis in commercial laboratories. The reported HPLC methods have short analysis times. Gradient elution is reported using water with acetic acid (0.075%) and methanol (20%) as the eluents. The reported time for complete derivatisation varies in the literature between 6 and 16 hours (or overnight).[12, 44, 68, 115]

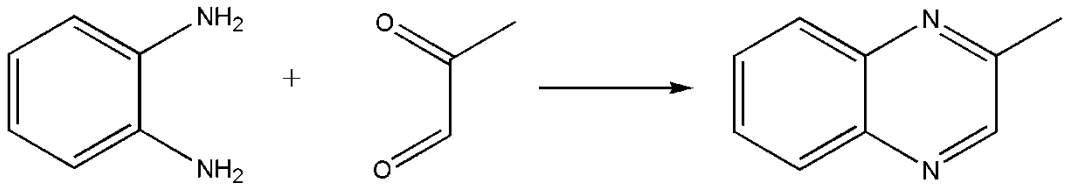


Figure 3.1 Reaction of OPD with MGO to form the stable, UV-active quinoxaline.

Direct detection of honey has been reported using a size exclusion column with RI detection.[12] This method has quick sample preparation (50% dilution) and uses water for isocratic elution, making it a cheap method. Sample preparation is quick and easy and the method can also quantify DHA. However, the chromatogram is prone to interference from co-eluting peaks, which may over estimate MGO for samples with low levels of MGO. In addition, Adams *et al.*[12] reported that the limit of detection for MGO was approximately. 50 mg/kg.

Adams *et al.*[12] compared analysis of MGO as a free compound, (i.e. direct analysis[*]) with OPD derivatisation. The direct analysis used a 50% (*w/v*) solution of honey compared to the OPD method which used a 30% (*w/v*) solution of honey treated with 2% (*w/v*) OPD in 0.5 M phosphate buffer; reactions were performed in the dark at room temperature for 16 hours before analysis. The study analysed 49 mānuka honeys and 34 non-mānuka honeys and reported that the results of the two methods were similar. However, in 2009 they published a corrigendum[89] which stated that a multiplication factor of 1.87 needed to be used for all samples analysed by the derivatisation method. With this factor applied, the difference in the data from the two methods increased; this increase was pronounced for samples with higher levels of MGO. They suggested that this may be caused by the deviation from linearity of the standard curve for the derivatisation method above ~800 mg/kg.[89]

Adams *et al.* reported that the Direct method had ambiguous results for non-mānuka honey and immature mānuka honey samples due to interferences in the matrix. The OPD derivatisation method was reported to have better peak resolution and a tighter fit for MGO concentration vs. NPA than the Direct method (R^2 = 0.98 compared to 0.92).[12] However, Atrott and Henle[13] state that below 10% equivalent phenol (determined by the well

[*] This method will be referred to as the Direct method throughout the text.

diffusion assay, WDA), there is limited sensitivity using the OPD method with analysis by RP-HPLC. They suggest that this limited sensitivity and accuracy may be the reason the corresponding regression lines for the MGO vs. antibacterial graph do not pass through the origin.[13] However, at low levels of NPA accurate measurement using the WDA is problematic. This may be the cause, rather than a poor limit of detection from the OPD method.

Lei *et al.*[116] also reported OPD as the derivatising agent for analysis of MGO in mānuka honey. They reported the LOD and LOQ were 0.02 and 0.06 mg/L respectively (0.03 and 0.09 m/kg respectively[*]). Recoveries were between 98.3 and 101.5% for spiked levels of 50-200 mg/kg. They reported the MGO derivative is stable up to 24 hours for concentrations between 50 and 200 mg/kg.

Stephens *et al.*[5] used a similar OPD derivatisation method to that reported by Adams *et al.*[12] and Mavric *et al.*.[44] The derivatisation was followed by headspace solid phase microextraction and analysis by GC-NPD instead of HPLC. Gas chromatography is a simple, low cost method and is a common instrument in laboratories. The authors reported that this method was sensitive enough to also detect MGO in nectar samples.

OPD is a known catalyst for the conversion of reducing sugars into MGO under certain reaction conditions. This occurs in a high sugar matrix. Homoki-Farkas *et al.*[118] (1997) heated (160-182 °C) sugars (glucose, dextrin and starch) in excess OPD and reported that the amino groups on OPD had a catalytic effect on forming MGO. In 2008, Wang and Ho[119] addressed the issue that OPD could generate MGO from monosaccharides under certain reaction conditions (high temperature and basic pH) due to the two amine groups on OPD which could catalyse the transformation of reducing sugars into MGO. Therefore in a sugar/amino acid/OPD mixture, OPD could play a dual role of catalysing the formation of MGO as well as a trapping agent for MGO derivatisation. After heating a fructose/OPD mixture at 150 ºC for 30 minutes it contained 0.1210 ± 0.0129 mmol/L MGO (12.37 mg/kg), which was 6-fold higher than the system containing only fructose. They stated that OPD is able to dramatically increase MGO generation in the Maillard reaction, depending on the reaction conditions.[119] Honey has a high sugar matrix so is susceptible to catalysis by OPD under certain reaction conditions. However, the

[*] Honey with a water content of 18% has a density of 1.4171 g/kg.[117] This value will be used as an approximation to convert mg/L to mg/kg in this chapter to compare the literature with the current research.

derivatisation procedure used in honey[12-13, 44, 115] occurs in an acidic pH at ambient temperature. Therefore the role of OPD should strictly be a trapping agent, and not a catalyst.

Analysis of MGO in honey by PFBHA derivatisation with HPLC-UV detection has been reported.[37, 120-121] PFBHA reacts with carbonyl groups (Figure 3.2) and the C=N bond in the derivatised product prevents rotation which leads to isomers of MGO. This derivatising agent is expensive and the separation requires acetonitrile, which is also costly. Derivatisation only takes 1 hour (samples were mixed with internal standard and water 1 hour prior to allow dissolution) and a good separation of MGO, as well as DHA and HMF is obtained in a 30 minute analysis. Recently a commercial laboratory have converted the method to UPLC and one analysis takes 7 minutes.[122]

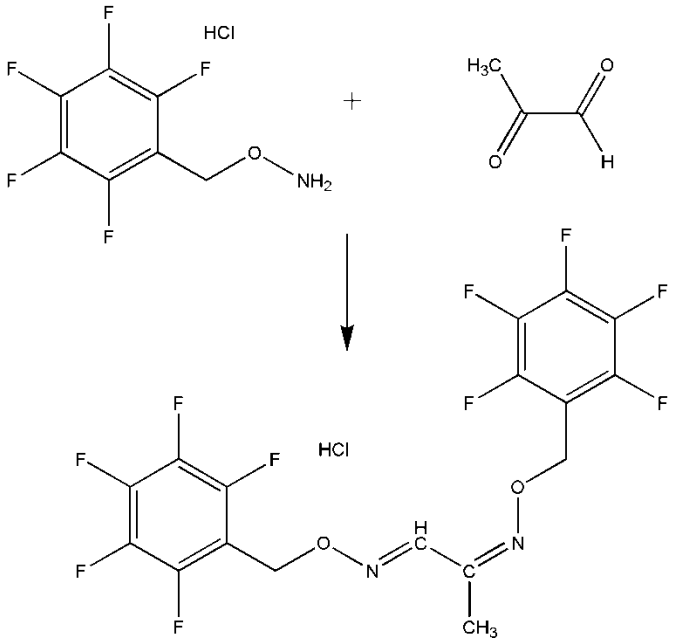


Figure 3.2 Reaction of PFBHA with MGO to form the stable, UV-active dioxime.

Nuclear magnetic resonance (NMR) has also been reported as a method for MGO detection. NMR is not often found in commercial laboratories. While the prospect of using NMR to quantify MGO is interesting, it does not have a practical use due to its inaccessibility. Donarsk *et al.*[90] (2010) were the first to describe the use of quantitative NMR for the measurement of MGO in mānuka honey; they obtained a ^{1}H spectra using a 500 MHz NMR. The authors reported that the results were comparable to analysis of MGO by OPD derivatisation with HPLC detection. This method analyses diluted honey, without the need of prior sample preparation. This method has the advantage that the honey does

not need to be chromatographically separated, but analysis of one sample takes one hour and five minutes; hence the technique is not viable for high-throughput. In 2012, Le Gresley *et al.*[123] reported the use of diffusion ordered spectropscopy (DOSY) to separate compounds in mānuka honey, without the prior separation, and quantify MGO using a 600 MHz NMR. DOSY is non-destructive and the analysis time was just over one hour.

Nemet[78] reported that commercially available MGO contains impurities and it is difficult to purify it due to its high reactivity and ability to polymerise. An advantage of NMR over HPLC or GC methods is that concentration of the MGO standard can be directly determined by the extrapolation of a graph of concentration vs. peak area for the ^{1}H signal of both the methyl peaks of MGO monohydrate and dihydrate corrected using the internal standard 3-trimethylsilyl[2,2,3,3,-D_4]propionic acid (TSP).[90]

3.1.2 Analysis of dihydroxyacetone

Literature on DHA analysis primarily covers sunless tanning creams and more recently honey matrices. Both direct analysis and prior derivatisation have been reported for sample preparation. HPLC and GC analysis are the common methods of detection for DHA. Due to limited literature on the determination of DHA in honey matrices, non-honey matrices will also be reviewed.

DHA analysis in non-honey matrices

Direct analysis of DHA using HPLC with UV or refractive index detection was reported in the early 1980s.[124-125] However, detection with derivatisation is more common. DHA has been analysed as the trimethylsilylated oxime with GC-MS detection in simple sugar matrices.[126] DHA in tanning solutions has been detected as its *bis*-2,4-dinitrophenylhydrazone (DNPH) derivative using RP-HPLC. The α-hydroxyketone reacts with DNPH to form an osazone via molecular rearrangement.[127] Samples were reacted with the derivatising agent at 50 °C for 20 minutes.

In 2007, Biondi *et al.* [128] used PFBHA as a derivatising agent for analysis of DHA in self-tanning creams using RP-HPLC with UV detection. The authors reported that derivatisation was complete after 5 minutes in citrate buffer (pH 4) at ambient temperature, and extra time did not affect the results. This method had good recovery (97-105%) of

DHA for spike recoveries of 25, 50 and 100 µmol. The method was reported to have high efficiency and resolution and was selective for DHA in self-tanning creams.
Becker *et al.*[129] studied two single-step derivatisations (per-trimethylsilylation, isopropylidenation) and four two-step derivatisation methods (ethoximation–trimethylsilylation, ethoximation–trifluoroacetylation, benzoximation–trimethylsilylation, benzoximation–trifluoroacetylation) for analysis by GC-MS of carbohydrate rich complex matrices (eight monosaccharides, glycoaldehyde and DHA). They reported that the ethoximation-trimethylsilyation derivatisation was the best for the biological and synthetic matrices tested.

DHA analysis in honey matrices

At present there is limited literature on analysis of DHA in honey because DHA was only discovered in mānuka honey in 2009.[14] Direct analysis and various derivatising agents (PFBHA, OPD) with HPLC analysis have been reported for detection of DHA in mānuka honey.

Direct analysis of a diluted honey solution using RP-HPLC with refractive index detection has been reported for determination of DHA in mānuka honey. This is the same method reported for MGO analysis. This method was used in the discovery of DHA in mānuka honey in 2009; however limits of detection for this method were not reported.[14]

In 2012, Windsor *et al.*[37] published a method for simultaneous analysis of DHA and MGO in Australian *Leptospermum* honey by PFBHA derivatisation, loosely based on the earlier paper by Biondi *et al.*.[128] This method has been discussed in section 3.1.1 (analysis of MGO).

Recently, DHA has been detected using OPD as a derivatising agent;[106] however, this has a different sample preparation than the previously reported detection of MGO using OPD derivatisation.[12] DHA reacts with OPD to give a stable quinoxaline (Figure 3.3). Detection of MGO is carried out in phosphate buffer (0.5 M, pH 6.5), while detection of DHA is carried out in acetate buffer (0.5 M, pH 4). Therefore the compounds cannot be simultaneously detected using OPD as the derivatising agent. Acid-induced formation of MGO-quinoxaline occurs at pH 4 in the honey matrix and would over estimate the true MGO concentration. On the other hand, at pH 6.5 reaction of DHA with OPD occurs too slowly for practical use. The authors noticed that this method caused a reproducible break-

down of glucose and fructose into DHA which was addressed by using an artificial honey matrix as a blank.

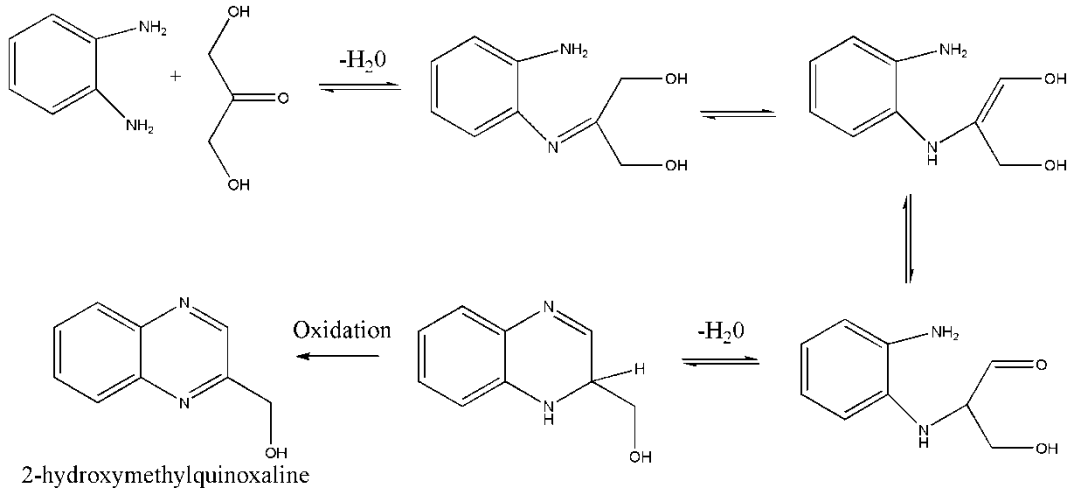


Figure 3.3 Proposed pathway by Atrott *et al.*[106] for the reaction of DHA with OPD to form 2-hydroxymethylquinoxaline.

3.1.3 Analysis of hydroxymethylfurfural

HMF is a potentially toxic material, which forms during heating or cooking of foods that contain sugars; the level of HMF is directly related to the treatment of the food. There are many articles containing detection of HMF in food and in the body because quality control and consumer protection have become more important. Due to the differences in sample preparation and analysis of HMF in biological samples and honey matrices, this review will not give a detailed account on detection in biological samples. Furthermore, due to the extensive amount of literature on detection of HMF in food matrices, this review will only focus on honey matrices. HMF is regulated in honey with a maximum allowed level of 40 mg/kg.[7]

The International Honey Commission (IHC) recommends three methods of analysis for the determination of HMF in honey; two spectroscopic methods and an HPLC method. One spectrophotometric method is the Winkler method which involves a *p*-toluidine-barbituric acid reaction with HMF and UV measurement. This is recommended as the official method in Spain.[130] However, to achieve stable and reproducible absorbance readings there must be a stringent control of reaction time and temperature. Salinas *et at.*[130] suggested the use of flow injection analysis to overcome this problem, because it allows the procedure to be automated. The authors reported the linear range of determination as 2.3-98 µg/mL. The

method has a good tolerance towards sugars in the samples. However, as *p*-toluidine is a recognised carcinogenic compound, it has been suggested that this method should be discontinued.[131] The second spectrophotometric method is the method by White[132] which involves measurement of UV absorbance of clarified aqueous honey solutions with and without bisulphite. The third method is a RP-HPLC method which requires the honey to be diluted then separated using an isocratic elution with water and methanol. This method allows HMF to be separated from other components in the honey; therefore interferences are eliminated.[133] Spano *et al.*[131] reported that the RP-HPLC official method of the IHC for determination of HMF in honey is unable to be used in strawberry tree honey (*Arbutus unedo*, a typical Sardinian honey) due to the interference of homogentisic acid and they modified the method to allow its quantification.

Zappalá *et al.*[133] compared the three methods for HMF determination recommended by the IHC using 14 unifloral honeys. They noted that eucalyptus honey behaved differently from all other honeys tested. While spectroscopic methods are fast, they may not have good specificity or sensitivity. The authors reported that the Winkler method has low precision and concluded that HPLC was the better method.

An advantage of HPLC is that it allows simultaneous analysis of other compounds. Furthermore derivatisation is not necessarily required (although this can significantly improve sensitivity), compared to analysis by GC. Sample preparation for analysis of HMF by HPLC without derivatisation requires dilution and filtration;[134-135] some methods also clarify the honey before analysis; for example 15% (*w/v*) potassium hexacyanoferrate solution (0.5 mL) and 30% (*w/v*) zinc acetate dehydrate solution (0.5 mL).[136]

There are various other HPLC methods reported in the literature for detection of HMF in honey. RP-HPLC-UV is the most common method; however, there is no consistency of eluents and the literature is divided between isocratic[134, 136 137-138]and gradient elution.[135, 139] Solvents include water, methanol and acetonitrile in various compositions for both isocratic and gradient elution.

Nozal *et al.* (2001)[140] developed an HPLC method for detection of HMF in honey using a C_{18} column operated at 30 ºC. This method was also optimised to detect related compounds (2-furoic acid, furan-3-carboxylic acid, furana-3-carboxaldehyde and methyl anthranilate). The authors reported that clean-up on a solid-phase extraction cartridge was required

before analysis to prevent matrix interference and to isolate the compounds, although many other methods for detection of HMF do not require a clean-up step. Nozal *et al.*[140] used gradient elution (1% aqueous acetic acid: acetonitrile, 97:3 and acetonitrile: water, 50:50) with UV detection of 284 nm for HMF and found that the retention time of HMF was influenced by the pH; optimum conditions required 1% acetic acid. They reported their detection limit as 0.04 mg/L with the linearity range as 0.13-100 mg/L.

In 2009, Spano *et al.*[135] expanded on their previously reported method[131] to simultaneously detect HMF, 2-furaldehyde, 3-furaldehyde, 2-furoic acid and 3-furoic acid in honey using RP-HPLC with UV detection. No prior clean-up step or derivatisation were required; samples were diluted 1:10 with water prior to analysis. The 23 minute analysis gave good accuracy and precision and the interference from strawberry tree honey did not affect the results. 97 ± 14% HMF was recovered and the LOQ was 0.01 mg/L with linearity up to 100 mg/L.

Windsor *et al.* (2013)[139] reported the use of PFBHA as a derivatising agent to quantify HMF. This was the same method used for simultaneous detection of MGO and DHA analysis. No prior clean up was required. The authors did not report the LOQ, recovery or linearity of the method.

There are very few reports in the literature detailing GC for analysis of HMF in honey; up until 2006 only a few papers were present in the literature. Horvath and Molnar-Perl (1998)[141] reported a GC-MS method for quantification of HMF with trimethylsilyl derivatisation and no prior isolation or clean up. Teixidó *et al.* (2006)[142] reported GC-MS analysis of HMF in honey and reported that N,O-*bis*-trimethylsilyltrifluoroacetamide (BSTFA) was the best derivatising agent. Sample clean up with a solid-phase extraction cartridge was required before analysis. A 15 minute reaction time was used and heat did not enhance the derivatisation yield. The analysis was complete within 30 minutes. They reported linearity between 25 and 700 ng/g and the LOD was 6 ng/g, which is about 100-fold lower than HPLC-UV methods reported in the literature.

Determination of HMF using near-infrared spectroscopy is also reported in the literature but analysis by this technique is deemed poor and unreliable. Qiu *et al.*[143] reported a high standard deviation for analysis of HMF and a poor calibration curve ($R^2 = 0.88$). The authors analysed a set of honeys by the spectroscopic method from AOAC and by near-

infrared spectroscopy. There was a large bias in the results and when plotted against each other the R^2 was only 0.66.

3.2 Experimental

Four HPLC methods were compared for analysis of DHA, MGO and HMF, these were the Direct method, OPD-MGO method, OPD-DHA method and PFBHA method. Details on the experimental conditions and sample preparation can be found in chapter 2.

3.3 Results and discussion

3.3.1 Overview of methods that were compared

Four methods were compared in this section of research to evaluate their advantages and disadvantages. One method required no derivatisation to detect DHA, MGO and HMF and has been called the 'Direct method'. Two methods required derivatisation by OPD; one method detected MGO and hence has been named 'OPD-MGO' and the other method detected DHA and has been called 'OPD-DHA'. The fourth method used PFBHA as the derivatising agent to analyse DHA, MGO and HMF and has been called the PFBHA method throughout this research.

3.3.1.1 Direct method

The Direct method is able to detect all three compounds of interest using UV and RI detection in tandem; minimal sample preparation is required which prevents sample contamination or loss. Derivatisation is not required; but as DHA and MGO both lack a strong chromophore group they are unable to be detected at low levels using UV. They are conventionally detected using refractive index (RI) with UV detection for peak confirmation.[12] RI is a universal detector but it is a relatively insensitive technique and low level detection is not obtainable. Previous work[12] has used the Direct method for MGO and DHA quantitation. The current research has also used UV detection in the Direct method to quantify HMF, which was not previously reported.

The Direct method uses isocratic elution on two size exclusion columns in tandem. One disadvantage of this method is that a single analysis takes approximately 60 minutes as HMF is very slow to elute. A typical RI chromatogram of a mānuka honey is shown in

Figure 3.4. The sugar peaks are very large and the chromatogram must be expanded to integrate MGO and DHA which are in the baseline (Figure 3.5). Furthermore, in some samples, the fructose peak tails and co-elutes with the MGO (Figure 3.6). Due to the use of isocratic elution the chromatogram cannot be optimised to prevent this occurring. Adams *et al.*[12] reported 0-135 mg/kg MGO was detected in non-mānuka honeys in the RI spectra, but noted that influence of the fructose was less noticeable in honeys with higher levels of MGO. To try and eliminate this complication, UV detection was examined as a way to quantify MGO because the sugar peaks are absent in the chromatogram. A typical UV chromatogram is shown in Figure 3.7. Details of the elution time for UV and RI detection and λ_{max} are found in Table 3.1. All analytes were analysed at their λ_{max} in the UV spectra.

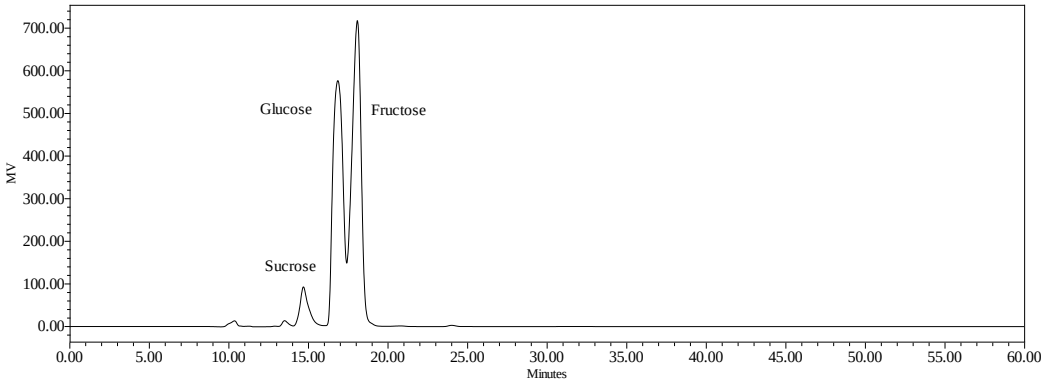


Figure 3.4 Typical RI chromatogram for a mānuka honey using the Direct method.

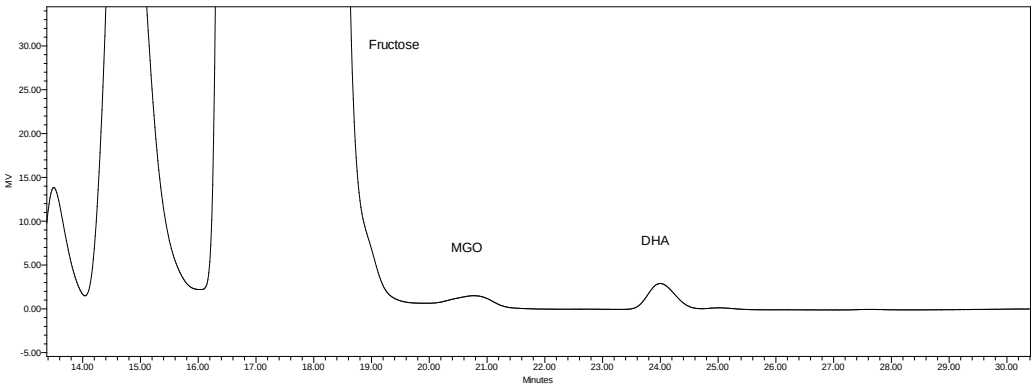


Figure 3.5 Expanded RI chromatogram for a mānuka honey using the Direct method. In this sample MGO elutes away from the tail of the fructose peak.

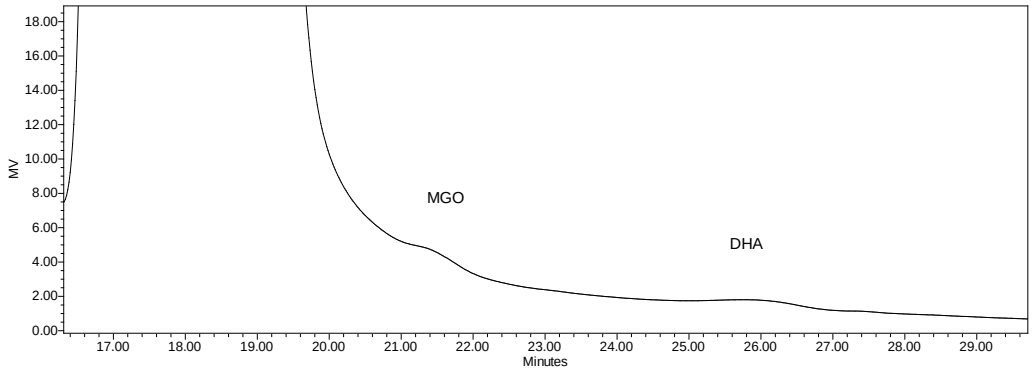


Figure 3.6 Expanded RI chromatogram for a mānuka honey using the Direct method. MGO elutes on the tail of the sugar peak.

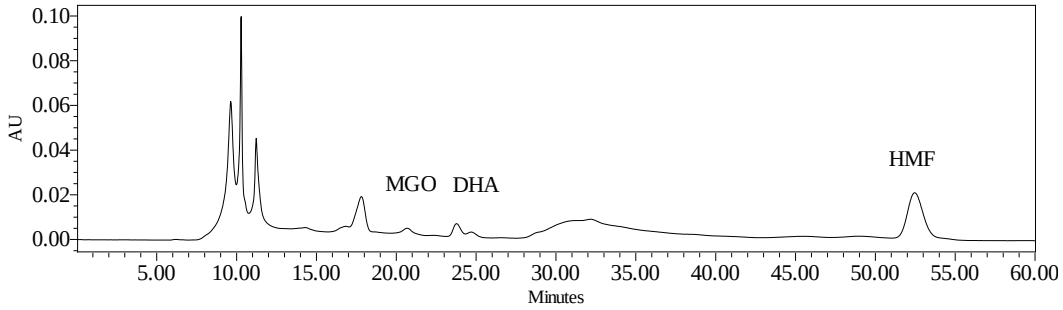


Figure 3.7 Typical UV chromatogram for the Direct method.

Table 3.1 Elution time and λ_{max} of compounds of interest for the Direct method.

Compound	Elution time for RI (min)	Elution time for UV (min)	λ_{max}
MGO	21.7	21.2	284
DHA	26.3	25.8	270.9
HMF	-	50.9	284

The reported concentration of DHA and MGO in each database sample (triplicate analyses) were compared when detected by UV and RI. The detectors were connected in series allowing unbiased results. HMF is not detected in the RI chromatogram, therefore could not be compared.

The reported concentration of DHA for the mānuka honeys measured by UV and RI gives a very strong positive linear correlation (R^2 = 99.0 %) when the concentrations are plotted against each other (Figure 3.8). All samples have less than 10% difference between the

reported concentrations for the UV and RI detection. However, in the UV chromatogram of clover honeys, an unidentified peak co-eluted with DHA. This peak has a different UV spectrum than that expected by DHA and prevented levels below 200 mg/kg from being analysed which was problematic as clover is a good matrix for standards and was used as a matrix in this research.

There was a weak correlation between the two detectors for MGO concentration (Figure 3.9). MGO was reported as below the minimum detection limit for eight samples; they have been left on the plot so that the reported concentration by the RI detector can be shown. With these samples the R^2 is 63.4%, compared to only 54.4% when they are removed. Most samples had more than 10% difference between the reported concentrations for the UV and RI detection. The low correlation is partially due to the high limit of quantification when analysed by UV and partially due to a peak of unknown origin which co-eluted with MGO in the UV spectra for some samples which increased the peak area. Hence the reported concentration was higher than that reported by RI detection.

Throughout this research the concentration of DHA and MGO was calculated using the RI peak area, while HMF was calculated from the UV peak area.

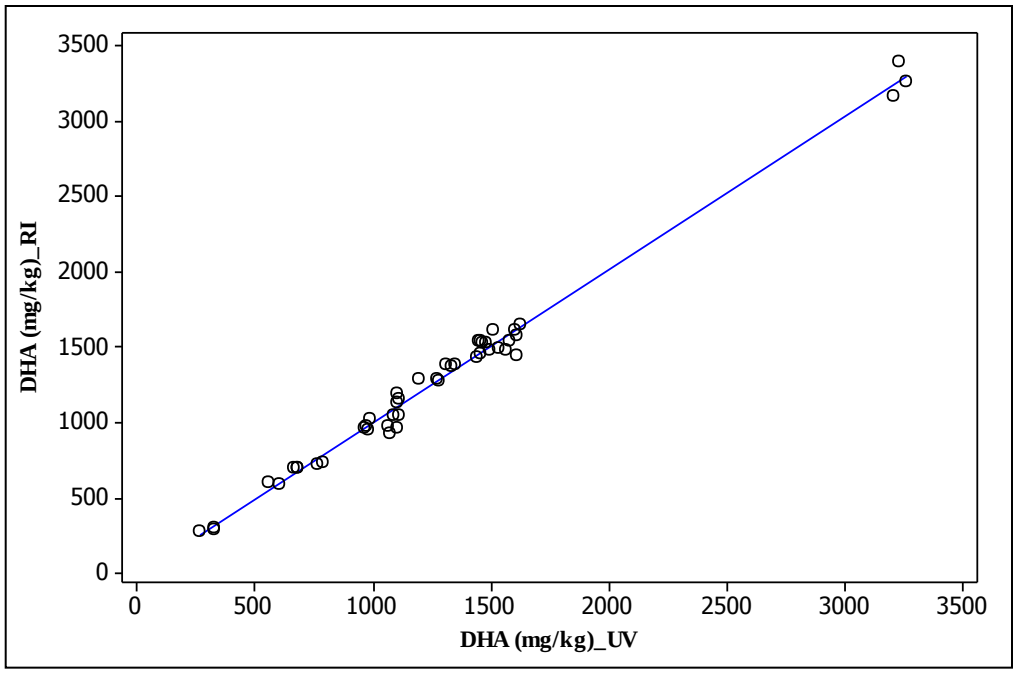


Figure 3.8 DHA concentration measured by RI vs. UV. The measured concentration has a positive linear correlation between the UV and RI detectors (R^2 = 99.0%) for mānuka honeys.

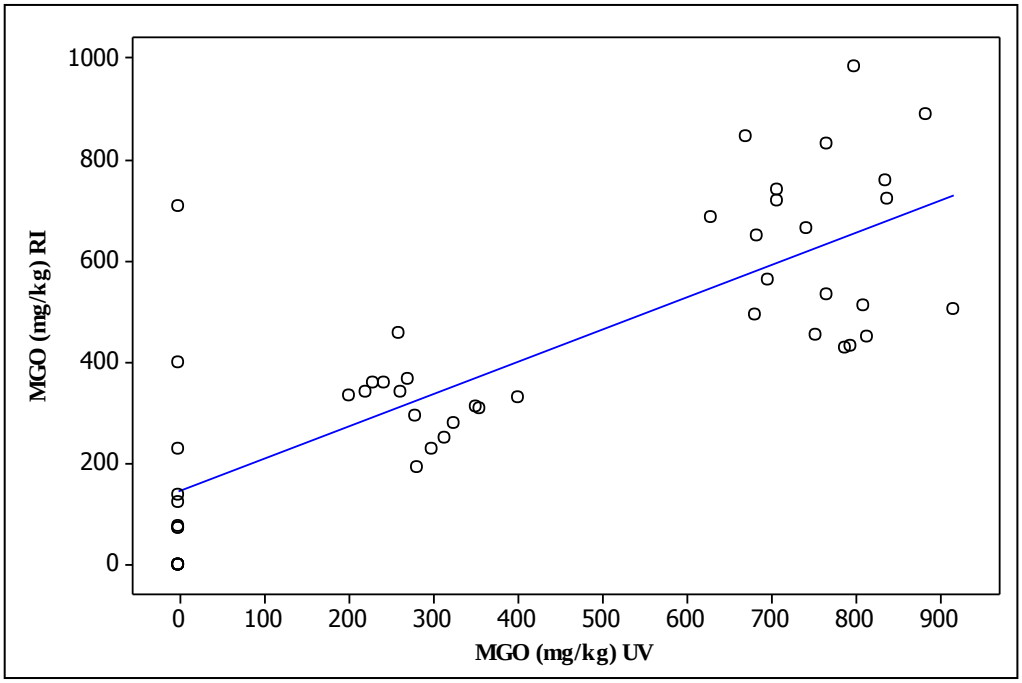


Figure 3.9 MGO concentration measured by UV vs. RI. The measured concentration is not linear between the two detectors (R^2 = 63.4% for all samples and 54.4% when samples below the MDL are removed). This is due to the low sensitivity of UV for MGO and a peak of unknown origin which co-eluted with MGO in the UV spectra for some samples. Samples that had MGO recorded as 'not detected' are shown as 0 mg/kg.

3.3.1.2 OPD method – MGO detection

The OPD-MGO method is used commercially to analyse the MGO content in mānuka honeys in New Zealand.[144] The main disadvantage of this method for this research is that it cannot detect DHA and HMF as well. It is becoming increasingly important to have a method that can detect DHA because the industry is starting to focus on predicting the final MGO concentration from DHA. A peak corresponding to DHA is present in this method; however, it cannot be used for quantitation (see below). In 2012, Attrot *et al.*[106] published a method that detected DHA using OPD derivatisation (OPD-DHA method), which is discussed in section 3.3.1.3.

In the OPD-MGO method MGO is derivatised with OPD so that it can be easily detected. The chromophore is detected by UV at 236 nm. The method uses a gradient elution, which allows optimisation of the MGO peak. A typical chromatogram of a mānuka honey is shown in Figure 3.10, with the MGO peak present at 31.49 minutes. Adams *et al.*[12] reported 0-24 mg/kg MGO in non-mānuka honeys but noted that this effect is swamped for higher activity honeys.

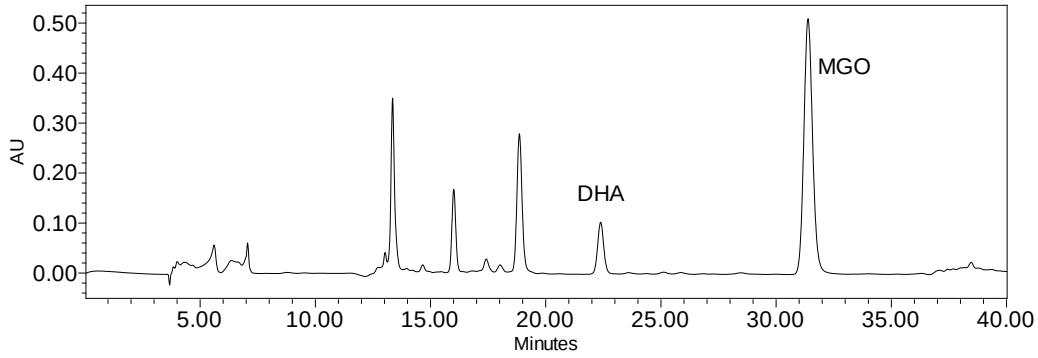


Figure 3.10 Typical chromatogram of a mānuka honey extracted at 236 nm. MGO elutes at 31.49 minutes. DHA elutes earlier (~22.5 min), but cannot be quantified by this sample preparation.

Derivatisation of MGO

The literature[12, 68, 114] states that samples require 8-16 hours incubation with OPD at room temperature before analysis. This requires samples to either be derivatised at the start of the day and run in the evening, or left overnight to complete derivatisation. This extended preparation time is not ideally suited to a

commercial setting. An experiment was carried out to see if the derivatisation step could be shortened; a sample was prepared and analysed hourly to monitor the change in peak area.

Initially, a sample of MGO (260 mg/kg) in water was analysed hourly for 16 hours throughout the derivatisation. This is a low- to mid- range concentration that naturally occurs in honey. Figure 3.11 shows the percentage difference of the peak area for each hour compared to the peak area at 16 hours, which is the recommended incubation time in the literature. Samples analysed at hours 1 and 3 have peak areas which are slightly below 100%. This may arise from experimental error (e.g. integration). The derivatisation appears to be complete at four hours.

The experiment was repeated using a mānuka honey sample, as it was thought that the derivatisation may occur more slowly in honey due to interfering compounds and/or the need for MGO to depolymerise or unbind from compounds, such as sugars. The sample was tested hourly for 22 hours. The results show that there is less than 2% difference between the peak area of the sample analysed in any hour compared to the mean peak area. There is also less than 2% difference between the peak area of MGO at any hour (other than 1 hour) and the peak at 16 hours (Figure 3.12).

The results show that the 16 hour derivatisation is not necessary. For this research, four hours was chosen for derivatisation. This allowed sufficient time for samples to react and is a practical time for commercial laboratories to use. Weigel *et al.*[68] used a 12 hour derivatisation but reported that derivatisation was complete after 6 hours.

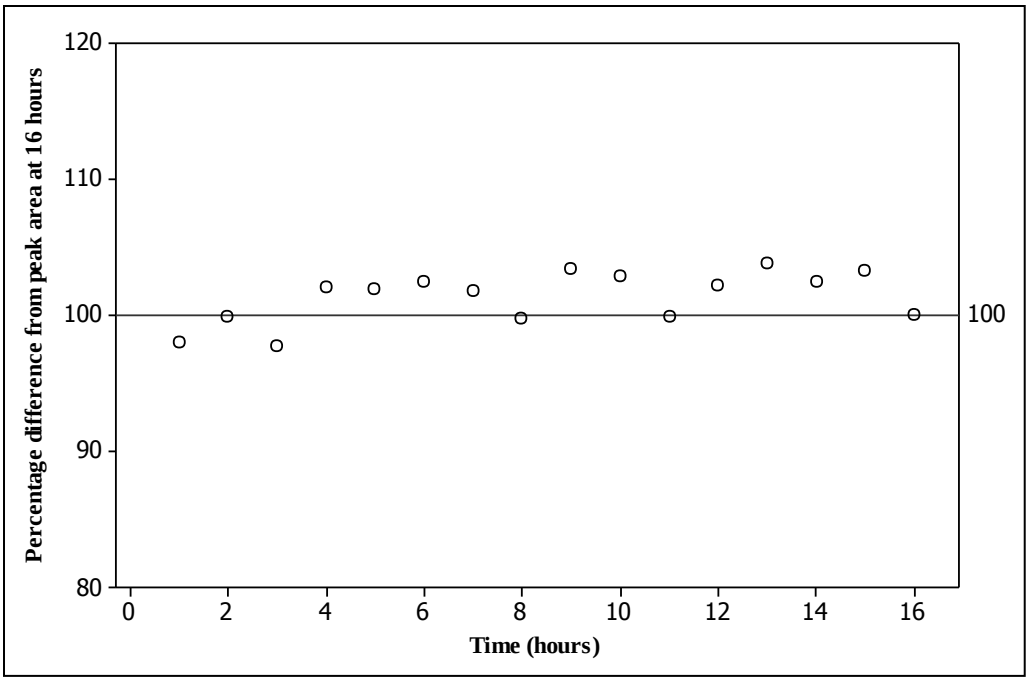


Figure 3.11 Percentage difference of MGO peak area in water at each hour during derivatisation compared to 16 hours derivatisation.

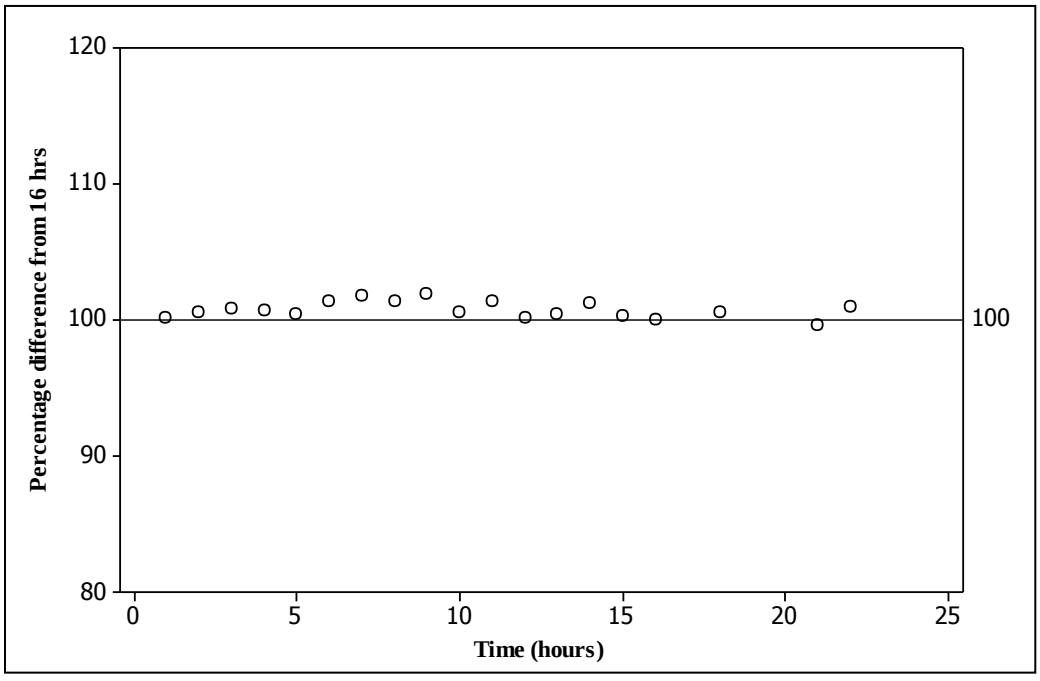


Figure 3.12 Percentage difference of MGO peak area in honey at each hour of derviatisation compared to 16 hours derivatisation.

Derivatisation of DHA

A peak corresponding to DHA eluted at 22.5 minutes. Initially the concentration of DHA was compared to the other methods, but no correlation was found between methods. Therefore the change in peak area during derivatisation time was analysed. A mānuka honey sample was analysed hourly for 28 hours during derivatisation. The DHA peak area continued to increase linearly over the 28 hours (Figure 3.13). This was also noted by Atrott *et al.*.[106] Therefore the DHA is unable to be measured by this method. Atrott *et al.*[106] modified the sample preparation so that DHA can be measured using OPD derivatisation (see section 3.3.1.3).

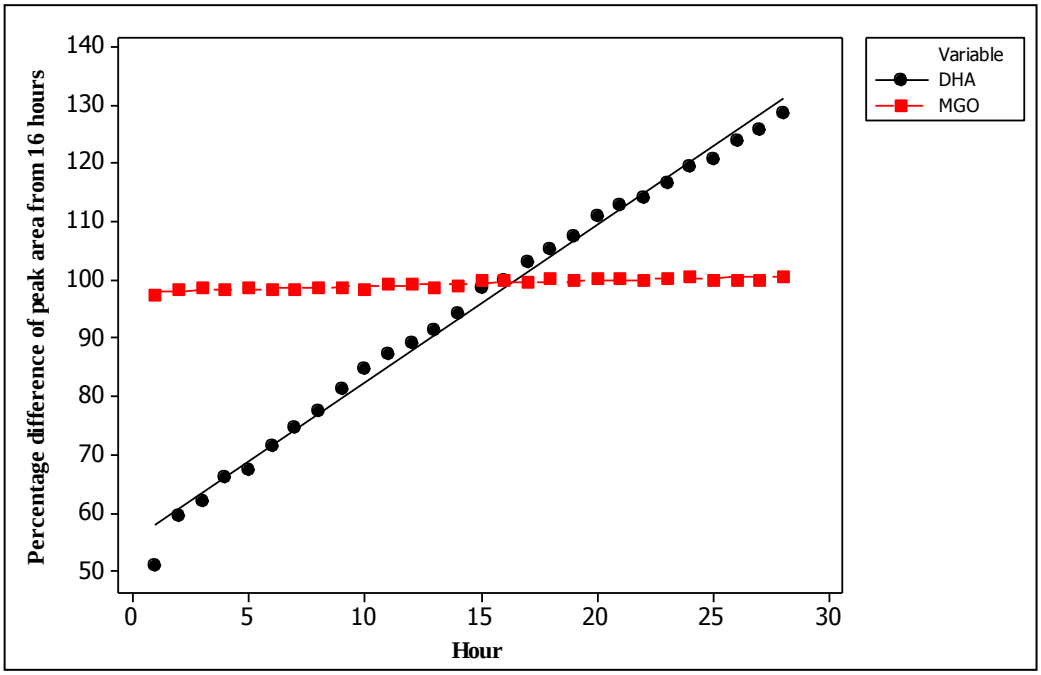


Figure 3.13 Percentage difference of the peak areas of DHA (circles) and MGO (squares) over 28 hours during derivatisation compared to the peak area at 16 hours. The peak area of DHA increases linearly over 28 hours of derivatisation, compared to the MGO peak area which is stable.

3.3.1.3 OPD method – DHA detection

In 2012 Atrott *et al.*[106] reported a new method for analysis of DHA, by derivatisation with OPD, which produced a stable DHA quinoxaline. RP-HPLC with UV was used for detection. DHA elutes at 12.5 minutes and the chromatogram is extracted at 312 nm (Figure 3.14). Acetate buffer (0.5 M, pH 4) was used instead of phosphate buffer (0.5 M, pH 6.5). This improved the reaction

for DHA, but could not be used for MGO detection as acid-induced formation of MGO-quinoxaline occurred.

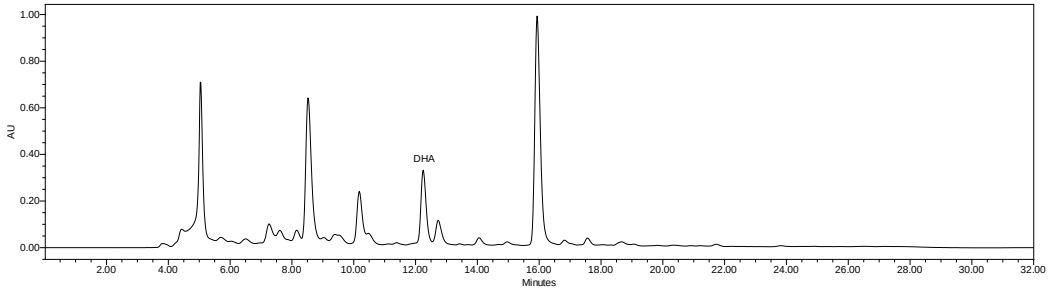


Figure 3.14 Typical chromatogram of a mānuka honey for the OPD-DHA method. The chromatogram is extracted at 312 nm.

Derivatisation time

Atrott *et al.*[106] reported that samples required 16 hours at 37 °C for complete derivatisation. A mānuka honey sample was prepared and kept at 37 °C in the autosampler and analysed hourly over 24 hours to monitor the progress of derivatisation. After 6 hours of incubation the sample exceeded the reported concentration of DHA as measured by the PFBHA method (981 compared to 921 mg/kg). After 16 hours the peak area continued to increase at a rate of ~0.022 mg/mL/hour (351 mg/kg/hour, Figure 3.15). This is a 19.3% increase from 16 to 24 hours. The increase in this peak may arise from the breakdown of glucose and fructose into DHA at the low pH of the solution, as suggested by Atrott *et al.*.[106]

An artificial honey with no added DHA was analysed hourly for 24 hours during derivatisation at 37 °C to investigate if a peak at the same time as DHA forms. A peak was observed after 1 hour of derivatisation and continued to increase linearly over the 24 hour period. The peak at 16 hours corresponded to a DHA concentration of 156 mg/kg. The rate of DHA increase was 0.0006 mg/mL (9.7 mg/kg) per hour.

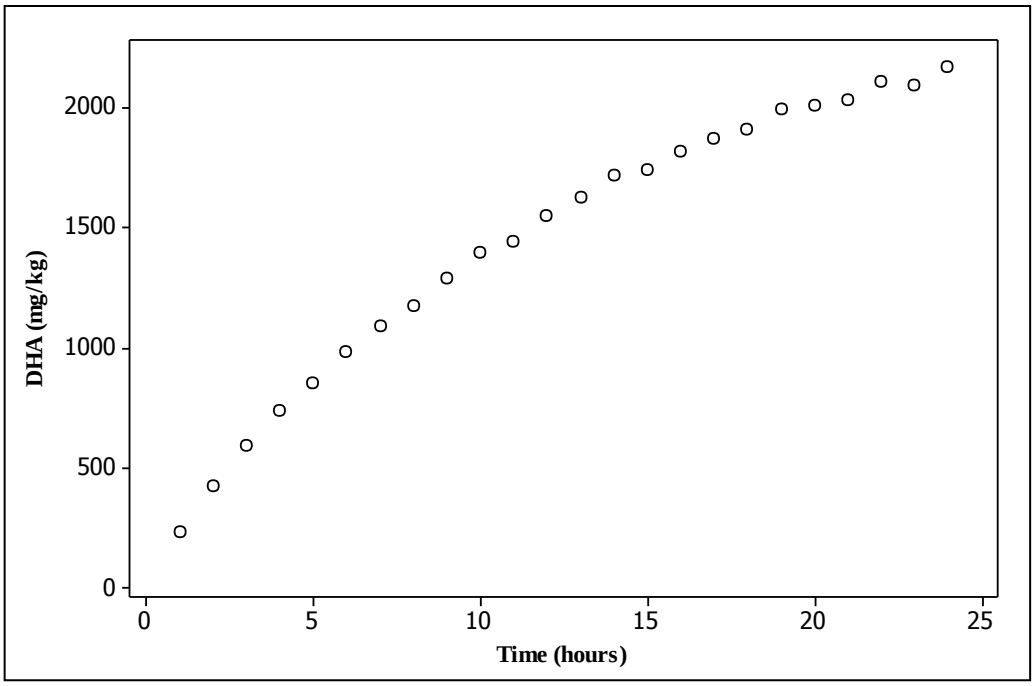


Figure 3.15 DHA (mg/kg) vs. time graph for a mānuka honey sample analysed hourly during derivatisation for 24 hours. The reported concentration of DHA continues to increase after 16 hours, suggesting that the breakdown of glucose and fructose is interfering with the true measure of DHA.

A second test was carried out with artificial honey where the sample was heated to 37 °C for the first 16 hours to simulate the incubation, then the temperature was reduced to 25 °C to simulate ambient temperature, in order to see if the peak ceased increasing at this lower temperature. There was a 4.47% increase in DHA (7 mg/kg) from 16 to 24 hours, which is only small and within experimental error. It suggests that the breakdown of glucose and fructose occurs at temperatures higher than ambient. These results indicate that it is critical that samples are incubated for a set length of time and an appropriate blank is subtracted from the peak area otherwise DHA would be over quantified.

A mānuka honey (triplicate) was analysed straight after incubation and again six hours later after sitting at ambient temperature. There was an overall increase of 96 mg/kg DHA (5.74%) in the average concentration. This may be a factor influencing the precision of the method – if samples are not analysed soon after they are derivatised the reported DHA concentration will be higher than for samples analysed straight after incubation. Due to these complications, this

method may not be suitable for a commercial laboratory, unless there is a completely automated system to ensure that there is a standard time between incubation and analysis for each sample.

3.3.1.4 PFBHA method

The PFBHA method allows simultaneous detection of DHA, MGO and HMF in a 30 minute analysis. Gradient elution allows the system to be optimised for the compounds of interest; each peak was sharp with no tailing and had baseline resolution.

Each compound is quantified at the λ_{max} (Table 3.2). The ratio of the compound of interest to the internal standard, HA, is used to calculate the concentration of a compound. The use of an internal standard is beneficial for this research; high levels of DHA (10,000 mg/kg) were used in some storage trials, which required excess PFBHA to be used to ensure complete derivatisation. In addition, some samples with high MGO required extra acetonitrile to dissolve the crystals of derivatised product (assumed to be MGO-PFBHA) before analysis. These alterations in volume would not be possible without the internal standard. The presence of PFBHA in the chromatogram confirmed that excess was added to the sample which allows for complete derivatisation of the analytes. If no PFBHA peak or a very small peak was present, it indicated that the derivatisation of all carbonyl compounds may not have gone to completion and in these instances, the sample was repeated with extra PFBHA.

A typical chromatogram of a mānuka honey, extracted at λ_{max} for DHA and HA (260 nm) is shown in Figure 3.16. Figure 3.17 and Figure 3.18 show chromatograms extracted at the λ_{max} for HMF (279 nm) and MGO (244.3 nm) respectively.

Table 3.2 Elution time and λ_{max} of compounds

Compound	Elution Time (min)	λ_{max}
DHA	6.52	260
PFBHA	7.88	260
HA	9.89	260
HMF Peak 1	11.19	279
HMF Peak 2	11.64	279
MGO Peak 1	18.53	244.3
MGO Peak 2	19.00	244.3

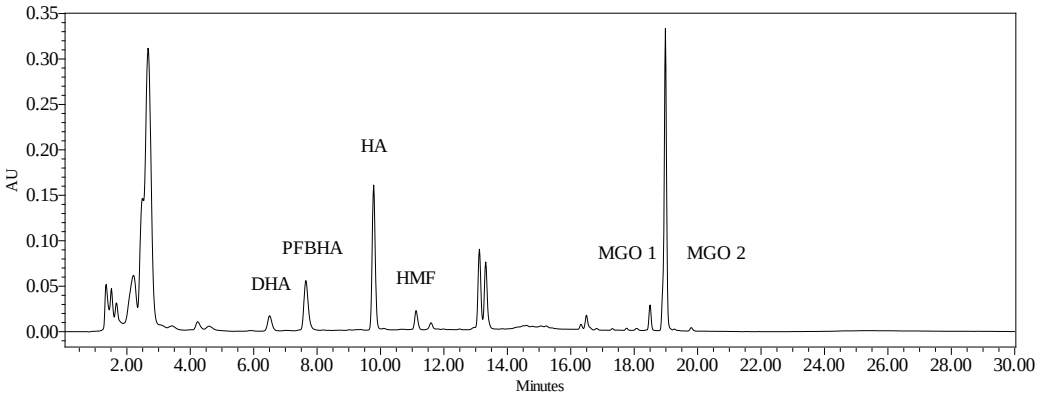


Figure 3.16 Typical chromatogram for PFBHA method of compounds in a mānuka honey matrix, extracted at 260 nm (λ_{max} for DHA and HA).

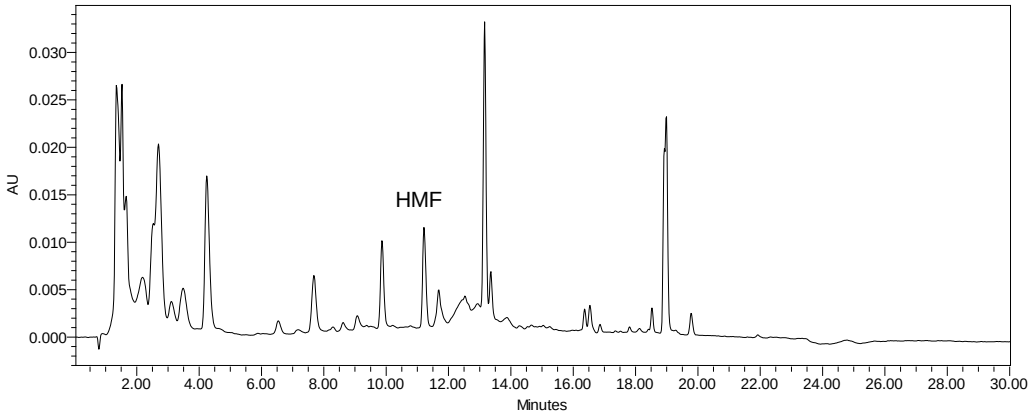


Figure 3.17 Typical chromatogram for PFBHA method of compounds in a mānuka honey matrix, extracted at 279 nm (λ_{max} for HMF).

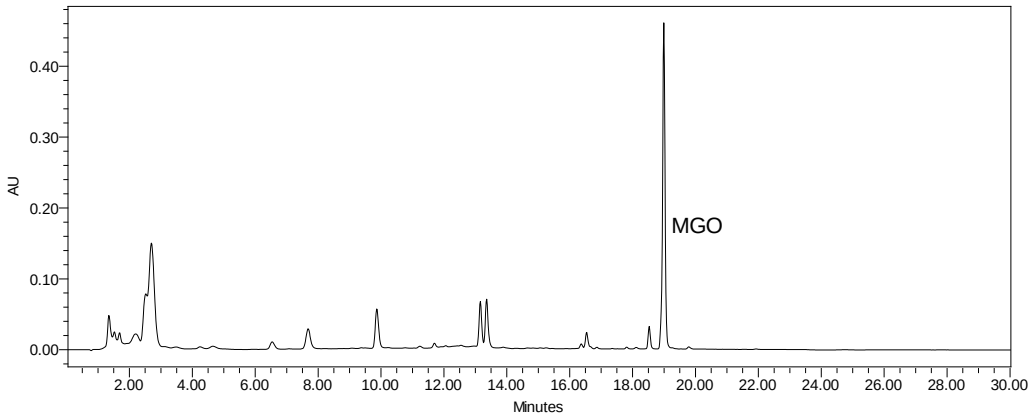


Figure 3.18 Typical chromatogram for PFBHA method of compounds in a mānuka honey matrix, extracted at 244.3 nm (λ_{max} for MGO).

The PFBHA method was reported in the literature for the analysis of DHA and MGO in honey by Windsor *et al*.[37] in 2012 and for HMF detection in 2013.[139] This method was used for analysing all samples in the database (chapter 4) and the storage trials (chapters 5, 6 and 7); further refinement of this method can be found in section 3.3.2.6.

Isomers of compounds

Compounds are derivatised by PFBHA through their ketone and/or carbonyl groups to give the corresponding oxime. The C=N bond restricts rotation and leads to isomers of MGO and HMF; DHA and HA are symmetrical molecules so do not have isomers.

MGO isomers

The ketone and aldehehyde groups of MGO both react with PFBHA. Due to the rigid N=C bond geometrical isomers are formed. Two different oximes, *Z* and *E*, can form from each carbonyl group (previously referred to as *syn* and *anti* respectively). Functional groups around the double bond are prioritised – if the two highest priority groups are on the same size it is the *Z* isomer, and if they are across the double bond it is the *E* isomer.[145]

There are four possible isomers that can form when MGO is derivatised with PFBHA (*Z* + *E*, *E* + *E* and *E* + *Z* and *Z* + *Z*). However, the *Z* + *Z* dioxime is not

observed,[146] most likely due to the steric hindrance of the large PFBHA molecules. Furthermore, only two peaks are observed in the chromatogram, possibly due to *Z* + *E* and *E* + *Z* isomers co-eluting. Figure 3.19 shows one of the four possible isomers.

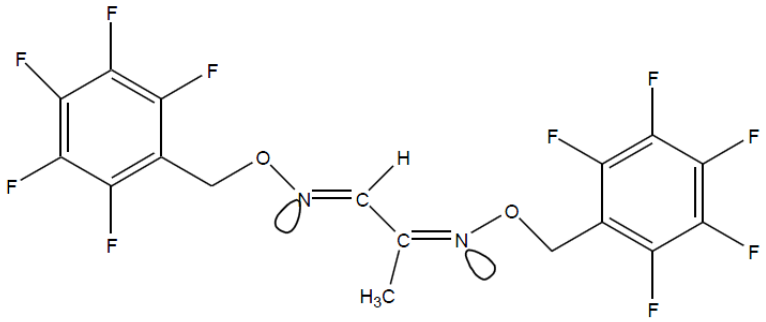


Figure 3.19 One possible isomer (*E* + Z) of MGO with two PFBHA compounds attached.

The ratio of the two peaks was examined to see if it was consistent between samples. Seven sets of data were examined. The RSD was 1.47%, showing that the ratio of the two peaks is consistent. The large peak accounts for approximately 95% of the two peaks. However, at very low concentrations of MGO only the largest of the peaks (MGO 2) is seen. Quantification of MGO was by the sum of chromatographic peaks for the *Z* + *E* and *E* + *E O*-PFB-dioximes; Lapolla *et al.*[146] also reported quantitation of MGO was achieved by the sum of the two isomers, compared to Windsor *et al.*[37] who only used the largest peak for quantitation of MGO.

HMF isomers

PFBHA reacts with HMF to form two isomers (*E* and *Z*) which are separated using the HPLC system. Windsor *et al.*[139] also reported the presence of the two HMF isomers in the chromatogram. The ratio of the peaks in the current research was examined to see if it was stable. Five calibration curves and two sets of samples were examined. The RSD was 4.61%, showing that the ratio of the two peaks is consistent. The large peak accounts for approximately 80% of HMF. Initially quantification of HMF was achieved by the sum of the peak areas of both *E*- and *Z*- isomers. However, some honey samples had an interfering compound co-elute with the second isomer. This caused a higher than expected concentration

for HMF of these samples. Therefore calibration of HMF was achieved using only the largest isomer (HMF 1). Further discussion of this decision can be found in section 3.3.3.3.

3.3.2 Comparison of the four methods

The four methods were compared for the linearity range, minimum detection limits, limits of quantification, accuracy, precision and repeatability. The length of derivatisation was also compared.

3.3.2.1 Linearity

Each method was examined to find the concentration at which the calibration curve deviated from linearity for each analyte. It is beneficial to have a working range which readily encompasses the concentrations of the analytes that would be found in real commercial samples. This reduces the need to add a dilution step for some samples.

Linearity was evaluated by least-squares regression analysis. A value of 0.995 was deemed to be the lowest acceptable correlation coefficient (R^2) for acceptable analysis. All calibration curves for all analytes in all four methods met this requirement. Table 3.3 summarises the upper limit of the working range for each analyte in each method. For the OPD-MGO and OPD-DHA methods, this was the highest concentration that could be detected before the calibration curve deviated from linearity. Linearity exceeded the upper limit presented here for the Direct method and PFBHA method.

Table 3.3 Summary of the upper limit of the working range for each analyte for the four methods.

Method	DHA (mg/kg)	MGO (mg/kg)	HMF (mg/kg)
Direct	11640*	4237*	525*
OPD-MGO	N/A	1880	N/A
OPD-DHA	1945	N/A	N/A
PFBHA	12125*	1970*	506*

* Linearity exceeds this concentration

Adams *et al.*[12] reported a calibration curve with a working range of 50-900 mg/kg MGO for the Direct method. The current research has shown that linearity extends to at least 4,237 mg/kg MGO. DHA and MGO also have high upper limits which covers the concentrations found in honey.

The OPD-MGO method deviates from linearity at 1,880 mg/kg in this research (Figure 3.20); some New Zealand mānuka samples exceeded the working range. Australian Jelly bush honeys have been reported to have higher levels of MGO (1,723 mg/kg) than generally reported for New Zealand mānuka honeys.[37] In addition, the storage trials in this current research used concentrations higher than the working range. Lei *et al.*[116] also reported a deviation from linearity for the calibration curve when OPD was used as the derivatising agent, but at a much higher concentration (6 g/L, 8,520 mg/kg). To overcome the deviation in the current study, samples that were reported with a concentration near the upper limit of the working range were re-run using half the amount of honey (0.3 g) when preparing them to bring the concentration of MGO back into the linear range. Weigel *et al.*[68] used a calibration curve between 0.07 and 2.2 mg/L for MGO (0.099 and 3.12 mg/kg[*]). Lei *et al.*[116] reported a much larger linear range of 1-50 mg/L (1.42-71 mg/kg).

The OPD-DHA method deviates from linearity at 1,945 mg/kg DHA. Atrott *et al.*[106] reported a similar working range (1,980 mg/kg). Naturally occurring DHA in honey rarely exceeds this level, but has been known to occur; Windsor *et al.*[37] reported 2,403 mg/kg DHA in one *L. polygallifolium* from Australia. In cases where the sample reported DHA near the upper limit, the sample was diluted and reanalysed.

The PFBHA method had a very large working range for all three analytes and was easily able to cover the concentrations of naturally occurring analytes in honey (Figure 3.21). DHA was linear beyond 12,125 mg/kg, which allowed the storage trials doped with 10,000 mg/kg DHA to be analysed without the need of a dilution. MGO was linear beyond 1,970 mg/kg; Windsor *et al.*[37] reported a working range for MGO between 20-1,800 mg/kg for the same method. HMF was linear beyond

[*] The conversion of mg/L to mg/kg is based on the density of honey with 20% moisture as 1.4171 g/kg.[117]

500 mg/kg for the PFBHA method. Honey should not exceed 40 mg/kg because it cannot be exported, therefore this working range is more than acceptable.

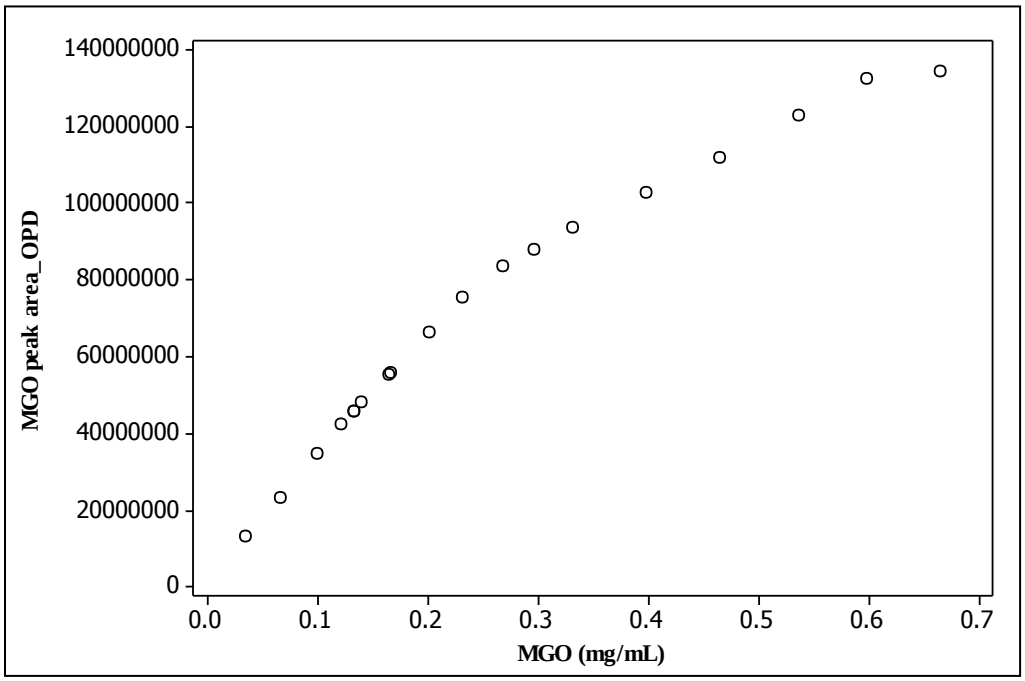


Figure 3.20 Calibration curve for MGO in the OPD-MGO method extended past the linear working range to show the deviation in the curve. The working range is up to 0.25 mg/mL (1,880 mg/kg).

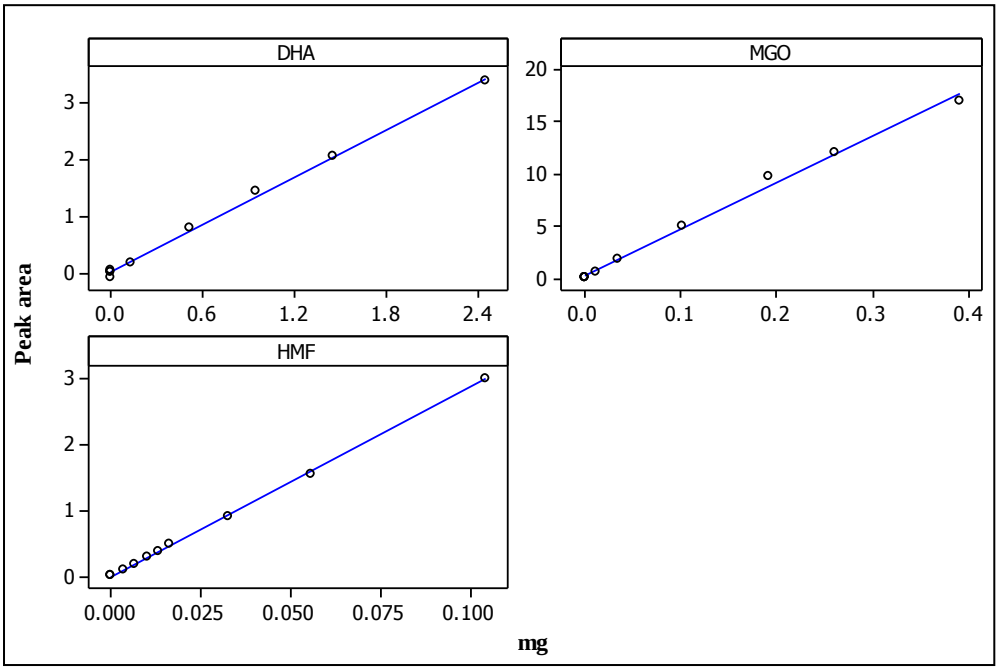


Figure 3.21 Calibration curves for DHA, MGO and HMF as detected by the PFBHA method. The DHA curve is equivalent to 0-11,665 mg/kg and MGO and HMF are equivalent to 0-1970 and 0-506 mg/kg respectively.

3.3.2.2 Chromatographic performance

The chromatographic performance was assessed by multiple analyses of one honey sample and integration of the analytes of interest. An autosampler was used for all injections. The average concentration, retention time, capacity factor, theoretical plates and peak area were assessed. Different honeys were used for each method, hence the average concentration cannot be compared.

The capacity factor (k) indicates how long each component is retained in the column. k was calculated using the following equation:

$$Capacity\ factor\ (k) = \frac{t_R - t_U}{t_U} \quad (3\text{-}1)$$

where:

t_R = Retention time of the compound of interest

t_U = Retention time of unretained peak

The number of theoretical plates (n) is a measure of the sharpness of the peaks and hence the efficiency of the column; the higher the plate number, the more efficient the column is. This was calculated using the following equation:

$$\text{Theoretical plates (n)} = 16 \left(\frac{\text{RT (min)}}{\text{W}_\text{b}\text{(min)}}\right)^2 \quad (3\text{-}2)$$

where:

RT = Retention time of peak of interest

W_b = Width of peak at base

Table 3.4, Table 3.5 and Table 3.6 summarise the chromatographic performance for DHA, MGO and HMF respectively for each method. %RSD is shown in parenthesis.

Table 3.4 Summary of chromatographic performance for DHA for the three methods that detect DHA. %RSDs are in parenthesis.

	Direct (n = 8)	PFBHA (n = 10)	OPD-DHA (n = 8)
Average (mg/kg)	1633 (7.40%)	2999 (1.42%)	1019 (2.92%)
Retention time (min)	26.29 (2.58%)	6.21 (0.25%)	13.25 (1.18%)
Capacity Factor (k)	1.50 (4.42%)	3.94 (0.28%)	1.46 (1.62%)
Theoretical plates (n)	53063 (0.62%)	2004 (17.38%)	10699 (11.63%)
Peak Area	74956 (3.09%)	834627 (2.28%)	1424213 (2.30%)

Table 3.5 Summary of chromatographic performance for MGO for three methods. %RSDs are in parenthesis.

	Direct (n = 8)	PFBHA-MGO 1* (n = 10)	PFBHA-MGO 2* (n = 10)	OPD-MGO (n = 8)
Average (mg/kg)	1439 (7.51%)	-	322 (1.0%)	111 (0.94%)
Retention time (min)	21.7 (2.9%)	18.49 (0.05%)	18.98 (0.05%)	31.49 (0.17%)
Capacity Factor (k)	1.07 (5.73%)	13.71 (0.37%)	14.11 (0.36%)	7.18 (0.22%)
Theoretical plates (n)	2434 (20.88%)	84855 (28.52%)	38929 (21.30%)	22388 (9.93%)
Peak Area	52026 (7.51%)	154495 (4.58%)	2809244 (1.51%)	7542811 (0.94%)

* The average MGO concentration for PFBHA was calculated by adding both isomers together.

Table 3.6 Summary of chromatographic performance for HMF for the Direct and PFBHA methods. %RSDs are in parenthesis.

	Direct (n = 8)	PFBHA-HMF 1 (n = 10)
Average (mg/kg)	10 (2.91%)	9 (4.34%)
Retention time (min)	61.41 (0.10%)	10.99 (0.13%)
Capacity Factor (k)	5.08 (0.24%)	7.75 (0.37%)
Theoretical plates (n)	3516 (12.56%)	12110 (26.45%)
Peak Area	769198 (2.91%)	43920 (4.84%)

The results show that the automated injection process and manual integration afford reproducible results. The variation in retention time for all analytes over all methods is very low (less than 5% RSD). The variation in average concentrations is also less than 5% RSD for all compounds in all methods, except for DHA and MGO in the direct method (~7.35% RSD).

The RSD (%) for the peak area was less than 5% for all compounds in all methods, except for MGO in the Direct method (7.51%). This had a greater spread due to the overlapping of the fructose peak with the MGO peak. Hence it was difficult to consistently integrate the start and end of the MGO peak.

The theoretical plates, which measures the sharpness of peaks were above 10,000 for most analytes in all methods. However they were very low for DHA in the PFBHA method (2,004) and MGO in the Direct method (2,434).

Peak resolution (R) measures the separation between two peaks. It is calculated as follows:

$$Peak\ resolution\ (R) = 2\left(\frac{(RT_A - RT_B)}{W_A + W_B}\right) \quad (3\text{-}3)$$

where:
RT = Retention time of compound of interest
W = Width of peak at baseline

Ideally peak resolution (R) should be >1.5, but >1 is acceptable. Peak resolution was not added to the above tables because it was only calculated for peaks that were very close to another peak. In the Direct method, the fructose peak and MGO peak overlap. The peak resolution of DHA from MGO is 3.97. The resolution for MGO in the OPD-MGO method is 1.55, which is satisfactory. In the PFBHA method DHA, HMF and HA are well separated and have a peak resolution >1.5. The isomers of both HMF and MGO are very close together; which is to be expected. The MGO isomers have a peak resolution of 1.43, which is good. The two HMF isomers have an average peak resolution of 1.11, which is acceptable.

3.3.2.3 Minimum detection limit

The minimum detection limit (MDL) is defined by the Code of Federal Regulations[147] as the minimum concentration of an analyte that can be measured and reported with 99% confidence. The limit of quantification (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy.[148] It is important for MGO to have a low LOQ for this research so that the initial formation of MGO from DHA can be measured in the storage trials. A low LOQ for DHA would show whether or not all DHA is consumed in the reaction. Additionally, guidelines state that HMF should not exceed 40 mg/kg in commercial honey; therefore it is important that low levels are able to be accurately determined.

The estimated detection limit (EDL) was obtained as three times the standard deviation of replicate instrumental measurements of the analyte (as determined in the chromatographic performance). The analytes of interest were spiked into an artificial honey matrix. The Environmental Protection Agency (EPA) procedure[147] states that analytes should be spiked 1-5 times the estimated detection limit. The MGO spike in the OPD-MGO method was slightly higher (20 mg/kg) than the recommended spike (3-16 mg/kg), but is less than 7 times the EDL. Therefore this should not affect the results. All other analytes were spiked within 1-5 times the EDL.

The MDL, with 99% confidence was calculated by multiplying the standard deviation by the Student's t value[147] and the LOQ was calculated as 2.5 times higher than the MDL.[147,149] Table 3.7, Table 3.8 and Table 3.9 summarise the MDL and LOQ for DHA, MGO and HMF respectively for all methods examined. Ten replicates were analysed for all analytes in the PFBHA method, and eight replicates were analysed for the other three methods. Overall, the PFBHA method has lowest LOQ for all three analytes compared to the other methods.

Table 3.7 Summary of MDL and LOQ for DHA in three different methods.

	Direct	PFBHA	OPD-DHA
Instrument variation	50.98	13.28	29.78
3 times σ (EDL)	152.94	39.83	89.34
5 times EDL	254.90	199.14	446.70
Spike (mg/kg)	268 ± 5	118.63	232 ± 2
Average measured (mg/kg)	208 ± 10	120 ± 10	360 ± 20
RSD (%)	5.54	6.82	7.66
MDL (mg/kg)	40.22	11.45	68.73
LOQ (mg/kg)	100.56	28.61	171.82

Table 3.8 Summary of MDL and LOQ for MGO in three different methods.

	Direct	PFBHA	OPD-MGO
Instrument variation	45.62	3.26	1.05
3 times σ (EDL)	136.86	9.78	3.15
5 times EDL	228.10	48.90	15.75
Spike (mg/kg)	620 ± 10*	24.00	20.00
Average measured (mg/kg)	130 ± 20*	29 ± 2	20 ± 1
RSD (%)	24.61	4.56	6.33
MDL (mg/kg)	111.31	2.12	2.19
LOQ (mg/kg)	278.27	5.29	5.48

*There were complications with measurement of MGO in the Direct method which have been discussed in the text.

Table 3.9 Summary of MDL and LOQ for HMF in the Direct and PFBHA methods.

	Direct	PFBHA
Instrument variation	0.15	0.37
3 times σ (EDL)	0.45	1.12
5 times EDL	0.75	5.60
Spike (mg/kg)	8.0 ± 0.1	4.51
Average measured (mg/kg)	6.9 ± 0.2	4.1 ± 0.4
RSD (%)	2.44	4.15
MDL (mg/kg)	0.59	0.51
LOQ (mg/kg)	1.48	1.27

The PFBHA and OPD-MGO methods had a similar LOQ for MGO. There was a problem obtaining the MDL and LOQ for MGO in the Direct method due to the MGO peak eluting on the tail of the fructose peak. At low concentrations the partial loss of the peak is enhanced. Furthermore it would be exacerbated by any small decline in chromatographic efficiency. These results highlight the variability of the method; when the database of honeys were analysed (see section 3.3.3.1) the tail of the fructose peak was not as large, hence the reported results were similar to the other two methods. Three different columns of the same type

(including one brand new column) were tested and all had the same result, hence the reason for the changing tail in the samples is unknown.

The EDL for the Direct method was 136.86 mg/kg; however, a spike at this level was not observed. Therefore a 620 mg/kg MGO spike was used to calculate the MDL (which is higher than the recommended 1-5 times EDL), but the average reported concentration was only 129.25 mg/kg, which is much lower than the spike due to partial loss of the MGO peak from co-elution with the fructose peak. The standard deviation was only 21.80 mg/kg, suggesting that at this level a consistent amount of MGO is lost under the fructose peak. Therefore the MDL was 111.31 mg/kg and the LOQ was 278.27 (equivalent to NPA 10). This is over 50 times higher than reported for the PFBHA and OPD-MGO methods (~5 mg/kg). Adams *et al.*[12] reported the LOQ for the Direct method was 50 mg/kg MGO, which is 5 times lower than found here.

Weigel *et al*.[68] reported the MDL for the OPD-MGO method as 0.04 mg/L MGO. Lei *et al.*[116] reported the MDL and LOQ for an RP-HPLC method using OPD derivatisation were 0.02 mg/L and 0.06 mg/L, which is similar to Weigel *et al*..[68]

The MDL for DHA in all three methods was lower than the estimated detection limit. The PFBHA method had the best LOQ (29 mg/kg) and was 3-6 times lower than for the other two methods. The reported MDL for DHA in the OPD-DHA method (68.73 mg/kg) is much higher than the MDL reported by Atrott, Haberlau and Henle[106] (10 mg/kg). They reported that the LOQ was 110 mg/kg which is lower than the LOQ reported here (171.82 mg/kg, 44% difference). This method has a much higher LOQ than the PFBHA method (29 mg/kg) which possibly arises from the alkaline breakdown of glucose and fructose into DHA during derivatisation in the OPD-DHA method, hence a blank was subtracted from the sample and a low level of detection cannot be obtained.

The PFBHA and Direct methods had approximately the same LOQ for HMF (1.5 mg/kg). This is a very low level and is suitable for HMF analysis in honey.

3.3.2.4 Accuracy

Accuracy shows the deviation between the mean reported value and the true value. A stock solution of each analyte was made up and a known amount was doped into artificial honey, which fell between 5 – 50 times the MDL. Multiple samples were analysed and accuracy of the method was tested by finding the percentage recovery of the analyte, with the expectation that it would fall between 80 – 120%.

Table 3.10, Table 3.11 and Table 3.12 summarise the accuracy of each method for DHA, MGO and HMF respectively. Percentage RSDs are shown in parentheses. All analytes for all methods fall within the expected 80-120%, except MGO in the Direct method. Complications in reported analysis of MGO in the Direct method are discussed in section 3.3.2.3.

DHA is slightly high (111%) for the PFBHA method, while the OPD-DHA method reads lower (91%). The same phenomenon is seen for the recovery of MGO – the PFBHA method reads slightly higher and the OPD-MGO method reads slightly lower. The PFBHA and Direct methods recover a satisfactory amount of HMF (99 and 105% respectively).

Lei *et al.*[116] used OPD derivatisation for MGO analysis by RP-HPLC with UV detection. They reported the recovery of MGO for spikes between 50 and 200 mg/kg were 98.3-101.5%, which has better recovery than the OPD-MGO method in the current research (90%).

Table 3.10 Summary of accuracy and precision for DHA in doped artificial honey.

	Direct (n = 5)	**PFBHA (n = 7)**	**OPD-DHA (n = 8)**
Average expected concentration (mg/kg)	1030 ± 10	72 ± 2	654 ± 5
Average measured concentration (mg/kg)	920 ± 50	81 ± 9	600 ± 30
Recovery (%)	89	111	91

Table 3.11 Summary of accuracy and precision for MGO in doped artificial honey.

	Direct (n=8)	PFBHA (n = 10)	OPD-MGO (n = 10)
Average expected concentration (mg/kg)	1020 ± 10	34.74	59.70
Average measured concentration (mg/kg)	260 ± 70	37.74 (5.37%)	53 ± 2
Recovery (%)	26	109	90

Table 3.12 Summary of accuracy and precision for HMF in doped artificial honey.

	Direct	PFBHA
Average expected concentration (mg/kg)	333 ± 5	21.17
Average measured concentration (mg/kg)	350 ± 20	20.97 (4.19%)
Recovery (%)	105	99

3.3.2.5 Repeatability

Repeatability is a way of monitoring the variation that occurs over a number of days when a single honey is prepared and analysed multiple times over multiple days. To assess the repeatability (precision) of the method, triplicate analyses of a honey were prepared and analysed each day over four days to introduce real world variability into the results (such as sampling, sample preparation and instrumental variation). Therefore the values recorded will be representative of what would be expected for the same sample analysed over time. Different honeys were used for each method, hence the average concentrations differ. Table 3.13, Table 3.14 and Table 3.15 summarise the repeatability results for DHA, MGO and HMF respectively.

Table 3.13 Summary of repeatability for DHA.

	Direct (n = 16)	PFBHA (n =12)	OPD-DHA (n = 10)
Average (mg/kg)	171	506	1572
Standard deviation	10	12	154
RSD (%)	5.77	2.43	9.77

Table 3.14 Summary of repeatability for MGO.

	Direct (n =14) high	Direct (n =12) low	PFBHA (n = 12)	OPD-MGO (n = 16)
Average (mg/kg)	848	76*	177	671
Standard deviation	18	17	7	29
RSD (%)	2.08	22.39	3.98	4.27

* Note that this is below the LOQ (278 mg/kg) and MDL (111 mg/kg).

Table 3.15 Summary of repeatability for HMF.

	Direct (n = 16)	PFBHA (n = 12)
Average (mg/kg)	2.80	52
Standard deviation	0.24	2
RSD (%)	8.69	3.30

As calculated previously, the MDL of MGO for the Direct method is high (278 mg/kg). The MGO repeatability for the Direct method was carried out for a high and low concentration of MGO to examine the difference. Unfortunately the low concentration was just below the MDL and therefore had varied results. However, the RSD for the higher MGO concentration was small and is in line with the other two methods.

Repeatability of DHA in the OPD-DHA method is the highest (9.77%). However, it is within 10% which indicates that the method is reproducible over time. Atrott, *et al.*[106] reported a RSD of 7.8%, which is similar to this research.

The repeatability of HMF is below 10% for both the PFBHA and Direct methods. The RSD is slightly elevated for the Direct method, due to a small concentration of HMF being reported.

3.3.2.6 Further refinement of the PFBHA method

The PFBHA method was deemed the most suitable method for analysis of DHA, MGO and HMF in honey in this research. It can simultaneously detect all three analytes to a low level. Furthermore, the working range is large, allowing artificial honey samples to be doped with 10,000 mg/kg DHA and tested without the added

step of dilution. This section provides extra information on this method; isomers, stability of standards and formation of MGO during derivatisation are covered.

The PFBHA method only requires 0.2 g honey sample for analysis, which could be argued as too small to be a representative sample for a commercial batch of honey. However, in this research the storage trials only contain 40 g of honey, hence a 0.2 g sub-sample is appropriate. Furthermore, the PFBHA method has been involved in inter-comparison trials to ensure the results for real honey samples is similar to other methods and other laboratories using the same method (see Appendix B).

Stability of standards after derivatisation

A set of standards were analysed on the day they were derivatised and then periodically afterwards to investigate their stability. Figure 3.22 shows the stability of MGO over three days. The results show that, if required, standards can be prepared then analysed three days later without any negative effects on the peak areas. In addition, standards run on day 1 and 2 showed small variation in the gradient. Changes in DHA and HMF were very small (0.69 and 2.80% respectively), while MGO had an 8.55% difference.

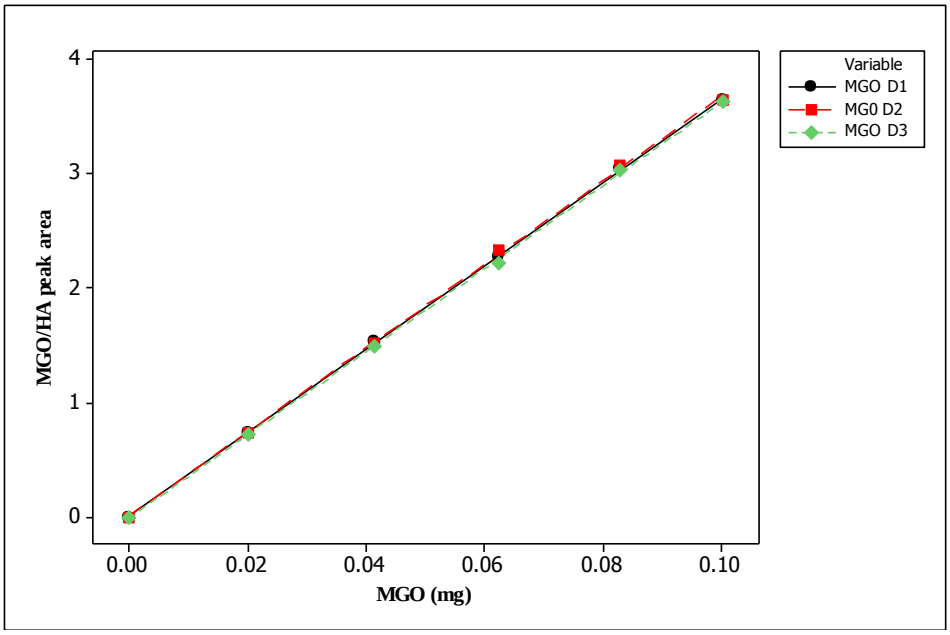


Figure 3.22 MGO calibration curve analysed daily over three days.

A set of standards run 17 days later showed MGO was still stable. The gradient of the line only changed by 3.5%. However, the peak shapes became much broader over time and are not as well resolved. This can be seen by comparing Figure 3.23 with Figure 3.24.

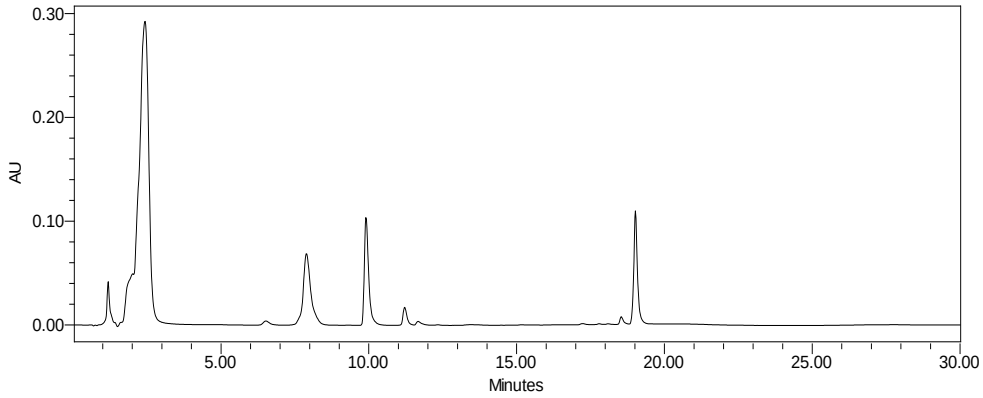


Figure 3.23 Sample analysed on the same day that it was derivatised.

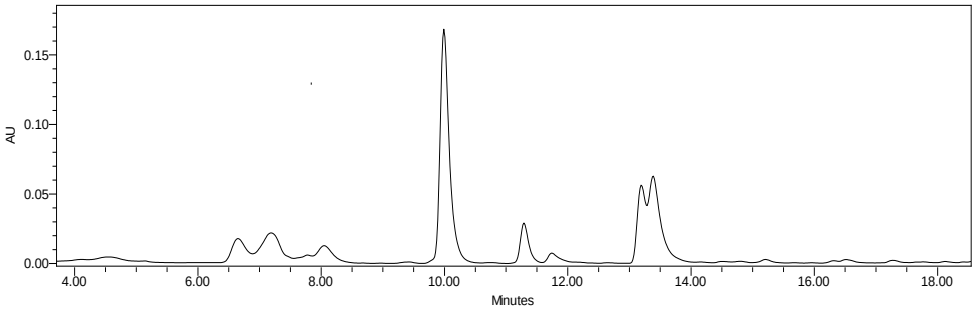


Figure 3.24 Sample run 24 days after being derivatised. The peaks are broader and the PFBHA has continued to react as it is not present in the sample (8 minutes).

Stability of samples after derivatisation

A sample with naturally occurring levels of DHA, MGO and HMF was tested periodically overnight to assess the stability of the derivatised products. The variation between samples was very small. This was repeated a further three times (up to 14 hours) with the same results. Table 3.16 summarises the results for one sample analysed every three hours for twelve hours. It can be concluded that the samples are stable for analysis over this period.

Table 3.16 Summary of one sample that was tested periodically for 12 hours.

	DHA (mg/kg)	HMF (mg/kg)	MGO (mg/kg)
Average	3325	8.58	317
σ	37	0.19	12
% RSD	1.10	2.27	3.87

During this research, some samples were doped with unnatural levels of DHA (10,000 mg/kg DHA, chapter 5) and after prolonged storage had unnaturally high concentrations of MGO. These samples could not be prepared too far in advance of analysis due to precipitation of the MGO-PFBHA derivative.

Formation of MGO from PFBHA derivatisation

The excess PFBHA in the sample may catalyse the conversion of DHA to MGO during the derivatisation step. A high, mid and low value of DHA in water were derivatised and the MGO concentration was recorded (Table 3.17). The sample with low DHA (515 mg/kg) had the same concentration of MGO as the blank. Samples with higher DHA (5,668 and 12,206 mg/kg) had elevated levels of MGO (16 and 21 mg/kg respectively) identifying that some of the DHA has already converted to MGO before analysis. However, a real honey sample would not have the mid-to high range of DHA present; therefore the initial conversion is not a problem when analysing samples.

Table 3.17 High, mid and low DHA derivatised in water.

	DHA (mg/kg)	HMF (mg/kg)	MGO (mg/kg)
Blank	0	1.04	9
Low DHA	515	1.35	10
Mid DHA	5668	1.26	16
High DHA	12206	1.17	21

Further tests were carried out where DHA was doped into water, artificial honey and clover matrices at levels between 100 and 3,000 mg/kg, which reflects the natural concentration seen in mānuka honey. There was no increase in MGO concentration in any of the DHA doped samples for any of the three matrices, confirming that there is no catalysis occurring at naturally occurring levels of DHA in the honey. The base level of MGO in water and artificial honey was 11

mg/kg on average, compared to 20 mg/kg for real honey matrices. The base level of MGO observed appears to be artificially augmented by the derivatisation procedure.

3.3.3 Comparison of DHA, MGO and HMF concentrations reported by four HPLC methods

Twenty Steens honeys (2008-2011) from the database were analysed by PFBHA, Direct, OPD-MGO and OPD-DHA HPLC methods so that the DHA, MGO and HMF concentrations could be compared.

3.3.3.1 Comparison of methods for analysis of MGO

MGO can be detected by three methods (Direct, OPD-MGO and PFBHA). Figure 3.25 shows a 3D plot of the reported concentration of MGO (in mg/kg) by the three methods; there is a positive linear correlation between the three methods. However, the PFBHA method has a consistently higher reading than the other two methods.

A plot of the concentration of MGO analysed by the OPD-MGO method vs. the Direct methods had a linear trend (R^2 = 94.6%, Figure 3.26). Twelve samples (55%) had less than 20% difference in reported concentrations between the two methods. The remaining eight samples had a higher reading when reported by the OPD-MGO method; in two instances (samples 673 and 802) this was due to the MGO concentration being below the LOQ for the Direct method (278 mg/kg), but above the LOQ for the OPD-MGO method (5 mg/kg, Table 3.18). It must be noted that during analysis of the database the tailing of the fructose peak in the Direct method was not as large as when the MDL and accuracy tests were performed (sections 3.3.2.3 and 3.3.2.4). Therefore the reported MGO concentration of samples with MGO concentrations close to the LOQ were not reported as substantially lower than the other two methods.

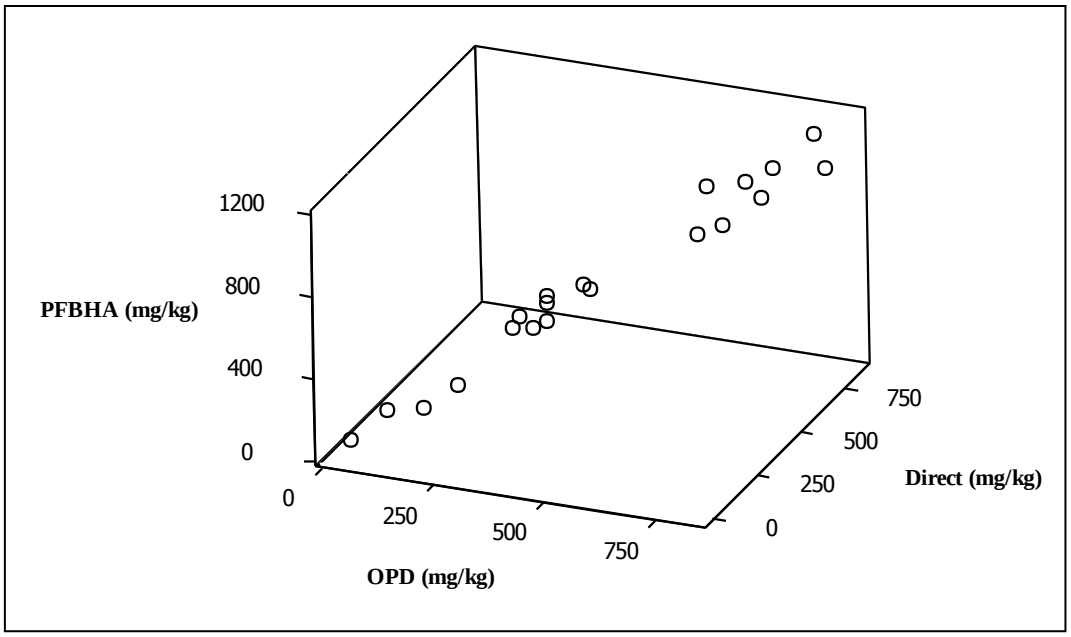


Figure 3.25 3D plot of MGO (mg/kg) for 20 mānuka honey samples analysed by Direct, OPD-MGO and PFBHA methods.

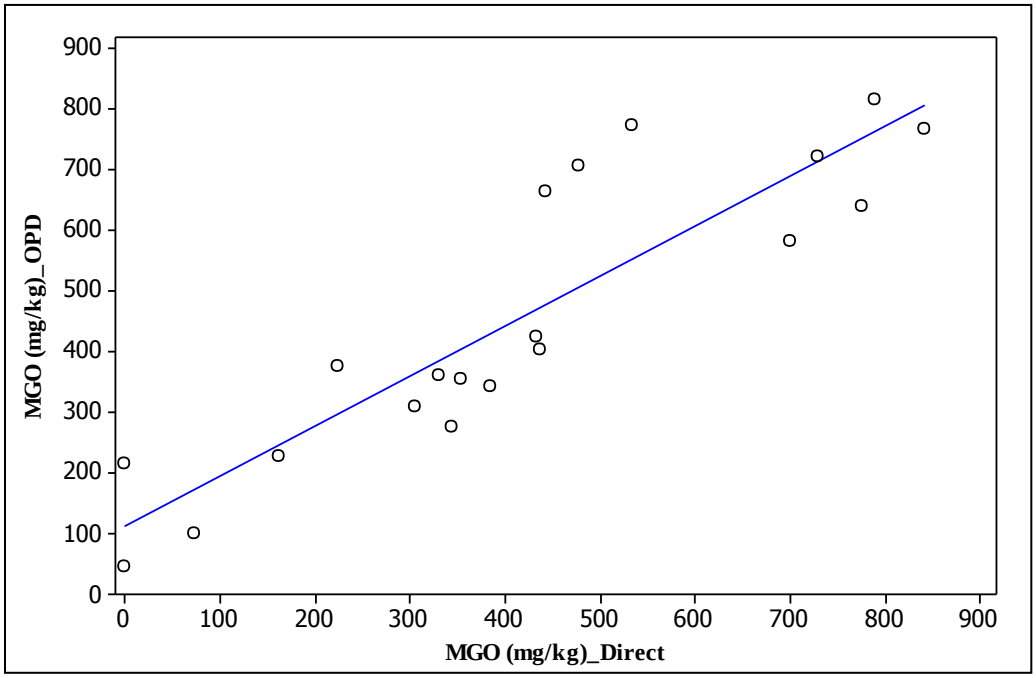


Figure 3.26 Plot of MGO concentrations of 20 mānuka honey analysed by OPD-MGO method vs. Direct method. There is a strong linear correlation (R^2 = 94.6%). However, the Direct method has a lower LOQ than the OPD method and is unable to report values for some samples.

Table 3.18 Reported MGO concentrations by the three methods for samples with low MGO.

Sample	MGO (mg/kg) Direct method	MGO (mg/kg) OPD method	MGO (mg/kg) PFBHA method
673	Below LOQ	216	299
802	Below LOQ	46	84

A plot of MGO concentration analysed by the PFBHA method vs. the Direct method showed a linear trend (R^2 = 82.8%). However, the PFBHA method reads higher for 60% (12/20) of the samples – two of which were below the LOQ for the Direct method.

There is also a strong linear correlation between the PFBHA and OPD methods (R^2 = 97.5%). However, the PFBHA method reads consistently higher for all samples; the samples are between 8-29% higher when analysed by the PFBHA method (Figure 3.28). The data and calibration curve were reprocessed using only the largest MGO isomer, not the summation of the two MGO isomers, to investigate whether or not this had an effect on the data; however, this made no difference to the reported values. The recovery of MGO in the OPD-MGO method is 89.53% and 108.65% for the PFBHA method (section 3.3.2.4) which accounts for 20% variation between the two methods. Dhar *et al.*[150] studied MGO in biological plasma samples and noted that there was a difference between the reported concentration of MGO when different methods were used.

If the derivatisation process of the PFBHA method caused formation of MGO (due to catalysis), samples with high DHA would also show a higher MGO concentration; however, there did not appear to be a correlation between the concentration of DHA and percentage difference of MGO. Furthermore catalysis of PFBHA derivatisation was disproven in samples with naturally occurring levels of DHA in honey in section 3.3.2.6. Another option for the high MGO reading in the PFBHA method is that the PFBHA method is able to release extra MGO so that it is available for detection. It is possible that some of the MGO is reversibly-bound to sugars and could unbind during sample preparation and hence be derivatised and quantified. Stephens and Schlothauer[151] reported that heat releases MGO that is bound to the sugars in honey. Hence, the derivatising agent may also be able to release the MGO.

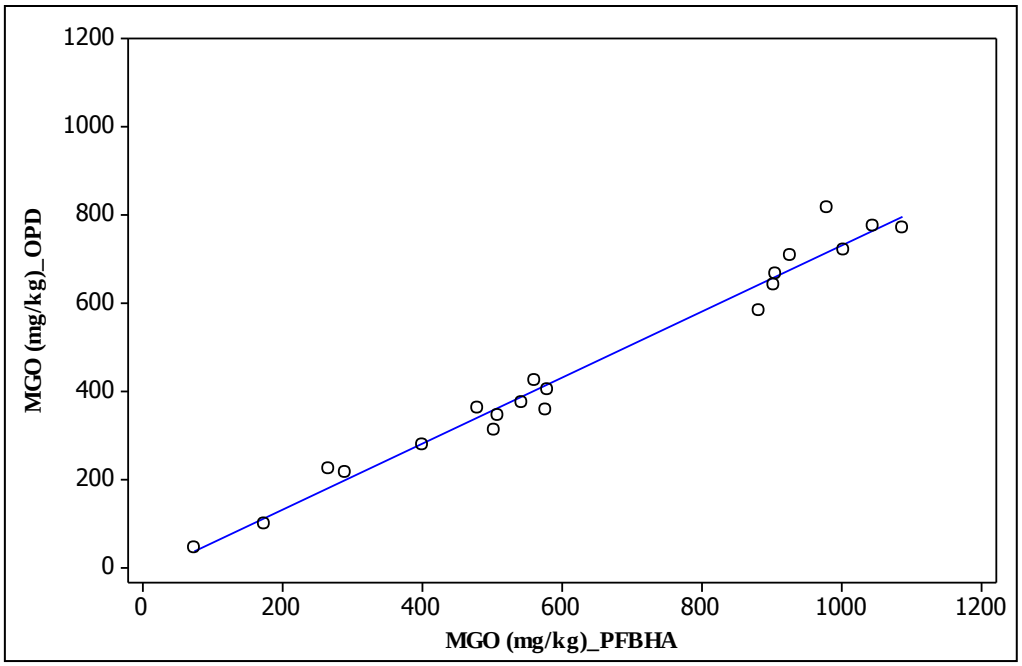


Figure 3.27 Scatter plot of MGO concentrations analysed by PFBHA and OPD methods. There is a good linear correlation (R^2 = 97.5%). However, the PFBHA method reads 10-33% higher than the OPD method for all samples.

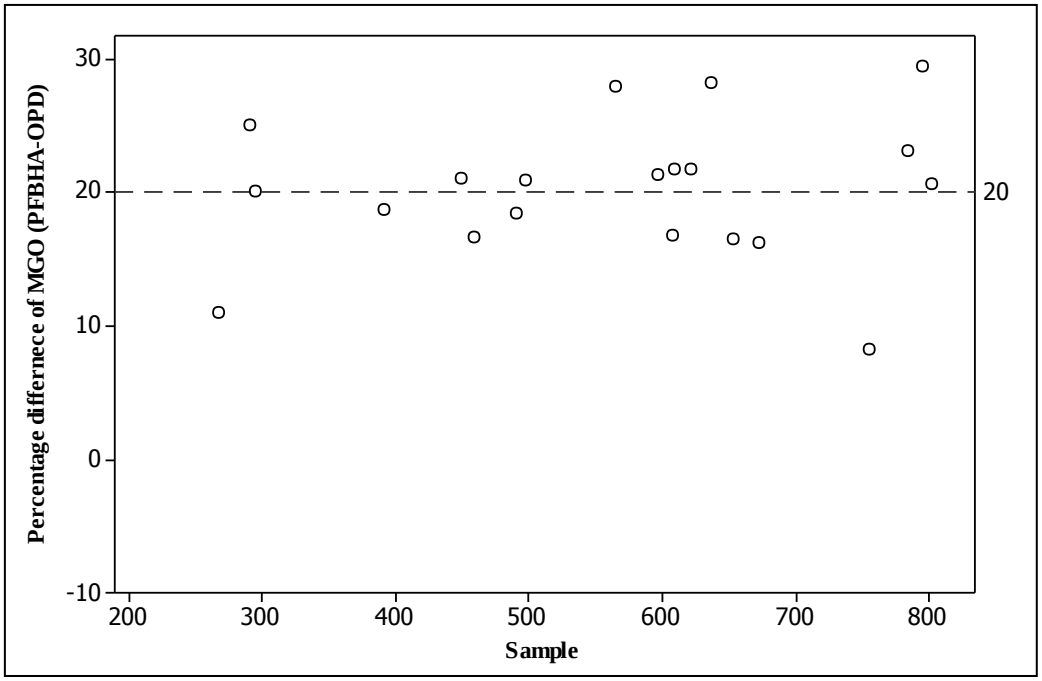


Figure 3.28 Percentage difference for MGO for the PFBHA and OPD methods. Samples are reported between 8-29% higher when analysed using the PFBHA method.

MGO is a very reactive compound and is known to react with amines and proteins. In biological systems it is known to bind both reversibly and irreversibly; irreversibly bound MGO is unable to be detected. Little is known about the binding habits of MGO in a honey matrix. In plasma samples, reversibly bound MGO in equilibrium with free MGO can be detected; when free MGO is derivatised the equilibrium would shift towards free MGO. MGO can reversibly bind to cysteine residues to form hemithioacetal adducts and to lysine and arginine to form glycoslyamine residues.[113] In addition, it can also bind irreversibly with lysine and arginine.[150] However, the type of binding/reaction depends on the pH of the matrix.

The OPD-MGO method uses OPD in a 0.5 M phosphate buffer at pH 6.5 to derivatise the samples for 16 hours (as seen in the literature,[12] or 4 hours in this research) compared to the PFBHA method which uses a citrate buffer at pH 4 to dissolve the PFBHA. It is possible that this difference in pH allows the PFBHA method to free more reversibly bound MGO for detection. Chaplen *et al.*[152] suggested that MGO will unbind in an acidic environment, allowing it to be detected. They focused on a plasma environment and suggested that the addition of perchloric acid (PCA) into samples would precipitate the proteins and the acidic environment would allow MGO to unbind from the proteins, allowing it to be detected. However, proteins are low in honey (0.058-0.786%[153]), so this would not have a large contribution because there would only be minimal MGO bound to protein. Dhar *et al.* (2009)[150] used OPD as the deriviatising agent (either 0.2, 1 or 10mM final concentration, pH was not reported) and treated plasma samples with PCA at different concentrations and incubation times. They reported that there were significant differences in the measured MGO.

The discrepancy in results shows that it is necessary to be cautious when directly comparing results that have been analysed by different methods. This is further discussed in the inter-laboratory comparison, which can be found in Appendix B.

Inter-laboratory comparison of MGO analyses

The reported MGO concentration for three honeys (946, 953 and 1349) were compared with another laboratory, known as Lab B. Lab B derivatised the samples with OPD and used solid phase micro extraction GC for analysis. The

OPD and PFBHA methods in this research (denoted as UoW) were compared to the results from Lab B; the results are shown in Table 3.19. Percentage differences are presented in Table 3.20.

Table 3.19 MGO (mg/kg) comparison between OPD and PFBHA methods.

Sample	PFBHA	OPD-MGO (UoW)	OPD-MGO (Lab B)
		MGO (mg/kg)	
946	67	45	51
953	246	159	180
1349	761	664	793

Table 3.20 Percentage difference between the methods for MGO.

Sample	OPD (UoW) – OPD (Lab B)	OPD (UoW) – PFBHA (UoW)	OPD (Lab B) – PFBHA (UoW)
		Percentage difference	
946	–12.50	–39.29	–27.12
953	–12.39	–42.96	–30.99
1349	–17.71	–13.61	4.12

The results show that the reported value of MGO is within experimental error (20%) when analysed using OPD in both laboratories. This gives confidence that the method is being applied correctly. There is similar discrepancy between the reported OPD concentrations from both laboratories compared to PFBHA results. Sample 1349 has the largest concentration of MGO, but had the smallest difference in reported concentration when the concentration measured by the PFBHA method was compared to the OPD results from both laboratories. The results for the three honeys fall in the same region of difference that was found when all database honeys were examined. This gives support to the idea that different derivatising agents recover different amounts of MGO from the honey matrix.

During this research, participation in three inter-laboratory comparison programmes for MGO detection was carried out with satisfactory results using the OPD-MGO method.[*] Refer to Appendix B for a brief discussion on the inter-laboratory comparison programme results for the three years.

[*] Due to confidentiality reasons, the author cannot identify their laboratory.

3.3.3.2 Comparison of methods for analysis of DHA

The reported DHA concentration was compared between the PFBHA and Direct method for all 20 honeys. The OPD-DHA method was not reported in the literature until after the analyses by the Direct and PFBHA methods were completed, hence there was only enough material for 9 of the honeys to be tested by the OPD-DHA method.

A comparison of DHA between the PFBHA and Direct methods shows a very strong linear trend between the two methods (R^2 = 99.7%, Figure 3.29). All samples (expect two) are within 10% of each other (Figure 3.30), which is an acceptable variation in sampling. The percentage difference was worked out as PFBHA – Direct. The discrepancy between the two methods is a lot smaller than for MGO. The better correlation between the two methods may be due to the DHA peak being well resolved from the fructose peak in the Direct method allowing the peak area to be integrated without interference.

Nine samples were used to compare the OPD-DHA with the PFBHA and Direct methods. 4/9 samples fell within 10% difference when compared with the PFBHA method and another three were within 20%. 6/9 samples fell within 10% difference when compared with the Direct method, with one more sample within 20% (Figure 3.31). The other two samples had between 25 and 53% difference, which may be due to a sampling error because sample 622 had a reported DHA concentration of 3,067 mg/kg when analysed by the OPD-DHA method which is unlikely. However, there was not enough material left to reanalyse the sample.

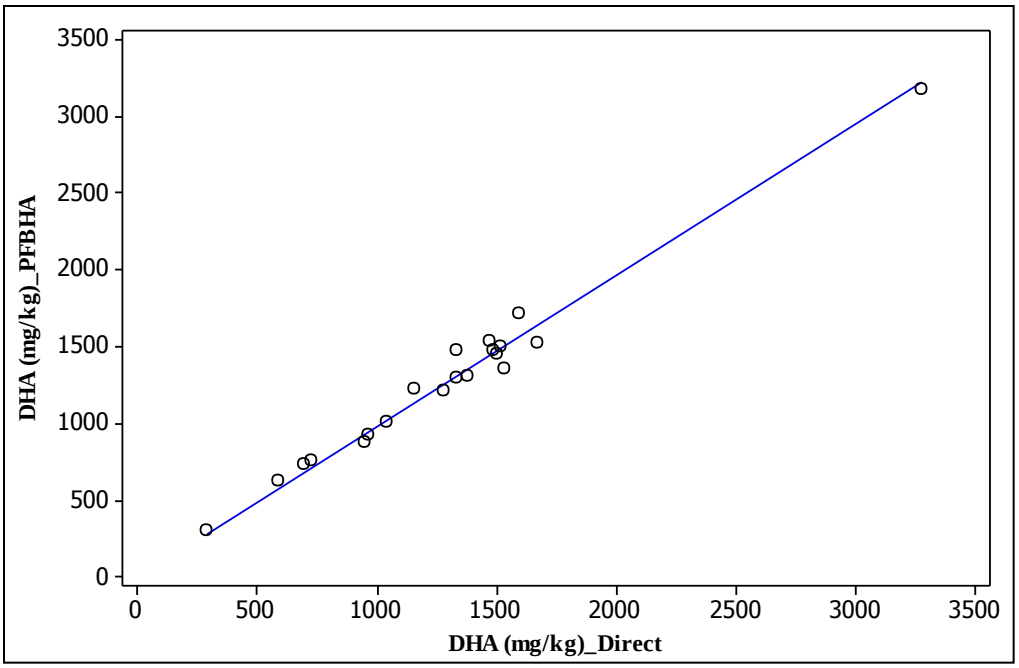


Figure 3.29 Concentration of DHA for 20 mānuka honeys analysed by PFBHA vs. Direct methods. There is a very strong correlation in the reported concentration between the two methods (R^2 = 99.7%).

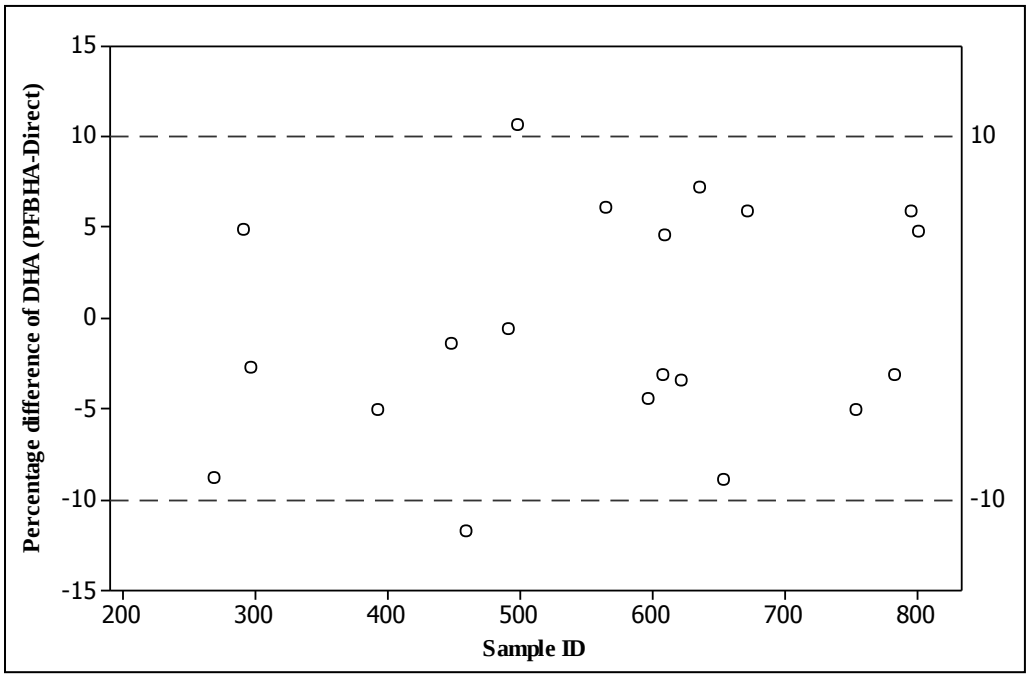


Figure 3.30 Percentage difference of reported DHA concentration between the PFBHA and Direct methods. All samples, except for 2, have less than 10% difference in the measured concentration.

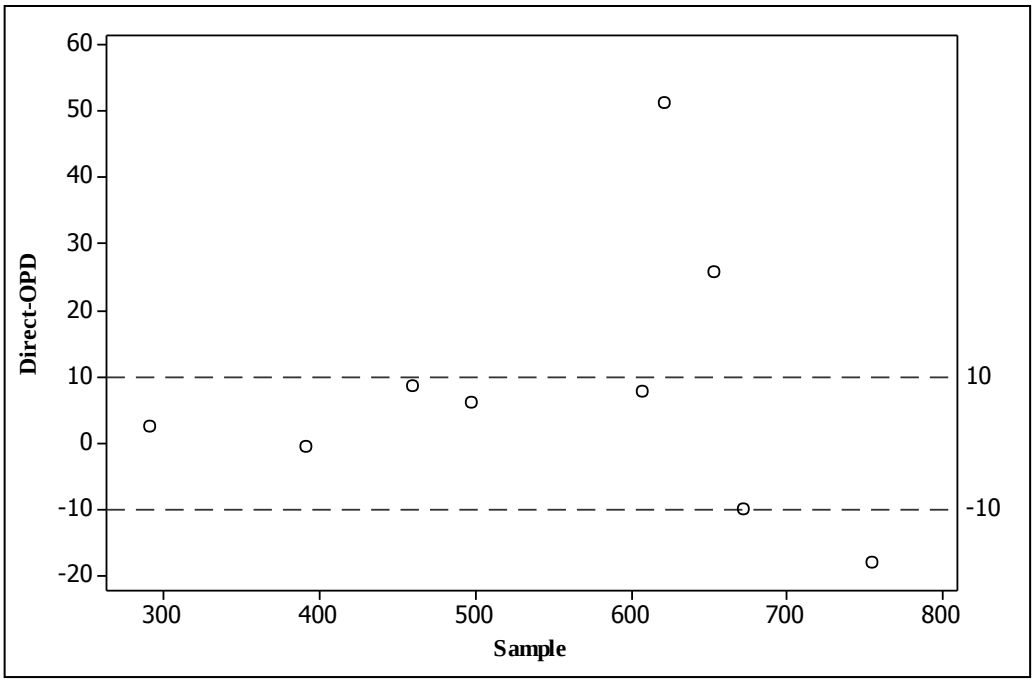


Figure 3.31 Percentage difference of DHA between the Direct and OPD methods. Six of the nine samples are within 10% difference. Two samples have over 25% difference, but this most likely arises from a sampling error.

3.3.3.3 Comparison of methods for analysis of HMF

HMF can only be compared between the PFBHA and Direct method because it was not detected in either OPD method. Originally both HMF isomers were used to report the concentration of HMF in the PFBHA. A plot of the concentration of HMF analysed by the PFBHA method vs. the Direct method gave a R^2 value of 86.3%. There was greater than 20% difference in the HMF concentrations for most samples; the PFBHA method had higher readings (Figure 3.32). Samples with a low concentration of HMF were affected the most due to an interfering peak co-eluting with the second HMF isomer in the PFBHA method, which was more prominent when the HMF concentration was low. It was decided that only the first HMF isomer would be used to calculate the concentration of HMF because this is free of interference. The ratio of the isomers is stable between samples, hence the results will not be affected. A graph of the reported HMF concentration when analysed by the PFBHA method vs. the Direct method gave an R^2 value of 91.5% (Figure 3.33), which is better than when both isomers were used for calibration. In addition, the percentage difference between the PFBHA

method and the Direct method was greatly lowered but not all samples were within the 20% range.

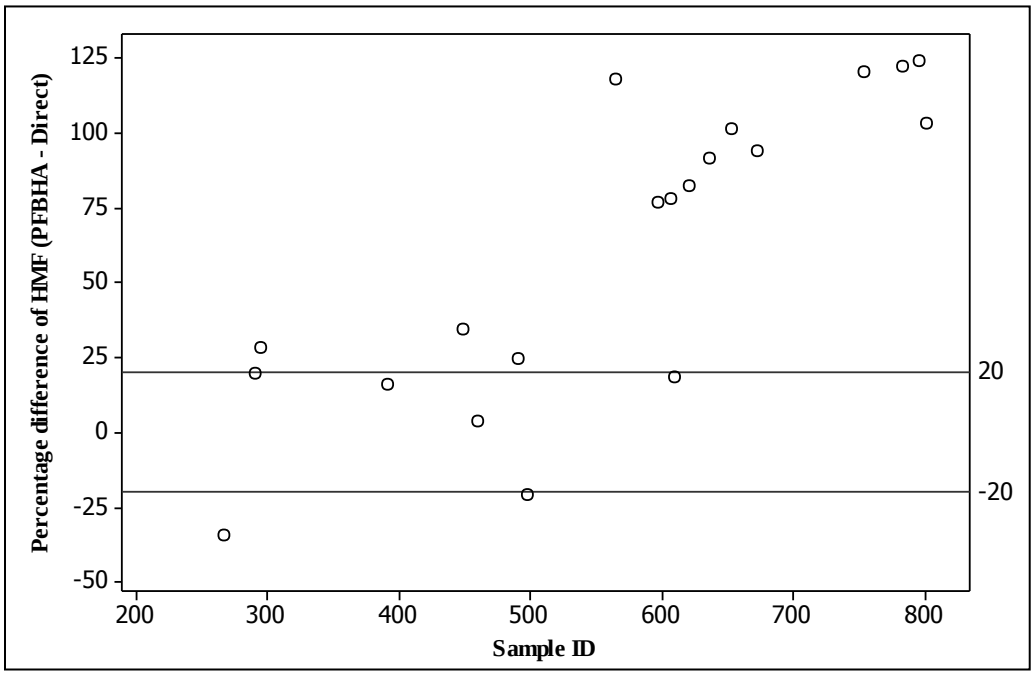


Figure 3.32 Percentage difference of HMF between the PFBHA (sum of two HMF isomers) and Direct methods. The PFBHA method mainly reports a higher concentration than the Direct method.

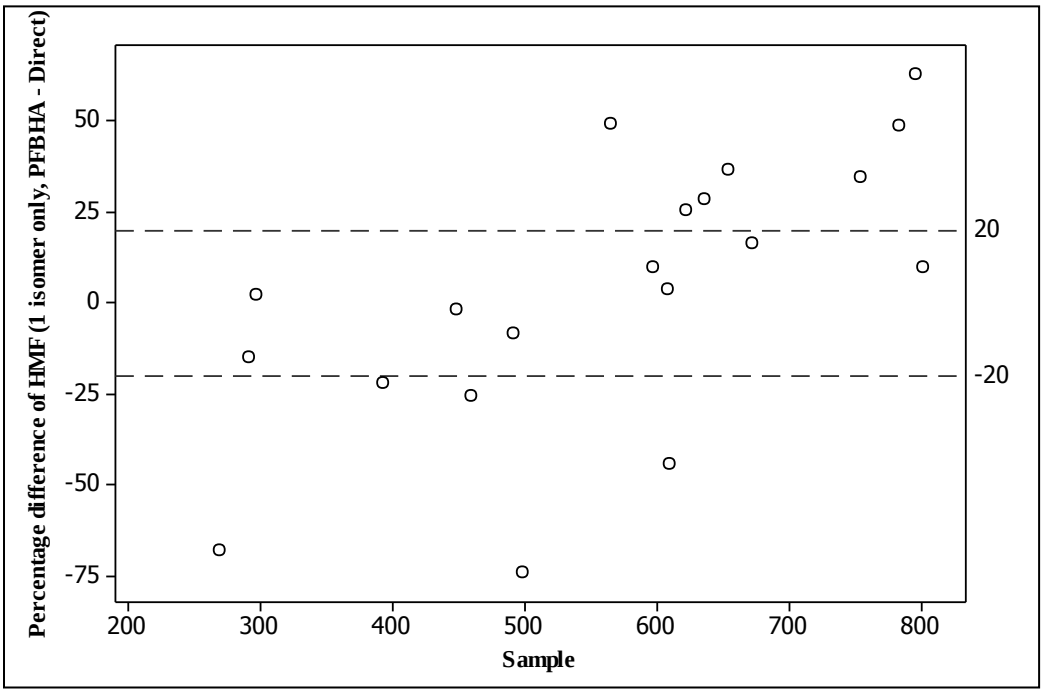


Figure 3.33 Percentage difference of HMF between the PFBHA (one HMF isomer) and Direct methods.

3.4 Conclusion

The four methods analysed had different advantages and disadvantages. The Direct method had a simple sample preparation and was able to detect all three analytes of interest, but it had a very high LOQ for MGO (278 mg/kg) and a long sample analysis time (~60 minutes). The MGO-OPD and DHA-OPD methods were each only able to analyse one analyte of interest which was not practical for this research.

The simultaneous detection of DHA, MGO and HMF by the PFBHA method was deemed to be the best method tested; it has a relatively fast, simple sample preparation as well as moderate analysis time (faster than the other methods examined) and low limits of detection for all three analytes. However, it uses the most expensive derivatising agent (over twenty times the price of OPD per gram) and solvent (acetonitrile). This method was used as the method of choice for the remainder of the research to analyse DHA, MGO and HMF.

4 Analysis of a database of mānuka and clover honeys

This section details the analysis of some of the components present in honey, with a focus on analysis of twenty three mānuka honeys. There is limited literature regarding the make-up of mānuka honey and no reports are currently available for a large database of mānuka honeys which have been analysed for multiple chemical parameters. It is assumed that the general composition of mānuka honey is similar to that of other honeys produced by *Apis mellifera*. However, floral and geographical origin will contribute to differences between mānuka honey and honey from other floral sources.

The chemical properties of mānuka honey are of key importance to this research because certain components may have a catalytic effect on the conversion of DHA to MGO. Furthermore these compounds may catalyse one or more side reactions of DHA or MGO, or bind to either compound of interest. This section gives an important overview of the honey matrix which is background information required for the analysis of the behaviour of DHA and MGO in the storage trials.

4.1 Literature review

4.1.1 General composition of honey

The composition of honey varies depending on factors such as the plant source, season and the bee species. There is currently difficulty in determining the portion of a honey that arises from mānuka nectar. There is no control over the foraging of the bee, hence other nectars may be present. Therefore the best compromise for scientific studies is to liaise with beekeepers to ensure that there is good practice of placing hives near flowering mānuka and removing them as flowering of mānuka stops; this will prevent bees from foraging from other floral sources and diluting the mānuka honey in the hive.

In July 2014, New Zealand Ministry of Primary Industry (MPI)[101] released a report detailing how mānuka honey should be classified until appropriate characteristics have been chosen. They stated that mānuka honey should be identified by having a colour greater than 62 mm pfund, conductivity range of 347-867 µS/cm, flavour that is mineral and slightly bitter, aroma typical of mānuka honey (damp earth, heather, aromatic) and presence of mānuka-type pollen,* DHA and MGO. If a honey meets these characteristics then it can be labelled as mānuka honey. Potential chemical markers have been identified and work is currently progressing to investigate these possible markers. Requirements for choosing a marker include: cost effective, easy analysis, and a compound that is consistently identifiable and stable over time.[154]

Since there is limited literature on mānuka honey, this review will focus on honey (of various floral sources) produced by the European honey bee, *Apis mellifera* which is the only bee used in New Zealand beekeeping.

The National Honey Board of the United States Department of Agriculture (USDA) have compiled a list of the average, range and standard deviation for the major constituents of honey (Table 4.1).[155] They did not state if this was solely floral honey or included honeydew honey. Furthermore, they did not report how many honey samples were analysed. This subsection gives a brief overview of the

* The pollen count may include kānuka pollen as the two are indistinguishable.

main compounds found in honey and those of interest in relation to this research; specific and more detailed reviews follow this section.

Table 4.1 Major constituents in honey (extracted from Sanford).[155]

Constituent	Average	Range	Standard deviation
Fructose/glucose ratio	1.23	0.76-1.86	0.13
Fructose (%)	38.38	30.91-44.26	1.77
Glucose (%)	30.31	22.89-40.75	3.04
Minerals (Ash) (%)	0.169	0.020-1.028	0.15
Moisture (%)	17.2	13.4-22.9	1.46
Reducing sugars (%)	76.75	61.39-83.72	2.76
Sucrose (%)	1.31	0.25-7.57	0.87
pH	3.91	3.42-6.10	-
Total acidity (meq/kg)	29.12	8.68-59.49	10.33
True protein (mg/100 g)	168.6	57.7-567	70.90

Sugars

Honey is comprised of approximately 75% of a mixture of glucose and fructose. The ratio of these two sugars does not vary significantly in honey. Analysis of 775 mānuka honey samples by HPLC reported the average sugar percentage as 29.7% glucose, 37.9% fructose, 1.2% maltose and 0.5% sucrose.[16] The glucose and fructose percentages lie within the range stated by the National Honey Board of the USDA (Table 4.1). Other disaccharides including maltose, kojibiose, turanose, isomaltose and maltulose are also present in low levels. Honey may also contain oligosaccharides, which contain more than three simple sugar sub-units.[156]

Isomaltose, kojibiose, turanose, nigerose and maltose have been reported as significant oligosaccharide components in mānuka honey, with maltose being the major oligosaccharide component. Active and non-active mānuka honeys displayed the same oligosaccharide compositions. In addition, clover honey has been reported to have an identical oligosaccharide composition to mānuka honey.[157]

In 1988, Tan *et al.*[31] proposed that mānuka honey is a honeydew honey. However, analysis by HPLC of the oligosaccharides showed that mānuka is derived from nectar, not honeydew. This was able to be differentiated because the oligosaccharide composition of honeydew honey is more complex and larger

oligosaccharides are present due to transglucosylation enzymes in the stomach of scale insects which act on sucrose and form oligosaccharides.[157]

Organic acids

Organic acids are present between 0.17-1.17% in honey. Gluconic acid (Figure 4.1) is the most prevalent organic acid, and is the most abundant component in honey after the sugars. It originates during the ripening of nectar in the hive; the glucose oxidase enzyme (GOx) catalyses the oxidation of glucose into D-glucono-1,5-lactone which hydrolyses to gluconic acid. Formic acid was identified in honey in 1908, while citric acid, malic acid (Figure 4.1) and succinic acid were not identified until 1931.[158] Other organic acids include acetic, butyric, lactic and pyroglutamic acids. [159] [159] [159]

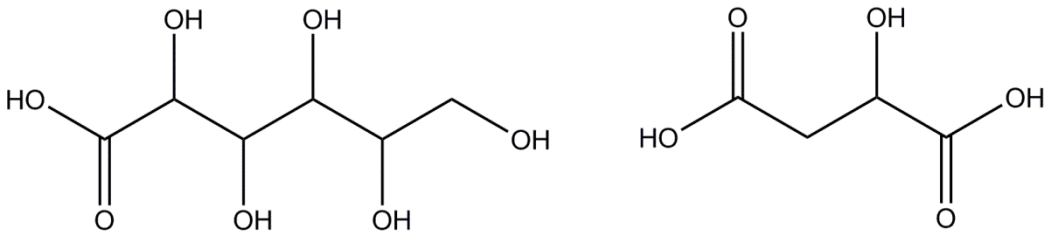


Figure 4.1 Gluconic acid (left) and malic acid (right).

Protein

Protein is the next most prevalent component, after gluconic acid, in honey. About 0.3% of honey is protein.[160] It mostly occurs in the form of enzymes generated by the bee for nectar digestion.[161]

Amino acids

Honey contains approximately 1% of free amino acids.[159, 162, 12] Proline is the most abundant amino acid comprising 50-85% of the total amino acids. Proline comes from the honey bee secretion during the conversion of nectar into honey.[162] Thus there is a similar concentration in honeys regardless of geographical or floral origin.[163] Proline is a secondary amino acid as it has a secondary amino group (-NH-) due to its cyclic nature – the N and α C atoms are part of a five-membered pyrrolidine ring. In comparison, lysine and serine (Figure 4.2) are classified as primary amino acids as they have a primary amino group ($-NH_2$)*.

* This relates to the amino group attached to the asymmetric carbon and does not take into account the functional groups on the side chain.

A detailed review of amino acids in honey and the analytical techniques used for their analysis can be found in section 4.1.6.

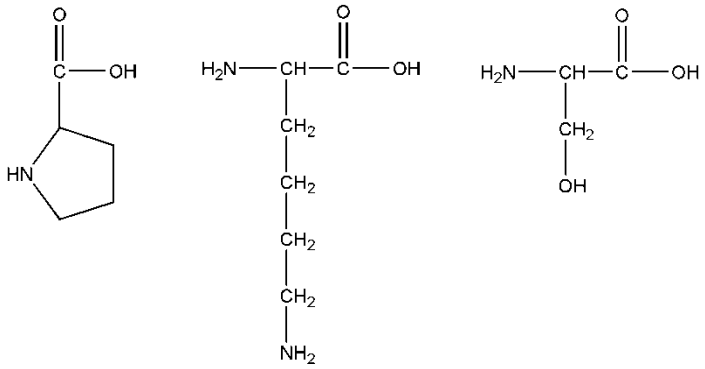


Figure 4.2 From left to right: proline (Pro, P), lysine (Lys, K) and serine (Ser, S).

Enzymes

Some enzymes are present in the nectar, while others are introduced by the bee's hypopharyngeal glands. The most significant of the enzymes are glucose oxidase (EC 1.1.3.4), invertase (EC 3.2.1.26), diastase (amylase, EC 3.2.1.1). Other enzymes include an oxidase, catalase (EC 1.11.1.6) and a phosphate hydrolysing glycerophosphatase.[158] Acid phosphatase may also be present and is likely to be derived from the pollens and nectar of plants.[55]

Invertase is added to the nectar by the bee and is responsible for most of the chemical changes that occur when nectar is ripened to honey. Invertase breaks sucrose into glucose and fructose.[164] At 30 ºC, the solubility of glucose in a fructose solution is increased if the fructose concentration is raised above 1.5 g per gram of water. This allows the gram of water to hold both 1.5 g fructose and 1.25 g glucose; this is 50% more than a dilute fructose solution can carry. The high solubility of glucose does not occur at higher or lower temperatures; in addition, sucrose does not display high solubility at this solution level. The conversion of sucrose into glucose and fructose allows the bees to produce a more concentrated supersaturated solution containing approximately 18% water. This prevents the honey from spoiling and is a energy rich food source which takes up minimal space.[10]

The honey bee adds diastase to the honey which converts starch to dextrins and mono-, di- and oligo-saccharide sugars. Glucose oxidase is also added by the bee and converts glucose to gluconic acid and hydrogen peroxide. The hydrogen peroxide protects the partly formed honey from bacterial decomposition until the sugar content is high enough to protect it.[10, 165] Catalase is present in some honeys and is thought to be derived from the nectar; it is able to decompose hydrogen peroxide.[165]

Vitamins

Vitamins are found at low levels in honey.[160] Reported vitamins include the B vitamins; thiamine (B1), riboflavin (B2), nicotinic acid (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B7) and folic acid (B9); ascorbic acid (vitamin C) is also present.[158] The fat-soluble vitamins, retinol (vitamin A), cholecalciferol (vitamin D), tocopherols (vitamin E) and phylloquinone (vitamin K) have been identified in honey.[55, 158] No reports of cyanocobalamin (vitamin B12) occur in the literature.

Minerals and trace elements

A range of minerals and trace elements are found in honey. Trace elements account for about 0.04-0.2% of honey. The elements vary depending upon the geographical location. A detailed review can be found in section 4.1.7.

Phenolic compounds

So called phenolic compounds are present in honey and contribute to the antioxidant activity, but do not individually or collectively contribute to the NPA of mānuka honey.[49, 166] Many phenolics in honey are derived from propolis; hence the levels depend on the amount and distribution of propolis in a hive. Various phenolics have been reported in mānuka honey. Some phenolic acids, such as caffeic, ferulic, syringic acid and benzoic acid and their derivatives are also common to other floral sources. A detailed review can be found in section 4.1.8.

4.1.2 Dihydroxyacetone

Dihydroxyacetone (DHA, $C_3H_6O_3$, Figure 4.3) is the simplest ketose and has no chiral centre; it is the tautomer of glyceraldehyde. DHA is the precursor to MGO

in mānuka honey and is found in mānuka nectar. The concentration in the nectar varies with mānuka varieties, geographical region and season.[34]

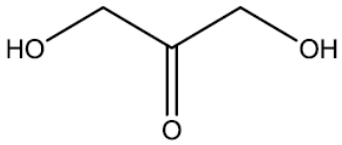


Figure 4.3 Dihydroxyacetone.

The first report of DHA in mānuka honey was by Adams *et al.* (2009).[14] The authors had noted a peak which eluted shortly after MGO in RP-HPLC, in previous work, whilst they were trying to isolate MGO. In 2009, they discovered that this peak, which was not bioactive, was DHA. They reported that it had a linear correlation to NPA; however, it was not as strong as the correlation of MGO and NPA. Analysis of fresh mānuka honey revealed high DHA and low MGO levels. The DHA concentration decreased and MGO levels increased over time when the honey was stored at 37 °C. They noted the same phenomenon also occurred when DHA was added to clover honey and at a slower rate when it was added to an artificial honey. Therefore they concluded that DHA is the precursor to MGO.

There are few publications addressing the concentration of DHA in mānuka honey. Values range in the literature and depend on a variety of factors including the age of the honey and its origin. A study of mānuka honey (n = 9) obtained from the hive showed various levels of DHA (1,192-5,099 mg/kg).[14] Another study of mānuka honey samples (n = 6) straight from the hive showed a range of 611 to 2,724 mg/kg (median 1,622 mg/kg) of DHA, compared to 18 store bought honeys which had a range of 127 to 1,563 mg/kg (median 735 mg/kg).[106] Meloncelli[30] reported 607-4,475 mg/kg DHA in mānuka honey (n = 7). A range of Australian *Leptospermum* honeys, pure and blend, were found to have levels between 412-2,403 mg/kg DHA in the honey.[167] A separate study reported 1,062 mg/kg DHA in a fresh jelly bush honey.[30]

The detection of DHA in honey has been addressed in chapter 3.

4.1.3 Methylglyoxal

Methylglyoxal, better known as pyruvaldehyde in biological sciences, is a three carbon dicarbonyl compound, which has both an aldehyde and a ketone functional group (Figure 4.4). MGO has a high hydrophilicity[83] and is a highly reactive electrophilic compound.[150, 168] Hence it can react with other compounds in the honey matrix.

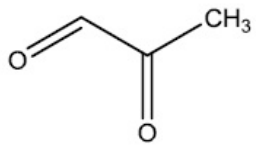


Figure 4.4 Methylglyoxal.

MGO accounts for the majority of NPA in mānuka honey.[37] High levels of MGO are not unique to mānuka honey and have been reported in honeys from other species of the *Leptospermum* genus.[37] MGO is also found in other food sources and physiological systems, but is formed by different pathways. In mānuka honey it is converted from DHA by a non-enzymatic pathway. MGO can be formed non-enzymatically during the caramelisation of carbohydrates and during the Maillard reaction between carbohydrates and amino acids (see chapter 6 for a review). MGO is found at low levels in numerous food substances (generally less than 10 ppm). For example, potatoes (n = 3, not detected-trace amounts), fruit juice (type not specified, n= 23, (not detected-2.2 mg/L), vinegar (n = 23, 1.7-53 mg/L), jam (n = 19, not detected-13 mg/kg).[115] In physiological systems MGO can be formed enzymatically via several biological pathways with three kinds of enzymes – methylglyoxal synthase (EC.4.2.99.11), cytochrome P450 IIE1 isozyme and amine oxidase.[169]

The MGO concentration varies in honey depending on a number of factors. This includes varieties of *L. scoparium* harvested by the honey bee, proportion of honey that is from mānuka nectar and age of the honey. Localised environmental factors or genetic differences between the *in stitu* wild varieties may also play a role.[5,23]

There is limited literature detailing the MGO content in mānuka honey since the discovery of MGO in mānuka honey is relatively new. The detection of MGO in honey has been addressed in chapter 3. Reported concentrations of MGO in mānuka honey vary between studies. Values from 0 to around 800 mg/kg[12, 44, 90, 106] are commonly reported. However, levels up to 1,490 mg/kg have also been reported.[5]

Windsor *et al.*[37] analysed MGO in mixed and monofloral Australian honeys (for example, *L. polygalifolium/L. liversidgei*, *L. polygalifolium/Guioa semiglauca*, *L. speciosum/L. semibaccatum* and *L. polygalifolium*). The MGO content was reported between 43 and 1,723 mg/kg; with the monofloral samples having the highest levels. This range is higher than that reported for New Zealand mānuka honey. A press release on 1 March 2011 from the University of Queensland, Australia made these results public.[170] Molan suggested that while the MGO levels are higher, the honey may not have a higher antibacterial effect as it may not have a synergistic effect like New Zealand mānuka honey.[171] However, this hypothesis has not been tested.

4.1.4 5-Hydroxymethyl-2-furaldehyde

Hydroxymethylfurfural (HMF) also known as 5-hydroxymethyl-2-furaldehyde, (Figure 4.5), is a cyclic aldehyde. HMF is not generally present in fresh food, but it is formed during heating or cooking of sugar-containing foods. It occurs in products where water co-exists with monosaccharides in acidic medium due to formation by the acidic decomposition of monosaccharides. It is also produced in the Maillard reaction by condensation of carbohydrates with free amine groups.[140] A review of HMF can be found in chapter 7, while detection of HMF has been addressed in chapter 3.

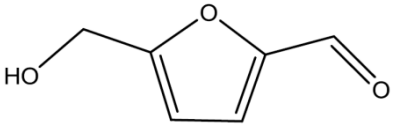


Figure 4.5 Hydroxymethylfurfural (HMF).

The level of HMF in food is directly related to the heat treatment it has received.[172] HMF has been identified in a range of foods and beverages including honey, apple juice, milk, breakfast cereals and tomato products. Roasted coffee contains high levels of HMF; one study found between 300-2,900 mg/kg HMF. Dried fruit can also contain high levels of HMF (2,200 mg/kg in dried plums).[173] HMF is generally not present in fresh honey.[133] However, honey that has been heated, stored in inappropriate conditions or adulterated with invert syrup may have high levels of HMF.[140] In addition, prolonged storage at room temperature can also increase the HMF concentration. Therefore detection of its presence in honey is critical to identifying honey that has been inappropriately treated.

The European quality standards and CODEX Alimentarius[174] have set a limit of 40 mg/kg HMF in honey. However, HMF levels in honey produced in subtropical climates can exceed this limit.[140] Therefore honey produced in tropical temperatures has a raised limit of 80 mg/kg. Furthermore, honey which has low enzymatic levels have a decreased limit of only 15 mg/kg.[131]

Honey is usually warmed to 32-40 ºC during processing to lower its viscosity for extraction, straining or filtration, which does not affect the honey during the short processing period. However, some honey samples are heated at higher temperatures for liquefaction or pasteurisation processes which can increase HMF levels.[175]

4.1.5 Proline

Proline found in honey mainly originates from the secretions of the honey bee. This was confirmed by Von der Ohe *et al*.[176] who fed bees either sugar syrup or honeydew/nectar/pollen. They concluded that the proline in honey comes predominately from the bee secretions, not the feed material; however, a small proportion of proline may also come from flowers.[177]

Amino acids account for 1% (*w/w*) of honey, with proline making up 50-85% of this.[162] Proline concentrations vary largely in honey; a minimum concentration of 180 mg/kg is accepted by the International Honey Commission (IHC) and values

below this suggest adulteration or un-ripeness.[104] Hence proline levels have been suggested as a parameter for detection of sugar adulterated honey.[178]

There is little reported literature on proline concentration in mānuka honey, but various concentrations have been reported for honey of other floral sources; Czipa *et al.*[179] analysed one mānuka honey and reported 809 ± 36 mg/kg proline. White and Rudyj[153] reported a mean concentration of 483 mg/kg proline in 482 honeys from America. Meda *et al.*[180] reported 989.5 ± 407.4 mg/kg proline in 27 honeys from Burkina Faso. Rebane and Herodes[181] collected 61 honeys from Estonian beekeepers and the Estonian Environmental Research Centre and reported proline ranged from 153.7 (polyfloral honey) to 487.4 mg/kg (heather honey). A range of 39 Spanish floral honeys have been reported to have a mean proline concentration of 575.5 mg/kg.[182] A study of various floral French honeys obtained from both independent honey producers and the supermarket had proline contents ranging from 208.7 mg/kg (rape, n = 28) to 592.3 mg/kg (chestnut, n = 38).[183] The large variation of proline in honey reported in the literature may be due to the amount of gland secretions that are added to the honey by the bees,[137] or due to the age of the honey, or treatment to which it has been subjected.

Czipa *et al.*[179] reported 2,283 ± 128 mg/kg proline in coriander honey which was substantially higher than levels found in any of the other samples analysed in their study and many other reported studies. They also found different concentrations of proline in honey from the same floral type that was harvested from different countries (e.g. vipers bugloss from New Zealand and Hungary). They concluded that proline may have been added because the levels are unnaturally high and such levels were only found in commercial samples, not directly from beekeepers. However, high proline concentrations have also been reported in other studies; sidder honey has been reported to have up to 2,800 mg/kg proline collected by *Apis mellifera.*[184] A study by Meda *et al.*[180] of 27 honeys from Burkina Faso also showed high levels of proline in some samples. The samples ranged from 437.8 to 2,169.4 mg/kg with an average of 989.5 ± 407.4 mg/kg. These high levels may occur because the honey bee may not be *Apis Mellifera*. While they did not mention the type of bee, it is possible that the African bee (*Apis mellifera scutellata*), which is a subspecies of the Western honey bee, collected the honey. In addition, high proline was reported in a Bangladeshi honey from *Apis dorsata*

(2,932.8 mg/kg).[185] Most of the literature does not state which species of bee collected the honey and this may be a factor in the wide range of concentrations reported. Therefore care needs to be taken when comparing the proline concentrations in honey collected by different bee species.

As honey ages, the proline content decreases, this trend is well known and can be used as a gauge of honey ripeness. The decrease of proline is accelerated at high temperatures (40 ºC and above).[179, 186] A study by Wootton *et al.* (1976)[187] on six Australian honeys stored at 50 ºC for 44 days had widely varying results; these ranged from a 10% increase to 85% loss of proline. It is possible that the initial increase in variation was seen due to the reversible binding of proline to other compounds, decomposition of bee saliva or an error during analysis. An American study by White and Rudyj (1978)[153] reported that the average loss of proline in honey stored at 37 ºC for 4 months is 18.6% (an initial increase would not have been noted in this case due to the extended time between samplings). A storage study conducted on honeys from Pakistan (ambient temperature) showed that proline decreased between 43 and 83% during 12 months. The results showed an increase in proline in one of the honeys (Churain) over the first three months before it decreased (Figure 4.6).[184] This concurs with results from 1978. Furthermore, Iglesias *et al.* (2006)[163] analysed the proline concentrations in 54 honeys (floral, honeydew and blend) stored at ambient temperature over 24 months. They also reported that the proline level initially increased and peaked at six months before declining.[163] It is plausible that the initial increase in proline could be due to a release of the proline from the bee saliva, which may contain proline-rich proteins. The usual methods of proline analysis only detects proline present in its free form; therefore as these proteins degrade, the proline concentration would initially increase. The decrease in proline may be due to it reacting with or binding to other components in the honey. Furthermore, Iglesias *et al.*[188] proposed that the increase in proline could be due to protease and/or peptidase activity of enzymes in the honey. More information is required for a full picture of the behaviour of proline over time.

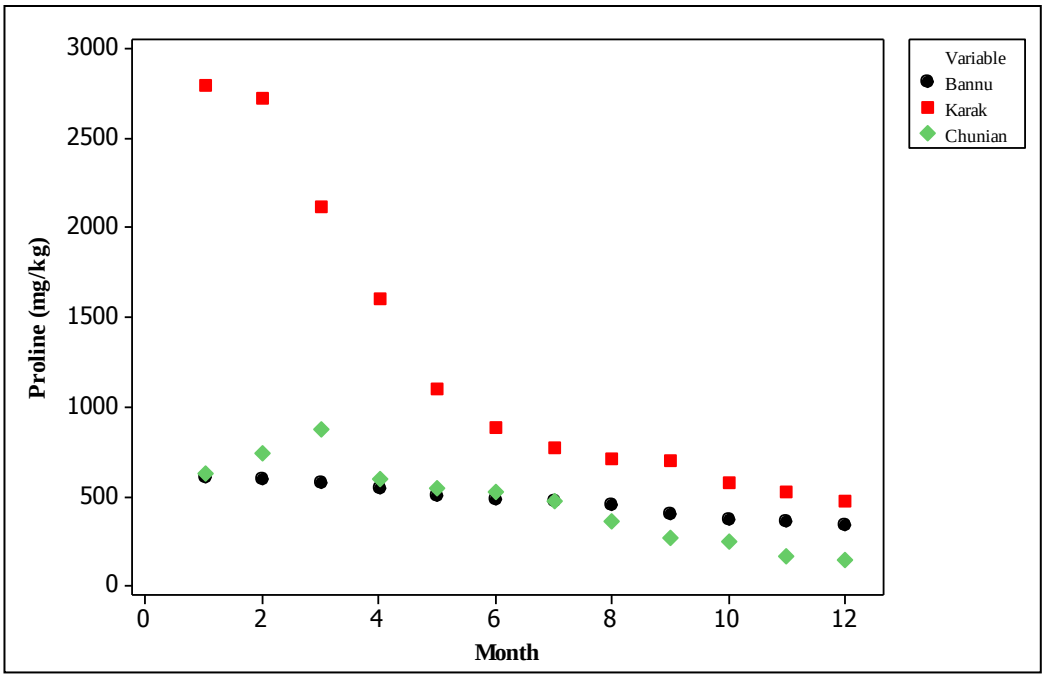


Figure 4.6 Loss of proline over time in Pakistan honeys. Data extracted from Qamaer *et al.*[184]

Analysis of proline can be difficult using HPLC because some derivatising agents are unable to derivatise secondary amino acids. Most literature uses a modified method of UV analysis by Ough.[109] Analysis of proline in honey is often carried out by an AOAC official method (979.20).[110] This method requires derivatisation with ninhydrin followed by UV detection. Another version of the Ough method is reported by IHC.[108] Truzzi *et al.*[189] compared the Ough, AOAC and IHC methods and reported that the AOAC method is the best method due to its accuracy and time saving compared to the other two methods. Linearity was reported up to 1,800 mg/kg and the LOD and LOQ were 20 and 61 mg/kg respectively.

Proline is the only amino acid that forms an orange/yellow colour when reacted with ninhydrin; all other amino acids develop a blue/purple colour. The principal interfering compounds (with relative responses compared to proline in parentheses) are lysine (5%), tryptophan (2%) and glutamine (1.4%). Lysine and tryptophan contribute to approximately 0.8 and 3.2% of amino acids in honey respectively; therefore the error from primary amino acids in proline analysis is about 0.2% and may be disregarded.[153]

4.1.6 Primary amino acids

The presence of primary amino acids in mānuka honey are of interest because there is indication that arginine and lysine can catalyse the conversion of DHA to MGO.[14] Knowledge of which amino acids are present and at what concentration may show a relationship with the conversion rate of DHA to MGO. Total amino acids account for 1% of honey and only 15-50% of this is primary amino acids.[162] 26 primary amino acids have been reported to occur in honey, but not all amino acids are in all samples. The main source of amino acids arises from pollen, therefore the profile may be characteristic of the botanical origin.[162] Free amino acids have been reported as a way to detect floral source.[162, 182-183, 188] There is no reported literature on the profile of amino acids in mānuka honey. Due to the aforementioned variation in amino acid content between floral sources, the profile of free amino acids in mānuka honey is likely to be different to the profile of honeys from other floral origins. Furthermore possible reaction of DHA and/or MGO with amino acids may decrease the concentration of some amino acids in mānuka honey over time.

Cometto *et al*. (2003)[182] compared the free amino acid (13 primary amino acids) profile of 56 honeys from three Argentinian regions. They reported that samples could be grouped into sampling regions, which had different flora, using cluster analysis. Samples with similar flora showed close grouping to one another, even if the sites were far apart. Proline was not used for analysis as there was no significant difference between regions. Hermosin *et al.* (2003)[162] studied amino acid content (22 analysed) in 31 Spanish honeys. They used principal component analysis to explain 64% of the variance with the first three principal components. They reported that the main primary amino acids were phenylalanine, tyrosine and lysine. Cotte *et al.* (2004)[183] reported the use of amino acids to detect different botanical origins, and used this to determine adulteration. Total primary amino acids (18 analysed) ranged from 108.5 mg/kg for fir honey to 1,278.5 mg/kg for lavender honey. The lavender honey had extremely high amounts of phenylalanine (1,152.8 mg/kg) compared to the other six varieties sampled (5.5-20.8 mg/kg). Sunflower honey had 312.7 mg/kg primary amino acids. Rebane and Herodes (2008)[181] analysed 20 primary amino acids in Estonian honeys; the total primary amino acid content ranged from 115.3 mg/kg in linden honey to 188.7

mg/kg in polyfloral honey. Perez *et al.*[190] determined the free amino acid content in Spanish honeys. They reported that the main primary amino acids were phenylalanine and tyrosine in floral honeys, which agrees with Hermonsin *et al.*.[162] Pereira *et al.*[191](2008) also reported that phenylalanine was the most abundant in a range of Madeira Island honeys, Portugal mainland honeys and Canary Island honeys; Rebane and Herodes (2008)[181] had a similar finding for 61 Estonian honeys.

Analysis of primary amino acids is commonly carried out by HPLC with fluorescence detection,[182-183, 188, 190-194] but photodiode array detection is also used.[162, 181] GC-FID and GC-MS are also reported in the literature.[195]

The literature is divided between simply diluting the honey sample before analysis[183, 188, 191, 193] and isolating the amino acids before analysis.[181-182, 192, 194]

Detection of amino acids was originally performed with HPLC using ion-exchange chromatography followed by post-column derivatisation with ninhydrin. However, this has been superseded by pre-column derivatisation because it is more flexible and does not require a dedicated instrument. There is a selection of pre-column derivatising agents used in the literature; the most common two are fluroenylmethyloxychloroformate (FMOC) and *o*-phthaldialdehyde (OPA). Diethyl ethoxymethylenemalonate (DEMM)[162, 181, 196] and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC)[197,196] have also been reported in the literature. Each derivatising agent has its own advantages and disadvantages.[193]

OPA was first recognised as a fluorigenic reagent for amino acids by Roth in 1971.[198] It is now the most common derivatising agent for primary amino acids and biogenic amines.[193] The reaction of OPA with amino acids requires a thiol-containing reducing agent and the solution must be alkaline for the reaction to take place. 2-mercaptoethanol (MCE) is most widely used as the thiol source in the literature. However, the isoindoles obtained from amino acids with OPD/MCE are unstable. Therefore the stability of the bulkier thiols *N*-acetyl-L-cysteine (NAC) and 3-mercaptopropionic acid (MPA) with OPA were examined by Molnár-Perl and Bozor.[199] The reported conditions used for derivatisation are widely varying for OPA/MPA and OPA/NAC. For example, the concentration of borate buffer

applied ranged from 0.03-0.45 M. Until 1998, there was little research regarding the characteristics and stability of OPA/MPA and OPA/NAC reagents. Storage of the OPA/MPA reagent varied between ambient temperature for three days[200] to refrigeration for up to one week; storage of OPA/NAC varied from two days to one month at ambient temperature. Molnár-Perl and Bozor[199] concluded that OPA/MPA and OPA/NAC have self-fluorescence which decreases to a stable blank after 90 minutes; this fluorescence is stable for 4 hours at ambient temperature and four days at 4 ºC.[199] Therefore the analyst must be aware of the age and storage conditions of the reagents to ensure acceptable results.

The major disadvantage of using OPA is that it does not react with secondary amino acids such as proline and hydroxyproline.[198, 201] This is a major shortcoming because proline makes up 50-85% of the amino acids in honey. Despite the drawbacks of OPA, it is a good derivatising agent which reacts rapidly, allowing analysis to occur within five minutes of the reaction taking place. Derivatisation of OPA does not require heat and is well suited to automatic derivatisation.[198, 201] Furthermore no extraction of amino acids is required. Therefore there is no potential loss of sample or need for an internal standard.[193]

FMOC has been used as a derivatising agent to overcome problems with OPA because it is able to react with both primary and secondary amines. FMOC requires quenching and also produces a large reagent peak in the chromatogram. The literature contains many different procedures for the use of FMOC which often contradict each other. For example, the concentration of the borate buffer ranges from 0.01 to 0.325 M and the pH ranges from 6 to 11.4.[202] Also, the solvents and their ratio vary. In 2009, Jámbor and Molnár-Perl published a literature overview on FMOC derivatisation for amino acids. This review aimed to clarify the wide range of reaction conditions found in the literature.[203]

Publications that use OPA by itself[191, 199, 204-206] and combined with FMOC[201] are present in the literature. The combination of OPA and FMOC allows simultaneous quantification of primary and secondary amino acids and of biogenic amines.[201] Herbert *et al.*[201] used OPA and FMOC to analyse amino acids and biogenic amines in musks and wines. They reported that proline co-elutes with late amine peaks, preventing its quantification. Therefore they made a second derivatisation

reaction which further diluted the sample to reduce the interference of other amino acids.

Many of the publications using OPA and/or FMOC have high chromatographic flow rates (up to 1.8 mL/min) and long analysis times (up to 120 minutes). Kelly *et al.*[193] developed a method based on those already seen in the literature with the aim of fast analysis time and solvent economy. They reported a method which did not diminish the integrity of the selectivity or sensitivity using OPA/NAC to detect 16 amino acids and seven biogenic amines in 35 minutes. Samples only required prior dilution before analysis due to the high sensitivity of the method; therefore an internal standard was not required. Proline, valine, tryptophan, α-aminobutyric acid, histadine and ornithine were not reported. Derivatisation of the sample was performed in-loop using OPA/NAC so there were no handling requirements. They reported that 1 OPA:5 NAC reagent gave the best peak height and shape. This method was used for honey samples; the coefficient of variation of honey data was reported to range from 1.94% for *gamma*-aminobutyric acid to 8.33% for glycine in a honey matrix.

4.1.7 Major, minor and trace elements

Trace elements account for 0.04-0.2% of honey.[175] Studies of elements and minerals found in honey have been undertaken around the world. However, there are only limited studies on New Zealand honeys, and even fewer on mānuka honey. The main inorganic elements in New Zealand honeys are of interest. Furthermore, levels of iron in honey may be important because there has been a belief in the industry that adding iron to mānuka honey will increase the non-peroxide activity. Knowing the natural concentration will help detect adulteration of elements in honey. Certain elements may also have a correlation with the rate of conversion of DHA to MGO in mānuka honey.

Elements in honey are dependent on the plant's absorption of minerals from the soil and environment. Elements vary due to the floral and geographical origin and have been suggested as a geographical marker for honey.[175] Therefore comparison of mānuka honey to honey of other floral origins and other countries will probably show large differences. Vanhanen *et al.*[207] reported that 27 minerals have been

tested in honeys across nine different countries, with no honey containing all 27 minerals. The major and minor elements (Ca, K, Mg, Mn, Na) are particularly dependent on the geographical origin.[207-208] For example, honeys from coastal regions may have an elevation of alkali metals by up to 10 times.[208] A review of honeys from different geographical regions, not including New Zealand, reports Cu, Fe, Zn and Mn are present in intermediate quantities.[208] Contamination of trace elements in honey may occur due to its acidic nature. Honey that has been left to ripen in steel containers may have higher levels of trace elements (due to metal release) than samples which are freshly collected and sealed in lined drums. Sn levels may be increased by its release from glass lids of containers in which the honey is stored.[209]

Vanhanen *et al.*[207] analysed 18 trace elements in ten New Zealand honeys, including one mānuka honey (the NPA rating was not specified). Samples were collected in autumn for analysis. They reported that K, P and Ca were the most abundant elements. They noted that these are also the most abundant minerals in honey from Canary Islands, Spain. K made up 73% of the minerals in New Zealand honey; this is similar to that found in Spanish honey from one study;[210] 34.8-3,640 mg/kg K was reported in the New Zealand study and 434.1-1,935 mg/kg in the Spanish study. A different Spanish study reported that K accounted for 48% of the minerals quantified; however, only six minerals were quantified.[211] The authors reported a range of 261-1,380 ppm for K in 25 Spanish thyme honeys. A Slovenian study also showed high levels of K.[212]

The New Zealand study reported that the heavy metals Cd (0.01-0.55 mg/kg), Pb (0.04-0.04 mg/kg) and Zn (0.00-2.46) were very low in honey. One sample did not contain Zn; this may be due to its deficiency in South Island soil.[207] In comparison, a study[213] on blossom, bends and honeydew honeys from Czech Republic identified higher levels of these elements; 0.5-77.4 mg/kg Cd, 0.02-1.0 mg/kg Pb and 0.2-22.9 mg/kg Zn.

Cr and Mo were not reported in the one mānuka sample,[207] which is similar to other honeys that were tested. The average Fe content for this mānuka honey sample was 1.86 mg/kg; this is close to the average Fe concentration of all honeys tested (1.71 mg/kg). Mn (2.15 mg/kg) was higher in mānuka than the average

(1.04 mg/kg), whereas Na (7.90 mg/kg) was much lower than the average (23.9 mg/kg). Na had a large range (1.10-110 mg/kg) over all samples tested. This is possibly due to its ubiquity and likelihood of contamination.

Cantarelli *et al*. (2008)[160] reported that Argentinean honey has more K, but less Ca and Mg than Turkish honey; honey from Italy was reported to have similar Fe and Zn concentrations, but higher Mn and Cr. Pisani *et al.*(2008)[214] reported that K (1,147 mg/kg), Ca (257 mg/kg), Na (96.6 mg/kg) were the most abundant elements found in honey from Siena County, Italy. Mg, Fe, Zn and Sr were found in the range of 1-5 mg/kg. Madejczyk *et al.*[215] reported that honeydew honeys have much higher Na and Mg levels than seen in rape honeys. But rape honey had a higher Ca content.

4.1.7.1 Methods for analysis of inorganic elements in honey

An overview of methods used for elemental detection is given here because the literature contains many different methods for analysis of trace elements in honey. A set of mānuka honeys were analysed for a various elements during this research.

Sample preparation

Honey is readily soluble in water; however, it is a complex matrix which is rich in organic matter. Therefore prior to elemental analysis, it is common practice to ash or wet-digest the samples; the residue is re-dissolved in water or acid solutions and the mineral components are transferred into solution. Pre-treatment disrupts the organic matrix of honey and extracts metal species from the complex sample matrix into solution. There are numerous advantages to treatment of the sample prior to analysis, including, elimination of variations in physico-chemical properties of samples and standards. Prevention of physical and chemical interferences in the plasma in ICP-MS and also prevention of accumulation and deposition of carbonaceous residues in the nebuliser are also advantages.[208]

Literature is divided between digesting[207, 209, 213, 216-217] and ashing[211, 218-220] for preparation of the honey. However, both are time consuming and prone to loss of analytes from sample overheating or analyte gain through contaminations.[208] Ashing may result in loss of elements from volatilisation during drying and

ashing.[221] Tuzen *et al.*[219] reported 40-93% recovery using dry ashing for 10 elements, compared to 80-97% and 96-102% for the same elements when wet digestion and microwave digestion, respectively, were used for sample preparation. Wet digestion is better suited to volatile elements such as Cd, Ni, Pb, Ni and Zn compared to dry ashing. Fredes and Montenegro (2006)[221] reported that Cd and Pb were 2.1% in the honey sample when using an ashing method. However, when an acid digestion was used they were reported as 14.9 and 34.0% respectively in the sample. In addition, a decrease in positive responses for Ni and Zn due to incomplete destruction of the organic matter during ashing was noted. They suggested that Cd, Ni, Pb and Zn should be analysed by acid digestion, but Al, Co, Cr, Cu, Fe, Mn and Sr should be analysed by the ashing method.[208]

One gram of honey is a common sample size for digestion.[214-215, 217, 222-223] Less than one gram has also been reported.[224-225] However, a sample of honey can be inhomogeneous, which may affect the validity of results. The sample size and the way a portion is collected are critical to give valid results. Caroli *et al.*[209] noted that replicates were significantly different due to the inhomogeneity of the honey. They resolved this by weighing out 10 g of honey and adding 5 g of water and heating the resultant solution to 50 ºC with sonication. A 1.8 g portion of this was then digested and used for analysis. Analysis of bulk-fluid material has also been cited elsewhere in the literature[208] and is a convenient procedure to eliminate heterogeneity.

Honey samples are easily decomposed with a HNO_3/H_2O_2 digestion, even at room temperature due to the high proportion of sugars.[226] HNO_3/H_2O_2 acid digestion has been used in a range of ratios.[209, 214, 219, 222, 224-227] Digestion using only HNO_3 has also been reported.[217] In addition, the dilution volume differs, including 50 ml,[224-225, 228] 25 ml,[209-210, 222, 229-230] 10 ml[219, 221, 226-227, 231] and 5 ml.[219, 223] This variation may be influenced by the instrumental technique used for analysis. Caroli *et al.*[229] compared plain water dilution and acid-assisted (HNO_3/H_2O_2) microwave digestion for sample preparation. The data from the two sample preparation methods were in good agreement and were reported to be equally effective. They adopted water dilution for further sampling for analysis of 11 elements due to the time efficiency and reduced contamination possibilities. A different study also compared dilution and digestion and found no significant

difference (95% confidence level) in the results. They adopted the dilution procedure for further analyses due to preparation time and the need for less sample.[222]

Closed vessel acid decomposition in a microwave oven system is preferable because it reduces or eliminates contamination or analyte loss.[232] However, hot plates have been used.[221] Elemental analysis of honey without prior digestion is also reported in the literature.[222, 229, 233-234] This is advantageous as it minimises the loss of volatile analytes and possibility of contamination. In addition, it is a faster sample preparation method.

Sample concentration is limited by instrument conditions because solution viscosity is increased due to the high organic matter content which may block the nebuliser. Furthermore formation of carbon compounds may affect the sensitivity. It has also been noted that samples are not stable for more than several hours because turbidity and flocculation may occur due to denaturation of proteins and microbial activity stimulated by the high content of carbohydrates in honey.[229, 233] Also, the matrix composition may change due to colloidal particles in the solution absorbing ions.[235]

Instrumental technique

Inductively coupled plasma (ICP) with mass spectrometry (MS), atomic emission spectroscopy (AES) or optical emission spectrometry (OES) has been reported for determination of elements in honey. ICP is a good technique as it is able to simultaneously detect all elements of interest, has high sensitivity and can quantify concentrations over several orders of magnitude.[209] One disadvantage of ICP-MS is the interference of atomic and molecular ions in the plasma produced from argon and constituents in the matrix. This includes $^{40}Ar^{12}C$ vs. ^{52}Cr, $^{40}Ar^{16}O$ vs. ^{56}Fe and $^{40}Ar^{35}Cl$ vs. ^{75}As.[209] However, these can be overcome by using a dynamic reaction cell (DRC)[236] or kinetic energy discrimination (KED).[237]

Atomic emission spectrometry (AES),[238] atomic absorption spectrometry (AAS)[213, 238] and particle-induced X-ray emission (PIXE)[239] have been recorded in the literature for metal determination in honey. Uncommon methods including anodic stripping voltammetry have also been reported as an alternative method for

determination of heavy metals. This cheap instrument can quantitatively measure trace levels.[240] Furthermore electrothermal atomic absorption spectrometry (ETAAS) has been discussed in the literature.[241] One complication is carbonaceous residue build up in the atomiser. This has been overcome with the addition of hydrogen peroxide to the honey solution; this addition also decreased the background noise.

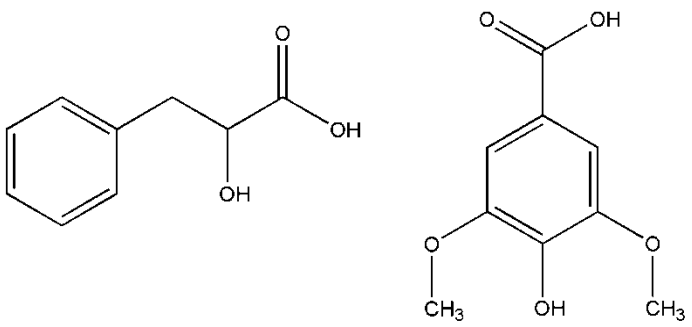
4.1.8 Phenolic and flavonoid compounds

A common problem with most of the literature discussed on analysis of phenolic compounds in honey is the small sample sizes and also use of commercially purchased honeys, hence details of the floral origin may not be known. This has been addressed further in section 4.3.8. The assembly of a large database of certified mānuka honeys (with known geographical location) is required and is currently being carried out by MPI.[154]

Various authors have reported that mānuka and kānuka honeys have a common phenolic profile, likely to occur due to the difficulty in distinguishing honey from the two plants. Phenyllactic acid, methyl syringate and a methoxylated benzoic acid are the primary shared compounds. Mānuka honey is reported to differ from kānuka with the presence of 2-methyoxybenzoic acid and trimethoxybenzoic acid.[5]

Tan *et al.* (1988)[31] analysed ether extracts of mānuka, kānuka and clover honeys by GC-FID. A range of acidic and phenolic substances were characterised. Both mānuka and kānuka honey contained 2'-methoxyacetophenone, 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid, Figure 4.7), 2-methoxybenzoic acid and 2-hydroxy-3-phenylpropionic acid (phenyllactic acid*, Figure 4.7), which were not present in clover honey. Phenyllactic acid was the most abundant phenolic compound.

* This can also be called 3-phenyllactic acid, but will be referred to as phenyllactic acid through this research.



Phenyllactic acid Syringic acid

Figure 4.7 2-Hydroxy-3-phenylpropionic acid (phenyllactic acid, commonly known as β-hydroxyphenyllactic acid (left) and syringic acid (right).

Wilkins *et al.* (1993)[242] reported a combination of phenyllactic acid and 2-hydroxy-3-(41-methoxyphenyl) propionic acid in concentrations greater than 700 mg/kg honey in mānuka honey samples (n = 14). Furthermore, syringic acid and 3,4,5-trimethoxybenzoic acid had a combined concentration greater than 35 mg/kg honey. Yao *et al.* (2003)[243] reported 140 mg/kg phenolic acids in mānuka honey (n = 2). The authors reported gallic acid as the most abundant phenolic in mānuka honey. In comparison, jelly bush had a lower gallic acid concentration and was higher in coumaric acid. Ellagic acid and caffeic acid were reported as 10.8 and 10.4% of the total phenolic acids respectively. Another study,[5] reported 903-2,706 mg/kg phenolics in seven mānuka honeys; 83-786 mg/kg of methoxylated phenolic compounds were also present.

There are varying reports on methyl syringate; Weston *et al.*[244] stated that methyl syringate (Figure 4.8) made up approximately 70% (*w/w*) of the phenolic fraction of mānuka honey. Methyl syringate was also reported in mānuka honey by Inoue *et al.*[245] and Kato *et al.*.[246] Meloncelli[30] reported the presence of methyl syringate in mānuka, kānuka and jelly bush honeys. Senanayake[247] reported that non-active mānuka honey contained a higher concentration of methyl syringate than active honeys. Recently Beitlich *et al.*[248] proposed that methyl syringate is a good marker for kānuka honey; hence the higher abundance observed in the non-active mānuka honey by Senanayake[247] may be due to a higher proportion of kānuka honey present in these samples. But the number of samples are limited and they did not test the DHA content and mono-florality cannot be guaranteed. Weston *et*

al.[244] stated that mānuka honey had the same phenolic compounds regardless of the NPA.

A novel glycoside of methyl syringate (leptosin, methyl syringate 4-O-β-D-gentiobioside, Figure 4.8) was also reported by Kato *et al.*[246] and subsequently remained leptosperin.[*249] Leptosperin ranged from 107 to 643 mg/kg in *L. scoparium* and *L. polygalifolium* honeys almost exclusively; trace amounts were found in other honeys from the Oceania region, possibly due to contamination from *Leptospermum* nectar. They suggested it may be a good chemical marker for mānuka honey. Leptosperin was positively correlated with the NPA concentration, but could only inhibit *S. aureus* at very high concentrations (25 mM). Beitlich *et al.*[248] also reported a high abundance of leptosperin in jelly bush honey (n = 1).

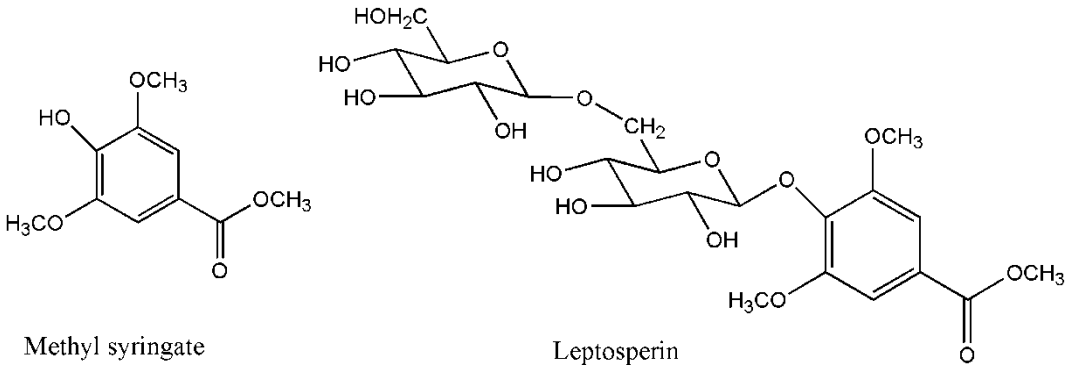


Figure 4.8 Methyl syringate (left) and methyl syringate 4-O-β-D-gentiobioside (leptosperin, right).

Phenolic compounds have been suggested as a marker for monofloral type and for geographical origin. Methyl syringate and phenyllactic acid have been proposed as floral markers of mānuka honey, but methyl syringate is found in high levels in rape, asphodel and other honeys,[250-251] while phenyllactic acid is common in European honeys, hence they may not good compounds for biomarkers of any floral honey.

Research by Oelschlaegel *et al.*[114] identified methyl syringate, 4-methoxyphenyllactic acid, phenyllactic acid and 2-methoxybenzoic acid as good markers for distinguishing mānuka honey (n = 40). In addition, they identified kojic acid, 5-methyl-3-furancarboxylic acid, unedone, lumichrome and 3-

[*] This was to avoid confusion with other unrelated leptosins.

hydroxy-1-(2-methoxyphenyl)penta-1,4-dione in mānuka honey for the first time and noted that they were also good markers.

Stephens *et al.* (2010)[5] reported that 2-methoxylated benzoic acid had a linear correlation with MGO in fresh mānuka honeys (n = 19). In addition, phenyllactic acid was reported to decline over time in honey from 90% of reported phenolic compounds in fresh mānuka honeys to approximately 70% in 5-year-old honeys. Therefore the total concentration and relative proportion of the methoxylated phenolic components increased throughout maturation. However, they did not see an inverse relationship between phenyllactic acid and 4-methoxyphenyllactic acids that had previously been noted by Wilkins *et al.*.[242]

Beitlich *et al*. (2014)[248] analysed eight mānuka honeys, seven kānuka honeys and one jelly bush honey by UHPLC-PDA-MS/MS and HS-SPME-GC/MS* and reported all three honeys could be discriminated from each other using both non-volatile and volatile compounds. Important compounds found in the HPLC profile of mānuka honeys were leptosperin, acetyl-2-hydroxy-4-(2-methoxyphenyl)-4-oxobutanate, 3-hydroxy-1-(2-methoxyphenyl)-penta-1,4-dione, kojic acid, 5-methyl-3-furancarboxylic acid, and two unknown compounds as well as 2-methylbenzofuran, 2′-hydroxyacetophenone, and 2′-methoxyacetophenone in the GC profile. Characteristic compounds in the HPLC profile of kānuka honeys were 4-methoxyphenyllactic acid, methyl syringate, *p*-anisic acid, and lumichrome as well as 2,6,6-trimethyl-2-cyclohexene-1,4-dione, phenethyl alcohol, *p*-anisaldehyde, and an unknown compound in the GC profile. The authors noted that jelly bush (n = 1) had a higher abundance of 2-methoxybenzoic acid, *cis*-linalool oxide, 3,4,5-trimethylphenol and an unknown compound and lower abundance of 2-methoxyacetophenone than mānuka honey.

Further discussion of the literature can be found in section 4.3.8.

Flavonoids

Flavonoids are a class of plant secondary metabolites; they are incorporated into honey through propolis, nectar and pollen. Flavonoids, including quercetin,

* HS-SPME-GC-MS = Head space solid phase microextraction gas chromatography mass spectrometry.

isohamnetin and luteolin have been found in mānuka honey. These compounds are in low concentrations and do not contribute to the antibacterial activity of mānuka honey.[166]

Yao *et al*. (2003)[243] analysed two mānuka honeys and reported that the average content of flavonoids was 30.6 mg/kg honey with quercetin (18.8%), isorhamnetin (12.9%), unknown flavonoid, F01, (12.7%) chrysin (5,7-dihydroxyflavone) (12.6%) and luteolin (12.6%), making up 64.6% of the total flavonoids. However, this may not be representative of mānuka honey due to the small sample size. The authors compared the flavonoid content of mānuka honey with jelly bush honey (n= 12). Jelly bush honey has a lower flavonoid content (22.2 mg/kg honey); but both had similar flavonoid profiles. Chan *et al.*[92] reported that the total flavonoid content of mānuka honey (n = 31) ranged between 5.9 – 22.4 mg/kg of honey, with an average of 11.6 ± 1.6 mg/kg of honey, which is lower than found by Yao *et al*..[243] In addition, the flavonoids reported by Chan *et al.*[92] also differed from Yao *et al*.;[243] 61% of the flavonoid content was made up of pinobanksin, pinocembrin, luteolin and chrysin. Other studies[49, 244] have identified pinobanksin, pinocembrin, chrysin and galangin in mānuka honey but report lower concentrations than found by either Chan *et al.*[92] or Yao *et al*.;[243] however, this may be due to the extraction method used. Chan *et al.*[92] also reported 2-formyl-5-(2-methoxyphenyl)-pyrrole in the flavonoid fraction was weakly correlated to the NPA. However, it was not active against *S. aureus in vitro*. Hence the reason for correlation with NPA is unknown.

4.2 Experimental

Samples were analysed for DHA, MGO, HMF, sucrose, glucose, fructose, proline, primary amino acids, water content, pH, acidity, a range of major, minor and trace elements and selected phenolic compounds. The methods and materials used for determination of the composition of honey can be found in chapter 2.

4.3 Results and discussion

4.3.1 Overview of the honey database analysed

As previously mentioned, there are many articles that discuss characteristics and properties of honey, but there is very little information on mānuka honey. The main compounds in mānuka honey (e.g. sugars, water content and pH) are expected to be similar to other honeys produced by *Apis melifera*. However, other compounds, such as amino acids and trace elements, may differ depending on floral source or geographical region. It is necessary to know what compounds are present in mānuka honey and their concentrations to help determine if they have an influence on the conversion of DHA to MGO. Therefore a database of honeys have been analysed for DHA, MGO, HMF, sugars, proline, major and minor elements, moisture content, pH, acidity and selected phenolic compounds. Mānuka honey is stored by beekeepers longer than honeys of other floral origins in order to increase the antibacterial activity. During this time some physical and chemical properties of honey may change. Therefore honeys that have been stored for different lengths of time were included in the database. This may give some insight into compounds or physical properties that may affect the conversion of DHA to MGO. Examination of the data by year may show depletion in certain compounds which could be due to a reaction with DHA or MGO. Due to limited mānuka honey studies or New Zealand honey studies in the literature, the samples analysed have been compared to a broader range of floral honeys.

Twenty three mānuka honeys from Steens Honey Ltd. were harvested between 2003 and 2012. These samples make up the main part of the mānuka honey database and were tested for all properties. Until the samples were taken, honeys were stored in drums at Steens Honey Ltd. at ambient temperature. Sample extracted from 2003-2011 were obtained for this research in early 2011 and the samples from 2012 were obtained in the same year as harvest. All samples were stored either at 3-5ºC or −18 ºC when not required for testing. A summary of the year of harvest and NPA after a period of storage for the 23 samples is shown in Table 4.2. NPA values reported here are the values supplied by Steens Honey Ltd. and will not necessarily reflect the NPA rating at the time they were received for the current research due to the conversion of DHA to MGO and subsequent loss of MGO. Fresh mānuka honeys were also obtained from Gibbs Honeys and New

Zealand Honey Traders (Table 4.3). They were analysed soon after harvest for a restricted number of properties, and have been included in the relevant sections. The number of storage years has been rounded to the nearest year before the sample was received. Samples from 2012, 2013 and 2014 were collected and analysed in the same year that they were harvested, but may have been stored for a number of months prior to being received.

Table 4.2 Summary of honey database samples from Steens Honey Ltd..

Sample	UMF*	Year honey harvested	Storage time before analysis (years)†	Activity*	Date activity tested*	Area of harvest‡
268	25	2008	4	23.0	Jun-11	1
291	20	2008	4	20.4	Aug-10	1
296	25	2008	4	23.0	Jun-11	1
392	20	2009	3	20.0	Jun-10	1
449	25	2009	3	24.0	Jul-10	1
460	20	2009	3	25.0	Feb-12	1
491	25	2009	3	23.5	Jul-10	1
498	20	2009	3	20.8	Dec-10	1
565	15	2010	2	12.4	Jan-11	1
597	10	2010	2	9.2	Aug-10	2
608	15	2010	2	13.5	Jan-11	1
610	15	2010	2	12.9	Jan-11	1
622	15	2010	2	13.6	Jan-11	1
637	10	2010	2	10.4	Aug-10	2
654	10	2010	2	10.6	Aug-10	2
673	10	2010	2	7.7	Aug-10	2
755	5	2011	1	8.3	Jul-11	2
784	5	2011	1	13.7	Jul-11	1
796	5	2011	1	6.7	Jul-11	2
802	5	2011	1	5.6	Jul-11	2
946	-	2012	0	7.2	Aug-12	1
953	-	2012	0	11.4	Aug-12	1
1349	-	2003	9	13.7	Mar-03	Unknown

* Samples tested for activity prior to 2009 were tested by one laboratory and samples after this were tested for MGO at a different laboratory. UMF and activity values were provided by Steens Honey Ltd.

†Storage years are rounded to the nearest year before the sample was collected. Samples from 2012 were collected and analysed in the same year, but may have been stored for a number of months prior to being received.

‡ Due to commercial sensitivity, the areas of harvest have been changed to numerical values.

Table 4.3 Summary of honey database samples from Gibbs honey and New Zealand Honey Traders.

Sample	Year honey harvested	Storage time before analysis (years)*	Area of harvest†
14	2012	0	3
16	2012	0	3
21	2012	0	3
25	2012	0	3
26	2012	0	3
31	2012	0	3
32	2012	0	3
41	2012	0	3
59	2012	0	3
66	2012	0	4
76	2012	0	4
78	2012	0	4
84	2012	0	3
1404	2014	0	5

*Storage years are rounded to the nearest year before the sample was collected. Samples from 2012 were collected and analysed in the same year, but may have been stored for a number of months prior to being received.

† Due to commercial sensitivity, the areas of harvest have been changed to numerical values.

4.3.2 Analysis of DHA, MGO and HMF

The database honeys were measured in triplicate using HPLC with PFBHA derivatisation to analyse DHA, MGO and HMF. Results are shown in Table 4.4. DHA concentrations ranged from 189 to 3,188 mg/kg and the average was 1,4940 ± 734 mg/kg DHA. MGO concentrations ranged from 44 to 1,085 mg/kg and the average was 455 ± 317 mg/kg MGO. The spread of DHA and MGO are large not only due to natural variation, but also the span of years of the honeys analysed. The HMF concentration ranged from 1.87 to 195 mg/kg. The lowest concentration is from a honey that was analysed soon after extraction. The maximum concentration of HMF is from sample 1349 which was harvested in 2003 and stored at ambient temperature until 2011; with this value removed the next highest HMF level is 31.80 mg/kg which is below the limit chosen by Codex Alimentaruis for HMF.[7] The average HMF concentration was 12 ± 8 mg/kg without sample 1349.

Table 4.4 Summary of DHA, MGO and HMF (mg/kg) and DHA:MGO for database honeys.

Sample	DHA (mg/kg)			MGO (mg/kg)			HMF (mg/kg)			DHA: MGO
268	870	±	24	977	±	78	24.26	±	2.89	0.9
291	761	±	3	881	±	13	31.80	±	0.71	0.9
296	1009	±	13	1003	±	25	30.67	±	1.42	1.0
392	1215	±	11	903	±	4	21.59	±	1.03	1.3
449	1497	±	34	1085	±	41	22.45	±	0.75	1.4
460	1360	±	35	926	±	22	21.03	±	0.25	1.5
491	1478	±	94	1042	±	92	23.74	±	1.20	1.4
498	1341	±	96	902	±	89	12.89	±	1.35	1.5
565	1229	±	27	500	±	8	13.02	±	0.73	2.5
597	921	±	29	399	±	5	12.74	±	0.47	2.3
608	1292	±	35	560	±	10	11.80	±	0.87	2.3
610	1540	±	28	540	±	6	10.97	±	1.09	2.9
622	1448	±	9	579	±	10	11.75	±	0.17	2.5
637	1713	±	9	575	±	10	16.23	±	0.09	3.0
654	1526	±	6	477	±	10	13.89	±	0.18	3.2
673	625	±	5	288	±	5	15.31	±	0.61	2.2
755	1311	±	14	265	±	2	8.55	±	0.06	4.9
784	3172	±	38	507	±	7	7.04	±	0.32	6.3
796	736	±	29	172	±	1	7.59	±	0.19	4.3
802	297	±	6	73	±	1	7.65	±	0.06	4.1
946	322	±	5	67	±	1	9.56	±	0.02	4.8
953	2675	±	97	246	±	10	10.07	±	0.47	10.9
1349	189	±	4	761	±	9	195.45	±	5.80	0.3
14	1612	±	179	179	±	180	7.30	±	0.81	9.0
16	1558	±	118	182	±	1	3.10	±	0.41	8.5
21	2091	±	19	274	±	36	2.94	±	0.16	7.6
25	2300	±	29	289	±	3	7.24	±	0.40	8.0
26	2095	±	19	236	±	51	2.73	±	0.18	8.9
31	1217	±	18	171	±	18	4.38	±	0.35	7.1
32	2378	±	106	123	±	1	4.09	±	0.27	19.3
41	3188	±	58	304	±	65	3.45	±	0.24	10.5
59	2899	±	77	359	±	5	8.50	±	0.58	8.1
66	1552	±	7	255	±	4	8.63	±	0.22	6.1
76	1322	±	3	217	±	1	7.54	±	0.51	6.1
78	1037	±	50	165	±	5	6.74	±	0.52	6.3
84	1968	±	18	302	±	4	8.45	±	0.26	6.5
1404	1539	±	87	44	±	4	1.87	±	0.36	35.0
Av.	1494	±	734	455	±	317	11.71*	±	7.74	5.76
Min	189			44			1.87			0.25
Max	3188			1085			31.80			34.95

* Sample 1349 was excluded from the average calculation.

Sample 784, harvested in 2011, and sample 953, harvested in 2012, have high DHA concentrations (3,172 and 2,675 mg/kg respectively). These samples were analysed in the same year that they were extracted from the hive so did not have as much time to convert to MGO as older samples. The MGO content of both honeys (507 and 246 mg/kg respectively) are reasonably high (ratings of 14.9 and 9.6 NPA respectively), suggesting that they will have high NPA once they have completely converted. Both samples were harvested from the same location showing that this area has high potential. In comparison, sample 946, which was collected from a different area in the same region, was harvested and analysed in 2012; it has a very low concentration of DHA (322 mg/kg), reiterating the variation of DHA between batches of mānuka honey.

Sample 1349 was harvested in 2003 and was stored at ambient temperature until 2011. The conversion of DHA to MGO is an irreversible reaction, therefore it is expected that all DHA will convert to MGO. However, some DHA is still present (189 ± 4), suggesting that there is some factor stopping complete conversion; the DHA may be reversibly bound in the honey and be unavailable for reaction, but the conditions of sample preparation may allow it to be detected as free DHA during analysis.

Windsor *et al*. (2012)[37] analysed DHA and MGO in a range of honeys from various *Leptospermum* species from New South Wales, Australia using HPLC analysis with PFBHA derivatisation. The samples were from years 2005 to 2010. The concentrations of DHA and MGO were similar to those reported in this current research. The samples (n = 34) had an average DHA concentration of 1,012 ± 434 mg/kg and MGO concentration of 493 ± 397 mg/kg and had large standard deviations due to the range of ages of the samples.

The ratio of DHA:MGO (Table 4.4) is used as a gauge to estimate how far the conversion of DHA to MGO has progressed. Freshly harvested honey has a high concentration of DHA and a low concentration of MGO. Over time, as MGO is formed from DHA this ratio decreases. A high ratio at the beginning of the reaction indicates that the honey has a high potential and will produce a high level of MGO. However, not all DHA will convert to MGO due to side reactions. Hence the starting level of DHA cannot predict the final level of MGO on a 1:1

basis (this is discussed in more detail in chapters 5 and 6). Sample 1404 was analysed soon after harvest; it has a very large ratio (35 DHA:1 MGO) indicating it is fresh and has a high potential. Sample 953, which was harvested and analysed in 2012 has a DHA:MGO ratio of 11:1, indicating the reaction also still has a long way to progress.

Some beekeepers sell their honey when the ratio of DHA:MGO reaches 2:1, while other beekeepers hold onto it for longer. The longer the sample has been stored the closer the DHA to MGO ratio is to 2:1; samples can progress past this point, but this progression occurs slowly due to pronounced side reactions of MGO at later times (this is discussed more in chapters 5 and 6). A scatter plot of the DHA:MGO ratio vs. the number of years the sample has been stored[*] was plotted (Figure 4.9). This set of samples reached a 2:1 ratio in their second year of storage at ambient temperature. Attainment of this ratio can be sped up by increasing the temperature the samples are stored at, but may also increase side reactions of DHA and/or MGO hence the potential of the honey will decrease. Sample 1349 was harvested in 2003 and analysed after 9 years of storage. This sample has a DHA:MGO ratio of 0.25:1; however, mānuka honey is not usually kept for this long, so it would be rare to see this in commercial honey.

Windsor *et al.* (2012)[37] analysed honeys direct from an apiary. They discussed the ratio of the two compounds; however, it was stated as MGO:DHA. They reported a steady proportional increase over time, similar to that seen with the honey in this research. In contrast to fresh honey, Atrott *et al.*[106] (2012) stated that all commercial honeys have a 2 DHA:1 MGO ratio. It is unlikely that all commercial honey have this ratio, but they may have purchased honeys of the same age which have been treated with similar storage conditions. On average, honey is held by the beekeeper for less than one year before it is sold. Therefore most honey purchased commercially would be of similar age. However, a number of factors will influence the ratio, such as the starting concentration of DHA, storage temperature, the length of time from extraction to packaging and how long the honey has been on the shelf. The conversion will continue while it is packaged and if a shop keeper has the sample in direct sunlight (such as a window or close

[*] Storage time is defined as the time it was at ambient temperature, not held at 4 or −18 °C.

to the door) the conversion of DHA to MGO or side products, and further reaction of MGO may be accelerated by the heat.

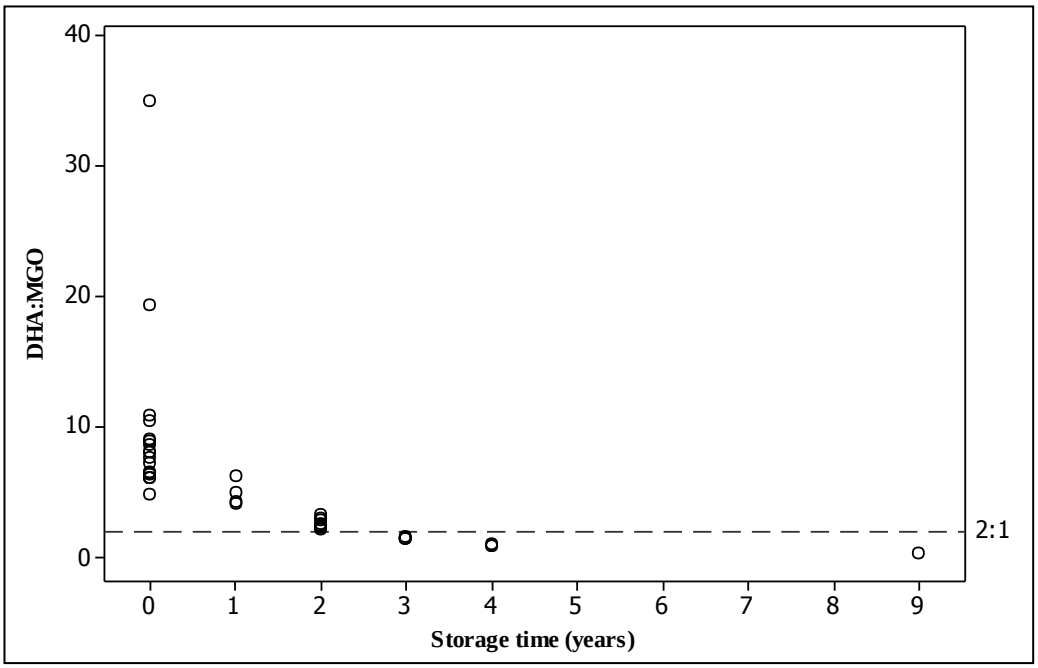


Figure 4.9 DHA:MGO vs. number of years honey was stored. The older the honey, the closer the ratio of DHA:MGO is to 2:1 (dotted line) as the conversion of DHA to MGO has had longer to progress. Sample 1404 has a high potential (35:1 ratio).

Atrott *et al.*[106] studied 18 commercial mānuka honeys, 6 fresh mānuka honeys and 17 various other honey varieties obtained from supermarkets. They reported a linear correlation between the DHA and MGO content of fresh and commercial mānuka honey ($R^2 = 0.8977$). No trend was observed when MGO vs. DHA was plotted for all of the database honeys in the current research. However, when the honeys were plotted by year of harvest a linear trend for each year was observed (Figure 4.10). All years have an R^2 value of at least 0.8. This suggests that Atrott *et al.* collected samples in the same year; this is also suggested by the 2:1 DHA:MGO ratio discussed above.

HMF is produced by acidic decomposition of monosaccharides; in addition, it can form from the condensation of carbohydrates with free amine groups.[140] HMF is able to form via both pathways in honey. Fresh honey contains no HMF and over time the concentration of HMF increases. Heating honey increases the rate at which HMF is formed, hence it is used as a measure of the honey quality. A

scatter plot of HMF vs. storage time shows the increase in HMF over time (Figure 4.11). With the exception of the 2003 sample, none of the samples were over the limit set by CODEX (40 mg/kg). Chapter 7 covers HMF in detail.

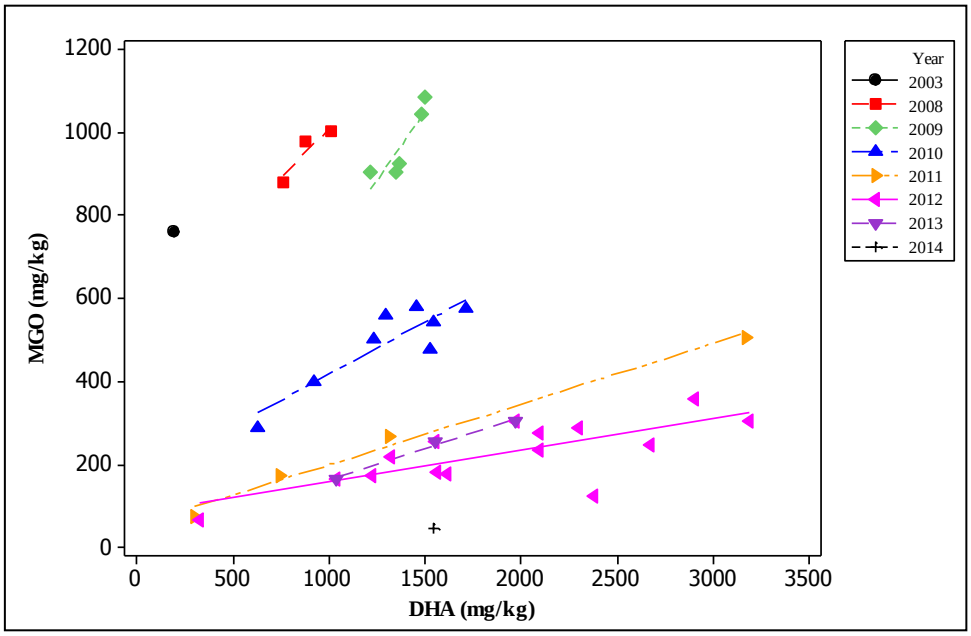


Figure 4.10 MGO vs. DHA concentration for samples grouped by year. There is a linear correlation within each year.

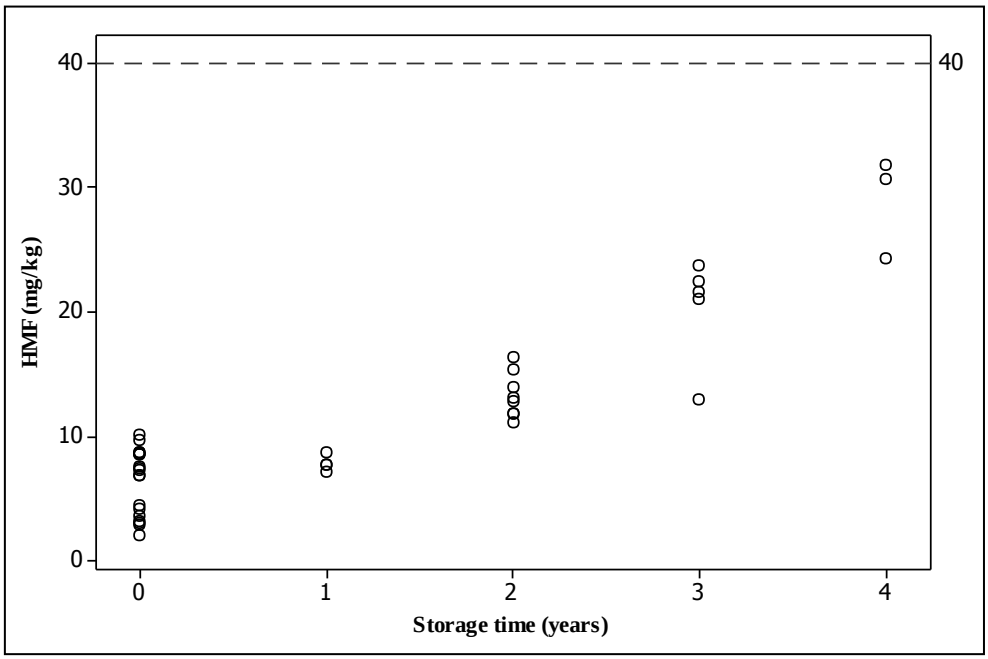


Figure 4.11 HMF vs. storage time (years). The concentration of HMF increases with length of storage. The 2003 sample has been left off the graph due to its extremely high value. All samples from 2008-2014 are within the threshold (40 mg/kg) allowed for HMF.

4.3.3 Analysis of sugars

Honeys were analysed by HPLC using the Direct method for sucrose, glucose and fructose concentrations. Samples were analysed in triplicate. Table 4.5 summarises the concentrations of the sugars for Steens honeys. A summary of the major sugars reported by the Honey Board[155] are shown in Table 4.6. The glucose and fructose in the mānuka honey database are close to the average of the literature values for the sugars and lie within the range stated in the literature. The sucrose concentration is higher than the average stated, but is within the range stated in the literature.

The average fructose:glucose ratio was 1.10 ± 0.03, which is similar to the ratio reported by the Honey Board,[155] and higher fructose is the general trend reported in the literature, however, the proportion of the two sugars largely depends on the nectar source.[175] Analysis of 775 mānuka honeys by Airborne Honey[252] had a slightly higher fructose:glucose ratio (1.28) than the mānuka honeys analysed here. Sporns *et al.*[253] reported that the average fructose:glucose ratio for Alberta, Canada honeys was 1.09. Ajlouni and Sujirapinyokul[136] reported a fructose:glucose ratio of 1.17 in Australian honeys, which is at the top end of the mānuka honeys analysed here. Zhang *et al.*[254] reported an even higher ratio (1.46) in acacia honey from China. Spanish unifloral honeys had a range of ratios from 1.12 (sunflower, n = 13) to 1.33 (forest, n = 16).[255]

Goss[256] reported a moderate correlation between the NPA rating and the glucose:fructose ratio of mānuka honey ($R^2 = 0.52$) in 38 samples. The relationship between the glucose:fructose ratio and NPA in this current research showed no correlation ($R^2 = 0.08$, Figure 4.12). Since NPA is a discrete value, the MGO concentration was used; the trend between the two continuous values was examined and no trend was found ($R^2 = 0.02$, Figure 4.13). The lack of correlation is presumably due to the small range of the glucose:fructose ratio, or because sugars do not influence NPA.

Table 4.5 Summary of sugars in Steens honeys for each sample.

Sample	Glucose (g/100g)	Fructose (g/100g)	Sucrose (g/100g)	Fructose: Glucose
268	33.20 ± 0.18	36.23 ± 0.29	4.26 ± 0.06	1.09
291	33.14 ± 0.10	35.95 ± 0.12	4.79 ± 0.03	1.08
296	33.19 ± 0.62	35.17 ± 0.31	3.92 ± 0.06	1.06
392	33.67 ± 0.35	37.06 ± 0.56	4.71 ± 0.06	1.10
449	33.94 ± 0.22	37.42 ± 0.07	4.36 ± 0.06	1.10
460	33.53 ± 0.12	37.06 ± 0.16	3.97 ± 0.06	1.11
491	33.00 ± 0.44	37.18 ± 0.51	4.39 ± 0.12	1.13
498	33.42 ± 0.58	36.11 ± 0.96	4.36 ± 0.21	1.08
565	32.07 ± 0.14	35.80 ± 0.27	4.82 ± 0.07	1.12
597	32.82 ± 0.32	36.64 ± 0.36	4.43 ± 0.05	1.12
608	33.25 ± 0.15	36.74 ± 0.08	4.24 ± 0.06	1.10
610	34.27 ± 0.62	37.79 ± 1.32	3.75 ± 0.05	1.10
622	33.10 ± 0.11	36.73 ± 0.05	4.61 ± 0.02	1.11
637	32.82 ± 0.21	36.48 ± 0.12	4.39 ± 0.16	1.11
654	32.85 ± 0.20	38.54 ± 0.23	4.09 ± 0.05	1.17
673	35.25 ± 2.02	37.34 ± 2.20	4.23 ± 0.36	1.06
755	33.43 ± 0.47	36.56 ± 0.21	4.64 ± 0.04	1.09
784	32.57 ± 0.13	37.30 ± 0.08	3.23 ± 0.10	1.15
796	31.79 ± 0.79	36.22 ± 0.67	6.31 ± 0.33	1.14
802	33.92 ± 0.23	36.39 ± 0.05	5.15 ± 0.12	1.07
Average	33.26 ± 0.03	36.74 ± 0.93	4.43 ± 0.61	1.10 ± 0.03
Minimum	30.94	34.82	3.12	1.05
Maximum	37.58	39.87	6.69	1.17

* Three replicates were analysed for each sample.

Table 4.6 Summary of major sugars in honey – literature values extracted from Sanford.[155]

Constituent	Average	Range	Standard deviation
Fructose/glucose ratio	1.23	0.76-1.86	0.13
Fructose (%)	38.38	30.91-44.26	1.77
Glucose (%)	30.31	22.89-40.75	3.04
Sucrose (%)	1.31	0.25-7.57	0.87

Majtan *et al.* (2012)[71] suggested that in mānuka honey there may be a loss of α-glucosidase, which converts sucrose into glucose and fructose, due to possible modification of the protein by MGO. However, they did not examine whether this modification had an effect on the function of the protein. In the current research, the spread of sucrose was small (3.2-6.3 g/100 g honey) and weak trends of DHA or MGO with sucrose were observed (R^2 = 38.8 and 13.9% respectively). Daglai *et al.* (2013)[72] studied the effect of MGO on digestive enzymes and reported that

there was no affect on the physiological activity of pepsin and pancreatin from carbonylation. This suggests that some proteins may still able to function even though they have been modified. However, further work by Majtan *et al.* (2014)[57] reported that MGO suppressed H_2O_2 generation by crosslinking with the enzyme glucose oxidase.

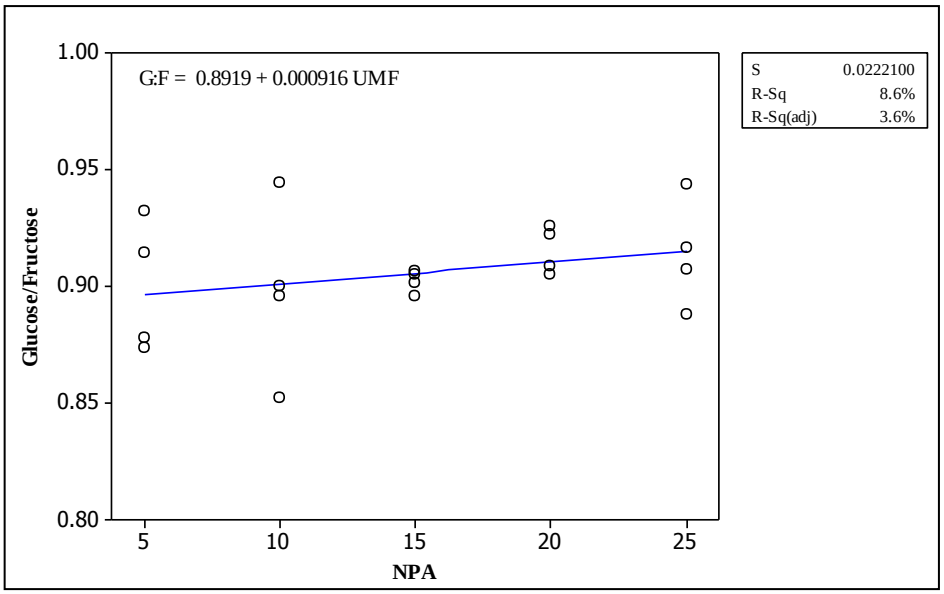


Figure 4.12 Glucose/fructose vs. NPA. There is no trend between the sugars and the NPA rating.

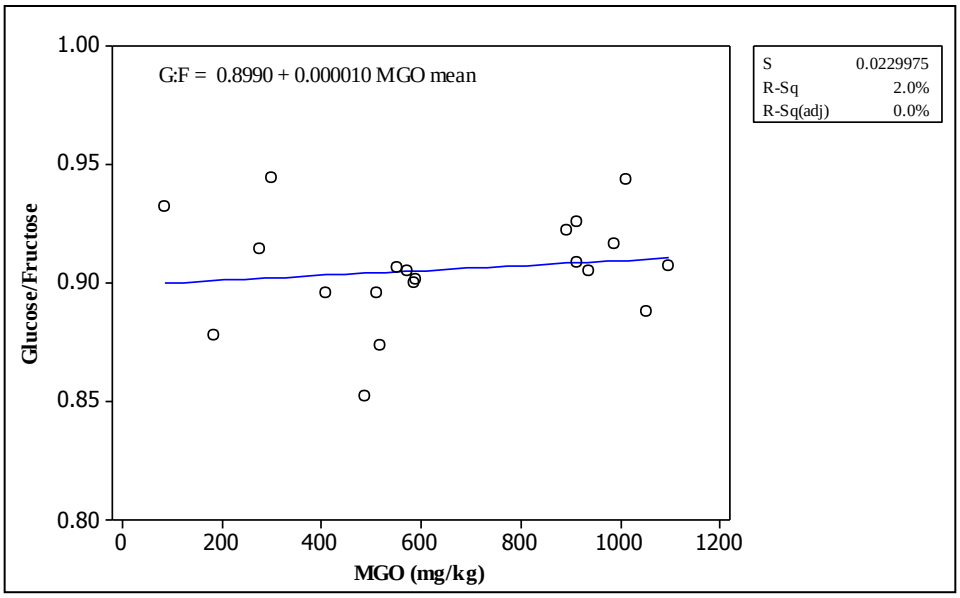


Figure 4.13 Glucose/fructose vs. MGO (mg/kg), measured by the PFBHA method. There is no trend between the ratio of sugars and MGO concentration.

4.3.4 Analysis of pH and acidity

The ability to accurately measure the acidity of honey has been a cause of complication ever since it was first attempted by titration. In 1958, White *et al.*[257] discussed the difficulty in obtaining a stable end-point when using phenolphthalein pink as the indicator of the end-point. In addition, reproducible results were hard to obtain. The official method was altered to produce better results for acidity and also to analyse for lactone content. There is a drift in the end-point of the titration due to lactone hydrolysis which causes problems in the reproducibility of the method. The IHC state that the RSD (determined by $100R/\bar{x} \times 2.8$) for free acidity of honey is 10.3-22.0.[104] This is very high and leads to doubt of whether the method is satisfactory. The end-point titration is used in most countries, and was the choice for this research to allow direct comparison with the literature.

The IHC method is based on the AOAC method. The IHC method states that the titration with NaOH should be completed within 2 minutes. This method was adopted for this research in an attempt to standardise the method. If the time limit is not adhered to, the pH drifts and excess NaOH is used in an attempt to reach pH 8.5. A clover honey was chosen to assess the reproducibility of the end-point titration method. The honey was analysed 6 times in one day. The %RSD is acceptable for pH and free acidity. However, it is extremely high for lactone, due to the complications with the method described above.

Table 4.7 Summary of reproducibility of end-point titration for the acidity of honey (n =31).

	pH	Free Acidity (milliequiv/kg)	Lactone (milliequiv/kg)	Total Acidity (milliequiv/kg)
Average	3.84	15.41	1.43	17.09
SD	0.05	0.86	0.62	0.78
% RSD	1.34	5.61	43.51	4.54

The summary of pH, free acidity, lactone and total acidity for 31 mānuka honeys are shown in Table 4.8. pH and free acidity are an average of three replicates. However, in some cases lactone is the determination of only one value, due to overshooting the end-point. The analysis could not be repeated due to limited sample size.

Table 4.8 Summary of pH, free acidity, lactone and total acidity for honey database.

Sample	pH	Free acidity (milliequiv/kg)	Lactone (milliequiv/kg)	Total acidity (milliequiv/kg)
268	3.68 ± 0.01	27.67 ± 2.47	3.05	32.46
291	3.49 ± 0.00	33.18 ± 2.23	9.21	40.81
296	3.48 ± 0.01	30.49 ± 1.77	8.47 ± 3.57	38.96 ± 1.80
392	3.49 ± 0.01	26.77 ± 0.50	12.45 ± 0.34	39.22 ± 0.84
449	3.52 ± 0.02	26.16 ± 0.01	11.79 ± 0.02	37.95 ± 0.01
460	3.47 ± 0.01	27.04 ± 0.22	13.17 ± 0.35	40.22 ± 0.57
491	3.56 ± 0.05	25.43 ± 1.34	8.87 ± 1.35	34.29 ± 2.69
498	3.56 ± 0.01	24.83 ± 0.82		
565	3.72 ± 0.03	23.33 ± 0.30	6.27 ± 0.35	29.60 ± 0.04
597	3.75 ± 0.01	22.71 ± 0.03	7.41 ± 0.05	30.13 ± 0.01
608	3.74 ± 0.05	21.14 ± 0.03	6.86 ± 2.06	28.00 ± 2.09
610	3.69 ± 0.04	20.06 ± 0.76	6.85 ± 0.59	26.91 ± 0.17
622	3.88 ± 0.01	20.44	3.37	23.81
637	3.63 ± 0.11	19.80 ± 0.82	7.19	26.41
654	3.54 ± 0.13	20.47 ± 0.67	11.85 ± 3.25	32.31 ± 2.59
673	3.63 ± 0.04	24.51 ± 1.38	8.98 ± 0.16	33.49 ± 1.54
755	3.82 ± 0.01	21.24 ± 1.85	5.02	27.58
784	3.53 ± 0.03	29.19 ± 0.62	8.49 ± 0.87	37.68 ± 0.24
796	3.94 ± 0.08	19.32 ± 0.32	5.91	25.46
802	3.68 ± 0.04	22.54	5.14	27.68
946	3.90 ± 0.04	34.75 ± 0.64	2.35 ± 0.21	37.10 ± 0.85
953	3.57 ± 0.00	35.34 ± 2.06	2.01 ± 0.13	37.34 ± 2.19
1349	3.57 ± 0.10	22.41 ± 8.83	1.96 ± 0.75	24.37 ± 9.57
25	3.76 ± 0.01	22.38 ± 1.28	12.19 ± 0.42	34.57 ± 0.86
32	4.77 ± 1.39	30.67 ± 11.75	12.46 ± 0.05	43.13 ± 11.80
41	3.70 ± 0.02	17.37 ± 0.01	11.42 ± 0.71	28.79 ± 0.72
59	3.71	17.25 ± 0.36	11.00 ± 0.71	28.24 ± 0.35
66	3.69 ± 0.04	20.11 ± 2.78	8.61 ± 1.82	28.72 ± 0.96
76	4.31 ± 0.01	16.15 ± 0.34	10.19 ± 0.34	26.34 ± 0.68
78	4.27 ± 0.01	15.40 ± 1.29	9.71 ± 1.89	25.11 ± 0.60
84	3.74 ± 0.02	24.23 ± 1.03	6.42 ± 0.12	30.65 ± 1.14
Average	3.73 ± 0.28	23.95 ± 5.25	7.96 ± 3.35	31.91 ± 5.61
Minimum	3.47	15.4	1.96	23.81
Maximum	4.77	35.34	13.17	43.13

It is difficult to obtain a true pH of honey because it needs to be diluted. This may affect the availability of the H^+ ions and alter the measured pH. Furthermore, the pH of honey is reported to reflect the buffering action of the inorganic cation constituents on the organic acids present rather than directly reflecting the total acid content.[258]

The pH of the honey matrix will influence the types of reactions that occur and also the state in which compounds exist. The average pH of the honeys was 3.73 ± 0.08, which was similar to other studies on various honeys.[254, 259] The one mānuka honey tested by Vanhanen *et al.*[207] had a pH of 4.21, which is higher than seen for the database. The ten New Zealand honeys had a pH range from 3.57 (Viper's bugloss) to 5.04 (honeydew). Australian honeys (n = 7, pH = 4.02 to 4.68)[136] and Spanish thyme honeys (n = 25, pH = 4.2 ± 0.33)[211] had a lower acidity than samples in this research. Various floral honeys from Latvia has a range of pH from 3.95 (various floral origin) to 4.63 (lime blossom).[260] Unifloral honeys from Spain had pH values from 3.86 (rosemary, n = 13) to 4.61 (forest, n = 16).[255]

Free acidity, lactone and total acidity vary throughout the literature. A wide range of European floral honeys have been analysed;[261] the free acidity ranges from 10.3 (brassica) to 37.2 meq/kg (thymus). Heather had the closest reported free acidity to mānuka honey. Cantarelli *et al.*[160] compared honey from Argentina, Spain, Italy and Turkey and reported a range between 1.56 to 16.61 meq/kg of lactone for the different countries. Indian honey has been reported to have 15-21.5 meq/kg lactone.[262] Sunflower and eucalyptus honeys were reported to have high lactone (40 and 33 meq/kg respectively).[263] The average total acidity of the database honeys is 31.91 ± 1.84 meq/kg which is below the limit proposed by the IHC[104] (50 meq/kg). However, due to the poor reproducibility of the method caution must be taken with samples that are near the limit. Total acidity of honeys from Latvia ranged from 11 (rape) to 35 meq/kg (wild flowers);[260] Australian samples (n = 7)[136] had a higher total acidity (33.5-53.5 meq/kg). Sidder honeys from various regions of Pakistan had large variation in total acidity; ranging from 6.5 to 39 meq/kg. Various floral honeys (n = 27) from Spain had a total acidity of 11.70-33.23 meq/kg.[188]

There was a slight correlation between free acidity and storage time (Figure 4.14); the general trend was for an increase over time. However, samples that had not been stored for a complete year had a wide spread of values which decreased the correlation. In addition, the sample stored for 9 years had a lower than expected value. Correlations were not observed between lactone or total acidity with storage time. This may be due to the variable results reported for the lactone concentration, which also affects the value of total acidity. A storage experiment[186] of citrus honey observed an increase in free acidity and lactone over 12 months for honey stored at both 20 and 40 °C, with a larger increase observed at the higher temperature. Furthermore a storage study carried out on fresh Sidder honey samples also showed the same trend in honey from three regions of Pakistan that were stored at room temperature (25-29 ºC for 12 months).[184] However, they also observed a decrease in pH, which was not seen in the former study. The pH decreased the most in the first 6 months, then the decrease slowed down in the following 6 months. For the three honey types tested the pH changed between 4.16 to 12.5% in 12 months. The acidity increased 128 to 346% over 12 months. The lactone content increased the most (400 to 800%) within 12 months.

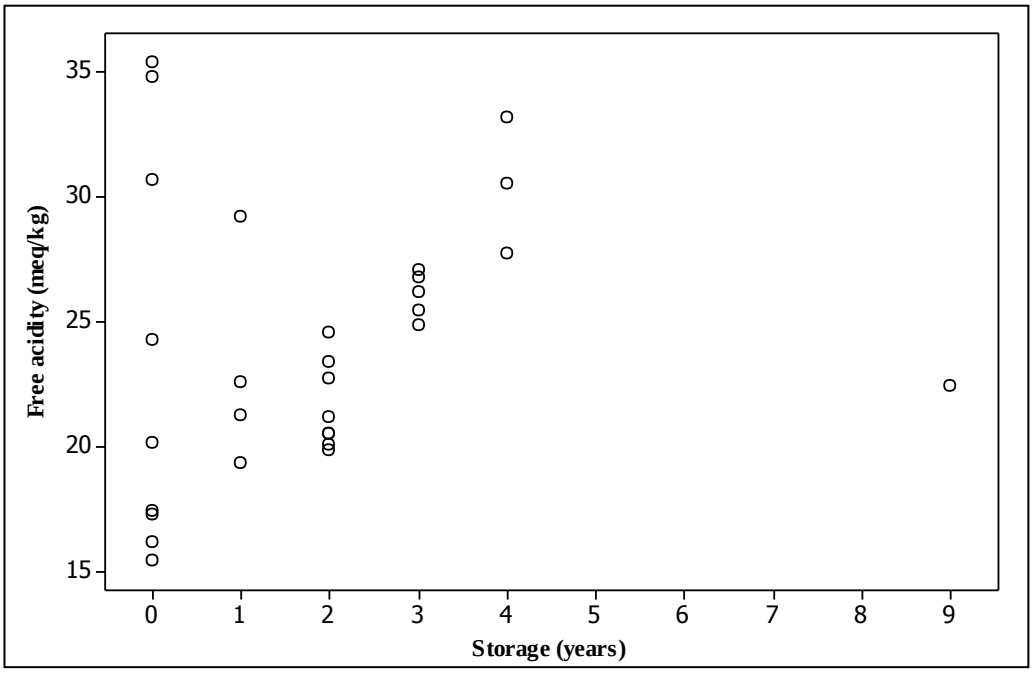


Figure 4.14 Free acidity vs. storage. There was an increasing trend for some samples, but the spread of values for the fresh samples was wide and decreased the correlation. The sample stored for nine years had a lower free acidity value than expected.

4.3.5 Analysis of moisture content

Food Standards Australia New Zealand (FSANZ)[264] state the moisture content of honey may be no more than 21%. The moisture content on the database honeys are summarised in Table 4.9; the average is 20.8 ± 1.4 % (n = 32). Many of the honeys have a moisture content higher than 21%. The moisture content of the database is higher than reported for New Zealand honeys (n = 10) in another study (16.4 to 18.0 %) when analysed by a refractometer.[207] Causes of the high water content may be due to the refractometer being incorrectly calibrated, the analysis temperature fluctuating or it may be a natural phenomenon due to the high rain fall during some seasons that the samples were collected which would increase the moisture content. Honey has a tendency to absorb moisture from the air due to the sugar content.[59] The high moisture content for honeys extracted in 2011-2012 is due to this season being wetter than average. Personal communication with three independent beekeepers indicated that their honeys had higher water content for these years. Sample 946 fermented which is also an indication that the water content was high. However, this does not account for the honeys from 2008-2010.

The refractometer was calibrated against Steens Honey Ltd. in-house method using a set of 35 mānuka honeys. In addition, 6 of the samples were also tested by a third laboratory. The results are shown in Table 4.10. Figure 4.15 shows the difference (%) between the results from the current research (UoW) and laboratories A and B.* There is not a large difference between the samples, with UoW values sitting in the middle of the other two laboratories.

The reported moisture content of honeys varies in the literature; an average moisture content of 17.2% was reported for 490 floral honeys analysed in the United States (1962). These samples had a range of 13.4-22.9%.[59] Moisture content from Australian honeys ranged from 10.6-17.8%.[136] A variety of Spanish unifloral honeys (including rosemary, lavender, sunflower and eucalyptus) had moisture contents from 15.8 (forest, n = 16) to 19.1% (rosemary, n = 13).[255] Multifloral honeys (n = 44) from Italy had an average moisture content of 17.65 ± 1.41%, but were found up to 21%.[224]

* The percentage difference was calculated as UoW – Lab A/B

Table 4.9 Water content of database honeys.

Sample	% Moisture
268	22.4
291	22.0
296	21.7
392	20.8
449	20.4
460	21.7
491	20.4
498	21.7
565	20.2
597	20.5
608	21.9
610	21.9
622	21.3
637	21.8
654	21.6
673	22.4
755	20.5
784	21.6
796	20.0
802	21.3
946	21.5
953	19.4
1349	19.7
25	22.0
32	21.8
41	20.5
59	17.2
66	20.1
76	17.2
78	18.4
84	22.0
1404	19.2
Average	20.8 ± 1.4
Minimum	17.2
Maximum	22.4

Table 4.10 Water content of honeys tested by three laboratories.

Sample	UoW	Lab A	Lab B
1239	17.7	18.0	
1240	17.8	18.1	
1241	17.8	18.0	
1242	17.3	17.6	16.3
1244	17.5	18.1	
1245	17.2	17.7	
1246	17	17.3	16.5
1247	17.2	17.3	
1248	17.2	17.3	
1249	17.6	18.3	
1250	17.6	18.3	
1251	17.5	18.1	
1252	17.6	17.9	
1253	17.6	17.8	17.0
1254	17.5	17.8	
1255	17.4	17.8	
1256	17.7	17.7	
1257	17.5	18.9	
1258	17.6	18.1	
1259	17	18.1	15.5
1260	17	18.1	
1261	17.7	18.6	
1262	17.3	18.8	
1263	16.5	17.7	
1264	17.9	18.1	
1265	18	18.2	
1266	18.1	18.4	17.1
1267	18.3	18.6	
1268	17.7	18.1	
1269	17.7	18.1	
1270	18	18.1	
1271	17.9	18.0	17.6
1272	17.8	18.1	
1273	17.4	17.8	
1279	17.9	18.4	
Average	17.5	18.0	16.7
SD	0.4	0.4	0.7
Min	16.5	17.3	15.5
Max	18.3	18.9	17.6

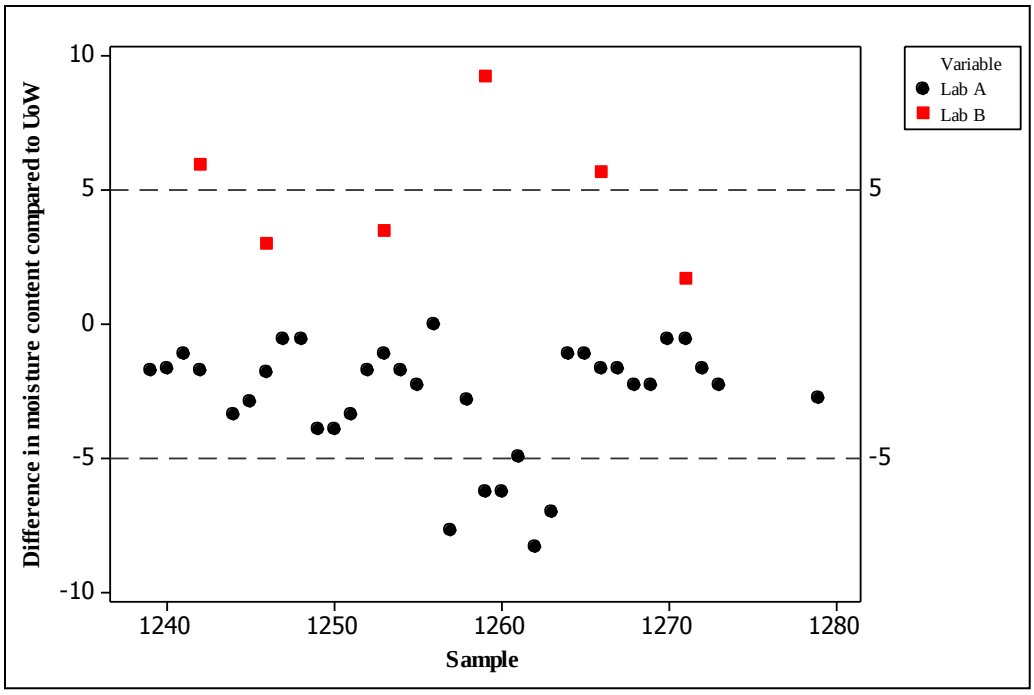


Figure 4.15 Difference in moisture (%) results for UoW samples compared to laboratory A (circles) and B (squares). Most samples are less than 5% different.

4.3.6 Analysis of proline content

The proline content was measured by ninhydrin for the honeys in the database. The concentration of proline ranged from 292 to 809 mg/kg. The results for all samples are shown in Table 4.11. The average proline concentration is 441 ± 123 mg/kg. The standard deviation is large due to the various ages of the samples. The average is similar to reported proline levels in a variety of other honeys (rape, wild, garlic and asclepias).[179] Cotte *et al.*[183] analysed 280 honey samples which had a mean proline content of 415 mg/kg and the result here is also similar to a study by White and Rudyj[153] on 482 American honeys (483 mg/kg).

Samples 946 and 953 were harvested and analysed in the 2012/2013 season and both have high proline concentrations. This was a wet season, hence the bees would have had to dry the honey more than normal which may have contributed to the high proline concentration. In comparison, samples from the drier 2013/2014 season had lower levels of proline. Interestingly, a study from 2006 reported that honey bees (*Apis mellifera*) prefer nectar that is rich in proline.[265]

Table 4.11 Proline concentration (mg/kg) for mānuka honey samples

Sample	Proline (mg/kg)	Sample	Proline (mg/kg)
268	397	**784**	473
291	470	**796**	632
296	442	**802**	380
392	476	**946**	809
449	495	**953**	746
460	513	**1394**	302
491	400	**25**	380
498	478	**32**	438
565	292	**41**	368
597	448	**59**	265
608	396	**66**	572
610	334	**76**	369
622	367	**78**	391
637	336	**84**	550
654	312	**1404**	372
673	551	**Average**	441 ± 123
755	371	**Minimum**	292
		Maximum	809

Proline is an indication of honey ripeness and can be used to detect sugar adulteration. A minimum value of 180 mg/kg for genuine honey is used as a control in some laboratories.[104] Sample 565 from 2010 has the lowest proline content of all samples analysed, but all samples, including the 2003 sample, are above the internationally accepted minimum proline content.[104]

Proline is known to decrease over time. Therefore the spread of proline concentrations in the database was examined as a function of year (Figure 4.16). There is a small decreasing trend of proline over the years; however, due to a wide variation in some years there is not a good correlation, but there is no obvious reason for the large spread. There is no information on the loss of proline in mānuka honey in the literature. However, it is expected to be different to other honeys due to the presence of DHA and MGO. Both compounds readily react with amino acids to form reversible or irreversible bonds. Therefore the loss of proline over time in mānuka honey may be different to the extent and rate of loss of proline in other honeys. It is possible that the initial loss in mānuka honey is greater due to the binding of free proline with DHA and/or MGO. Refer to section

4.1.5 for a literature review on the change in proline concentration over time in other floral types.

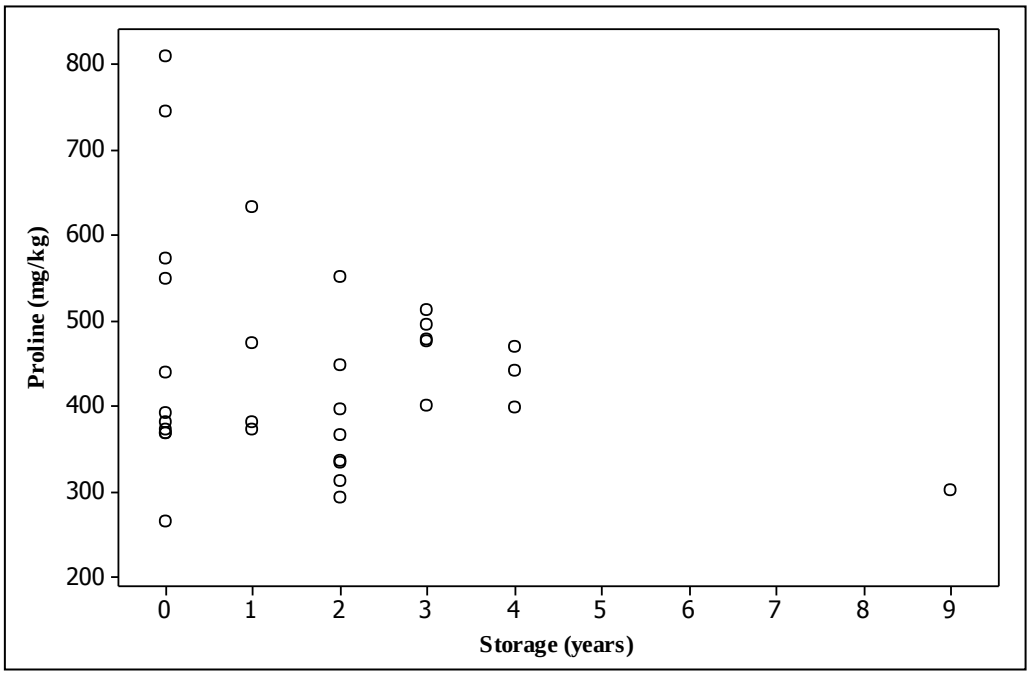


Figure 4.16 Proline vs. storage times. Samples analysed in year 0 have a large spread of reported proline concentrations.

Czipa *et al.*[179] analysed mānuka honey (809 ± 36 mg/kg), tawari (717 ± 9 mg/kg) and vipers bugloss (848 ± 22 mg/kg) from New Zealand. These were purchased commercially and no indication of their age or the NPA rating for the mānuka honey was provided. The reported values by Czipa *et al.* are about twice the concentration of the average found during this research for the database honeys, but have a similar content to the freshly collected mānuka samples. The lowest proline concentration reported by Czipa *et al.* in Hungarian honeys was 252 ± 38 mg/kg in an acacia honey. This is only slightly lower than the lowest reported in this study.

There is no apparent relationship between proline and DHA or MGO in the database (Figure 4.17). This may be due to multiple reactions between DHA/MGO and other sugars. In addition, it is most likely that the DHA and proline had irreversibly bound long before the samples were measured. There is also no apparent relationship between HMF and proline (not shown). Chapter 5 reports that DHA and MGO both bind to proline in a honey matrix; chapter 6

reports a relationship between DHA loss and the initial amount of proline in the sample. A trend is not expected in this database of honeys as this is only a snapshot of proline, DHA and MGO, rather than following the reaction over time.

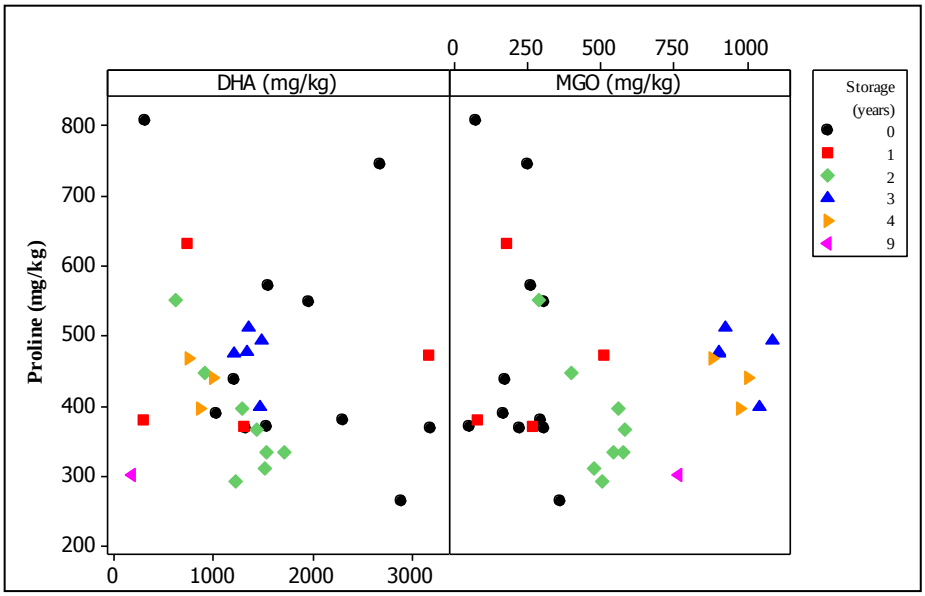


Figure 4.17 Proline vs. DHA (left panel) and proline vs. MGO (right panel). There is no apparent relationship between DHA or MGO with proline in these samples. The legend shows the number of years the honeys have been stored.

Proline was also analysed by HPLC together with the primary amino acids for 10 mānuka and clover honeys used in the storage trials (chapter 6). A summary of the data can be found in Table 4.12. The HPLC analysis was carried out approximately two years after analysis of proline by ninhydrin; however, all samples were stored in the freezer during this time so the level of proline should be reasonably stable.

Five of the samples were reported to have the same concentration within 10-20%. However, four of the samples were 25-70% higher when reported by ninhydrin and the last sample was 55% larger when reported by the HPLC method. Sample 1404 had the largest reported concentration difference between methods (70%). The LOD was not calculated in this research but Truzzi *et al.*[189] reported the LOD and LOQ as 20 and 61 mg/kg respectively for the AOAC method, which is very similar to the method used in this research;[108] the reported LOQ is well below the minimum level of proline found here. Therefore the large difference in reported concentration may be due to experimental error.

Table 4.12 Proline concentrations for samples detected by the ninhydrin method and HPLC method.

Sample*†	Proline (Ninhydrin, mg/kg)	Proline (HPLC, mg/kg)	Difference between ninhydrin and HPLC (%)
Happy Bee (C)	422	328	25
Airborne (C)	361	640	–56
Hollands (C)	480	379	24
Katikati (C)	637	552	14
946 (M)	809	709	13
953 (M)	746	675	10
1404 (M)	372	179	70
66 (M)	572	465	21
78 (M)	391	326	18
84 (M)	550	356	43

* C = clover honey, M = mānuka honey

† All honeys were analysed within a year of storage at ambient temperature.

4.3.7 Analysis of primary amino acids (free)

Sixteen free primary amino acids (aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, arganine and tryptophan) were analysed in ten honeys used in the storage trials (chapter 6), this consisted of six mānuka honeys from the database and four clover honeys. There are currently no reports of amino acids in New Zealand mānuka honey, nor any New Zealand honeys in the literature. Amino acids in honey are reported as a good indication of floral origin.[162, 182-183] Therefore it is difficult to directly compare amino acids in mānuka honey to honey from overseas. It is acknowledged that six mānuka honeys is a very small representative sample of mānuka honey. However, work is currently underway in separate research to find a rapid analytical method for amino acid detection; this study will also include analysis of a larger number of mānuka honeys.

A summary of the individual amino acids for each sample is shown in Table 4.13. A summary of the average individual amino acids are shown in Table 4.14 for mānuka honeys and Table 4.15 for clover honeys. These tables also include the percentage of each amino acid with respect to total primary amino acids.

Table 4.13 Summary of amino acids (mg/kg) in mānuka and clover (C) honey matrices used in the storage trials.*

Amino acid	946	953	1404	66	78	84	Happy Bee (C)	Airborne (C)	Hollands (C)	Katikati (C)
Aspartic Acid	20.2	16.9	31.1	4.6	5.9	4.6	11.0	11.4	9.0	11.4
Threonine	6.9	6.0	3.4	2.8	1.9	1.9	1.8	3.6	3.8	3.0
Serine	16.0	13.8	6.6	10.4	7.1	10.9	8.4	11.8	13.9	9.2
Glutamic Acid	15.1	14.9	22.9	9.6	10.3	8.8	14.5	15.2	13.6	10.0
Glycine	4.6	4.5	1.7	2.2	1.5	2.4	2.2	3.6	3.4	3.0
Alanine	20.7	18.3	5.0	11.6	7.3	12.3	7.0	10.8	9.6	9.7
Valine	10.9	12.2	4.9	7.2	4.8	8.0	5.1	9.2	9.0	8.1
Methionine	0.5	0.5	0.6	0.2	0.2	0.4	0.1	0.2	0.4	0.2
Isoleucine	6.6	7.5	2.3	4.6	2.8	4.8	2.6	5.6	4.5	6.1
Leucine	7.2	5.0	1.7	3.0	2.0	2.8	2.7	5.8	6.3	9.6
Tyrosine	20.1	17.5	2.8	13.6	7.1	10.0	7.5	12.6	65.4	33.9
Phenylalanine	87.8	54.3	5.9	42.1	19.2	19.6	27.1	47.8	480.8	135.7
Histidine	19.9	18.9	4.7	5.5	2.4	3.1	5.7	9.8	6.2	5.7
Lysine	23.9	22.2	7.5	16.1	6.8	10.2	9.3	24.4	12.3	14
Arginine	13.7	8.9	4.3	5.2	3.5	1.2	5.2	8.1	5.3	9.4
Tryptophan	1.1	0.2	0.2	0.2	0.2	4.1	0.6	0.5	9.5	0.1
Total primary amino acids	275.2	221.6	105.6	138.9	83.0	105.1	110.8	180.4	653.0	269.1

*****Honeys were analysed in the year that they were harvested.

Table 4.14 Summary of individual primary amino acids in mānuka honey (n = 6).

Amino acid	Average (mg/kg)	Standard deviation (mg/kg)	Minimum (mg/kg)	Maximum (mg/kg)	% of PAA*
Aspartic Acid	13.9	10.8	4.6	31.1	9.0
Threonine	3.8	2.1	1.9	6.9	2.5
Serine	10.8	3.7	6.6	16	7.0
Glutamic Acid	13.6	5.3	8.8	22.9	8.8
Glycine	2.8	1.4	1.5	4.6	1.8
Alanine	12.5	6.1	5	20.7	8.1
Valine	8.0	3.1	4.8	12.2	5.2
Methionine	0.4	0.2	0.2	0.6	0.3
Isoleucine	4.8	2.0	2.3	7.5	3.1
Leucine	3.6	2.1	1.7	7.2	2.3
Tyrosine	11.9	6.5	2.8	20.1	7.7
Phenylalanine	38.2	29.9	5.9	87.8	24.6
Histidine	9.1	8.1	2.4	19.9	5.9
Lysine	14.5	7.4	6.8	23.9	9.3
Arginine	6.1	4.5	1.2	13.7	4.0
Tryptophan	1.0	1.6	0.2	4.1	0.6

* PAA = Primary amino acid

Table 4.15 Summary of individual amino acids in clover honey (n = 4).

Amino acid	Average (mg/kg)	Standard deviation (mg/kg)	Minimum (mg/kg)	Maximum (mg/kg)	% of PAA*
Aspartic Acid	10.7	1.1	9.0	11.4	3.5
Threonine	3.1	0.9	1.8	3.8	1.0
Serine	10.8	2.5	8.4	13.9	3.6
Glutamic Acid	13.3	2.3	10.0	15.2	4.4
Glycine	3.1	0.6	2.2	3.6	1.0
Alanine	9.3	1.6	7.0	10.8	3.1
Valine	7.9	1.9	5.1	9.2	2.6
Methionine	0.2	0.1	0.1	0.4	0.1
Isoleucine	4.7	1.6	2.6	6.1	1.5
Leucine	6.1	2.8	2.7	9.6	2.0
Tyrosine	29.9	26.3	7.5	65.4	9.8
Phenylalanine	172.9	210.6	27.1	480.8	57.0
Histidine	6.9	2.0	5.7	9.8	2.3
Lysine	15.0	6.6	9.3	24.4	4.9
Arginine	7.0	2.1	5.2	9.4	2.3
Tryptophan	2.7	4.6	0.1	9.5	0.9

* PAA = Primary amino acid

On average, the primary amino acids account for 26 ± 6% of all amino acids (primary and secondary) in the six mānuka honeys. This is similar to 3 of the 4 clover honeys analysed. Proline is reported to account for 50-85% of amino acids in honey.[162] Hence the levels of primary amino acid reported here are within the correct range. Primary amino acids in Hollands clover honey accounted for 63% of all amino acids. This is higher than the average amount of primary amino acids in honey due to the exceptionally high level of phenylalanine present in this sample (480.8 m/kg). Katikati clover honey also had high phenylalanine (135.7 mg/kg). Clover honeys are usually a blend of floral honeys which will affect the amino acids present. Lavender honey has been reported to have high phenylalanine content[183, 192] and could be the source of high phenylalanine.

The average concentration of primary amino acids in the six mānuka honeys is 154.9 ± 76.5 mg/kg. This is similar to that reported by Cotte[183] for honeys from various floral sources (acacia, chestnut, rape, fir, linden and sunflower) from France, Hungary, China and Morocco. It is also similar to Estonian honeys.[181] A different study[193] of chestnut honey reported a lower primary amino acid concentration (50.3 mg/kg). However, it cannot be ruled out that the concentration of amino acids in mānuka honey has been lowered due to reactions with DHA or MGO. The average primary amino acid concentration in the clover honeys is 303.2 ± 242 mg/kg which has a very large standard deviation due to the concentration of phenylalanine.

Phenylalanine and tyrosine had the most noticeable differences in concentration between the mānuka and clover honeys. The mānuka honey samples had 38.2 ± 29.9 mg/kg phenylalanine, while the clover honeys had a much larger average phenylalanine concentration (73.9 ± 210.6 mg/kg). Phenylalanine was the largest single primary amino acid detected in mānuka honey; it accounted for 25% of the primary amino acids. Phenylalanine was also reported as the most abundant in honeys from Portugal,[191] Estonia,[181] and Spain.[162, 191] A study[183] of lavender honey (n = 53) showed that phenylalanine accounted for 91.7% of total amino acids (the same amino acids as detected in this study, expect tyrosine). Whereas it was low in ilex[*], oak, chestnut and heather honey analysed by Paramas *et al.*;[194]

[*] Ilex is also known as holly and is a genus of 400-600 species of flowering plants in the *Aquifoliaceae* family.

phenylalanine was the most abundant in the heather honey (the amino acids differed slightly to those reported in the current study). Interestingly, phenylalanine was not detected in any of the 5 honeys from France analysed by Kelly *et al.*.[193] Tyrosine was more abundant in clover honeys than mānuka honeys (29.9 ± 26.3 compared to 11.9 ± 6.5 mg/kg). Levels of tyrosine reported by Rebane and Herodes[181] and Kelly *et al.*[193] were similar to that in the mānuka honeys, but varied in honeys analysed by Pereira *et al.*.[191] Tyrosine was not analysed by Cotte *et al.*[183] All other amino acids had similar concentrations in the mānuka and clover honeys analysed.

Phenylalanine, lysine, aspartic acid and alanine each account for at least 8% of the primary amino acids in mānuka honey. In comparison, only phenylalanine and tyrosine each account for over 8% of primary amino acids in the clover honeys due to the high concentration of phenylalanine. Cometto *et al.*[182] also reported phenylalanine and tyrosine were the most abundant primary amino acids in Spanish floral honeys (n = 39). Rebane and Herodes[181] tested honeys from six different floral origins; the three most abundant amino acids for 4 of the 6 honeys were phenylalanine, glutamic acid and lysine; with the exception of methionine, the set of amino acids used to calculate this were the same amino acids used in this study. Aspartic acid and glutamic acid were the most abundant amino acids in the majority of floral honeys tested by Cotte *et al.*.[183] These two amino acids reported for acacia, chestnut, rape, lavender, fir and linden honeys had a similar concentration to the mānuka honeys in this current study. However, sunflower honey had much higher concentrations of both amino acids (42.1 mg/kg aspartic acid and 69.6 mg/kg glutamic acid, n = 36).

Lysine accounted for 9.3% of the primary amino acids in the mānuka honeys, and is the second most abundant amino acid in the mānuka honeys tested. In comparison it was the third most abundant primary amino acid in the clover honeys (4.9%). However, due to the large amount of phenylalanine in the clover honeys the actual concentration of lysine in both samples was the same within experimental error; 14.5 ± 7.4 mg/kg lysine was reported for the mānuka honeys compared to 15.0 ± 6.6 mg/kg for clover honeys. Lysine has been reported in similar concentrations by a number of authors,[181, 183, 191] but was higher in oak honey (77 mg/kg).[193]

Arginine was observed in low levels in both the mānuka (4.0% of primary amino acids) and clover honeys (2.3% of primary amino acids) reported here; but the average concentration was the same within experimental error. Arginine has also been reported in low levels in studies of other floral sources.[181, 183, 194] Pirini *et al*.[266] reported that it is absent in acacia, citrus fruit, rhododendron, rosemary and lime-tree honeys.

Lysine and arginine were added to clover honey by Adams *et al*.[14] as potential catalysts for the conversion of DHA to MGO. They found that both amino acids accelerated the conversion, but high levels of the amino acid consumed MGO at later times. Lysine is a very reactive amino acid due to the ε-amino on the R group and is more readily lost in the initial stages of the Maillard reaction in food due to this group.[267] However, this usually occurs at high temperature such as when baking food. The detection of lysine in the honeys analysed indicates that it has not all reacted in the fresh honeys.

Methionine was the least abundant amino acid in both mānuka and clover honeys analysed (0.3 and 0.1% of primary amino acids respectively). Similarly, Hermosín,[162] Rebane and Herodes[181] and Cotte[183] also found low levels of methionine in samples from Spanish, Estonian and French honeys, while Pereira[191] and Kelly *et al*.[193] did not detect it.

4.3.8 Analysis of selected phenolic compounds

Selected phenolic compounds were investigated that may have a catalytic role in the conversion of DHA to MGO, or may consume DHA or MGO. Eight mānuka and four clover honeys were analysed for syringic acid, methyl syringate, leptosperin,[*] phenyllactic acid,[†] 4-methoxyphenyllactic acid, 2-methoxybenzoic acid and luteolin (Figure 4.18) by HPLC high resolution mass spectrometry.

Leptosperin, methyl syringate, 4-methoxyphenyllactic acid, phenyllactic acid and 2-methoxybenzoic acid have been reported in mānuka honey by various authors,

[*] Leptosperin was previously called leptosin, but was changed to avoid confusion with other unrelated leptosins isolated from a marine fungus.
[†] Phenyllactic acid is also known as 2-hydroxy-3-phenylpropionic acid and hydroxyhydrocinnamic aicd. Furthermore it may be 3-phenyllactic acid. Throughout this research it will be referred to as phenyllactic acid.

and some have been suggested as good markers for distinguishing mānuka honey from honey of other floral types.

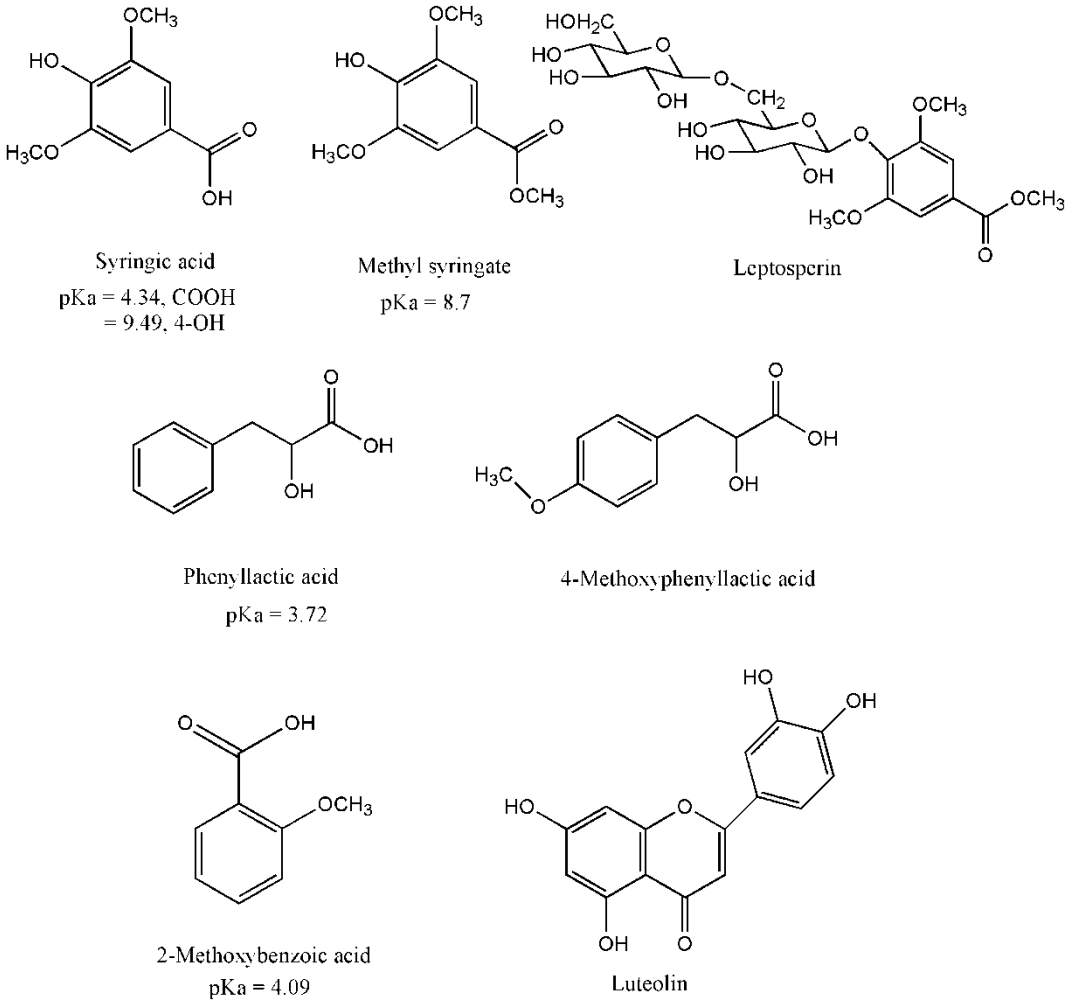


Figure 4.18 Phenolic compounds analysed by UPLC-DAD-MS.

When reviewing the literature, problems associated with the reporting of phenolics were noted; these are:

1. Sample size – this is often very small (<10) and in some cases as low as one.
2. Guarantee of monofloral honey – often samples are purchased commercially and there is no guarantee that the honey is predominately mānuka or kānuka honey. Since there is no way to distinguish the two honeys, either may be contaminated with the other. Furthermore, since active mānuka honey is in high demand and there is only a limited supply it is possible that active Australian jelly bush honey may have been blended with New Zealand mānuka honey.[268]

3. Geographical location – compounds in honey may vary with region,[151] similar to that seen for DHA. Hence if only one region is sampled, a false conclusion may be drawn.
4. Species of *L. scoparium* – compounds in samples may vary with species of the tree, similar to that seen for DHA.

Due to the above complications, extreme care must be exercised when extrapolating from small sample sizes of honey that are not absolutely guaranteed to be mono-floral. Compounds that arise from mānuka honey may be present due to a contribution from Australian honey or kānuka honey and vice versa. Further work is required on larger sample sizes of honeys from known regions.

A further complication in reporting concentrations of phenolic compounds arises from the sampling method. In plants, only a small fraction of phenolic compounds exist as the 'free acid', the majority are linked to molecules such as proteins, flavonoids or smaller organic molecules such as glucose and malic acid.[269-270] Hence various analysis methods may give different results. Furthermore, sample preparation may influence whether a compound or the methylated analogue is reported or whether artefacts generated by Strecker degradation or the Maillard reaction are observed.[271] For example, phenolics in honey are often analysed by GC after methylation. Therefore it may not be easy to determine whether the methyl ester is naturally occurring or an effect of the sample preparation.[251] More recent reports in the literature use solid-phase extraction with GC analysis instead of derivatisation, which prevents detection of artefacts.

Syringic acid is a common plant constituent but the methylated analogue is seldom observed. In addition, the levels of phenolic acids are known to vary during stages of maturation of plants and are affected by temperature. Hence, phenolics that originate from the nectar may vary depending on these factors.[269, 272]

Table 4.16 and Table 4.17 summarise the concentrations of the selected phenolic compounds in mānuka and clover honeys respectively. All compounds analysed were in much lower abundance in clover honey than in the mānuka honey. Tan *et al.*[31] also reported that mānuka honey contains phenolic compounds that honeys of

other floral sources do not contain. Compounds that are in high abundance and can readily donate a proton are likely to have an influence on the conversion of DHA to MGO by affecting the equilibrium constant of DHA dimer and monomer. Side reactions of DHA may also be increased if there is a larger pool of free DHA. Furthermore, donation of a proton to MGO may increase side reactions and cause a larger loss of MGO at later times. It is unlikely that the phenolic compounds investigated here will bind with DHA or MGO.

Syringic acid was found at low levels in both mānuka honey and clover honey (0.32 ± 0.23 and 0.01 ± 0.01 mg/kg respectively). Syringic acid has previously been reported in both mānuka and kānuka honey, but absent in clover honey.[31] This difference may arise from differences in detection limits or sample preparation. Oelschlaegel *et al.*[114] analysed 40 mānuka honeys and divided them into three groups according to their chemical profile. The authors reported high syringic acid in one group of mānuka honeys (n = 20), but did not report concentration; this group was also characterised by high methyl syringate and 4-methoxyphenyllactic acid. Differences in the concentration may arise from geographical location and variety of mānuka tree, or blended honey. Tan *et al.*[31] did not detect syringic acid in the nectar of mānuka flowers (sample number was not reported). The 4-OH has a pK_a of 9.49, which would not act as an acid in the honey matrix, whereas COOH would act as a strong acid (pK_a = 4.34) and could donate a proton. Loss of a proton would be stabilised by resonance structures and −I from the two OCH_3 groups and OH group. However, the levels in mānuka honey are not significant.

Table 4.16 Selected phenolic compounds (mg/kg) in mānuka honeys (n = 8).

Sample	Area of harvest*	Syringic acid	Methyl syringate	Leptosperin	Phenyllactic acid	4-Methoxyphenyllactic acid	2-Methoxybenzoic acid	Luteolin
					mg/kg			
946	1	0.81	197.93	99.27	855.27	875.00	1.88	0.96
953	1	0.51	61.27	451.00	780.59	319.83	3.29	1.20
1404	5	0.09	9.10	149.56	621.77	1.63	18.27	1.37
66	4	0.25	39.96	523.02	720.82	321.33	4.35	1.60
78	4	0.19	18.43	345.99	455.98	181.29	4.09	0.94
84	3	0.28	47.06	573.53	967.83	207.17	5.65	1.72
25	3	0.26	34.38	735.83	727.74	498.45	4.61	2.00
41	3	0.15	22.16	304.69	632.21	22.23	9.36	0.97
Average		0.32	53.79	397.86	720.28	303.37	6.44	1.35
σ		0.23	60.59	215.72	156.07	282.76	5.25	0.40
%RSD		73.47	112.64	54.22	21.67	93.21	81.56	29.51
Min		0.09	9.10	99.27	455.98	1.63	1.88	0.94
Max		0.81	197.93	735.83	967.83	875.00	18.27	2.00

* The area of harvest was assigned in chapter 4

Table 4.17 Selected phenolic compounds (mg/kg) in clover honeys (n = 4).

Sample	Syringic acid	Methyl syringate	Leptosperin	Phenyllactic acid	4-Methoxyphenyllactic acid	2-Methoxybenzoic acid	Luteolin
				mg/kg			
Happy Bee	0.11	3.96	11.52	36.59	10.38	0.94	0.13
Airborne	0.09	3.41	5.30	19.59	16.20	0.20	0.11
Hollands	0.08	1.46	4.06	5.77	3.12	0.53	0.23
Katikati	0.07	3.59	2.13	14.81	24.07	0.56	0.05
Average	0.09	3.10	5.75	19.19	13.44	0.56	0.13
σ	0.01	1.12	4.06	12.94	8.88	0.31	0.07
%RSD	16.29	36.12	70.56	67.42	66.07	54.62	56.07
Min	0.07	1.46	2.13	5.77	3.12	0.20	0.05
Max	0.11	3.96	11.52	36.59	24.07	0.94	0.23

Methyl syringate varied from 9.10 to 197.93 mg/kg in mānuka honey but this high level was only seen in sample 946; with this sample removed the maximum was only 61.27 mg/kg. In clover honey, methyl syringate ranged from 1.46 to 3.96 mg/kg. Russell *et al.*[64] reported the presence of methyl syringate in mānuka honey obtained from aparists, but did not report the concentration. Samples were methylated with etheral diazomethane for 2.5 minutes before GC-MS analysis; Tan *et al.*[31] reported that longer derivatisation caused progressive methylation of some phenolic hydroxyl groups. Dahar and Gülaçar[273] reported that methyl syringate and phenyllactic acid were two of the most abundant compounds (along with *o*-methoxyacetophenone) found in the solid phase micro extraction extracts of mānuka honeys, but did not report absolute concentrations (n = 2, purchased from a local market in Switzerland). In comparison, the present research found the relative abundances of the two compounds reversed. Weston *et al.*[244] reported that methyl syringate made up 70% (*w/w*) of the phenolic fraction of mānuka honey (n = 22, honey obtained from the honey research unit at University of Waikato), but did not test for any of the other compounds reported in the present research. The authors used methanol during sample preparation which may have caused methylation of syringic acid. Kato *et al.*[249] reported 25.89 to 144.51 mg/kg methyl syringate (n = 20 commercial mānuka honeys), which is similar to the concentrations reported in the current research. They also reported it in 14 commercial honeys obtained from China, Japan and NZ in a later study.[249]

Beitlich *et al.*[248] studied mānuka (n = 8), kānuka (n = 7) and jelly bush honey (n = 1) supplied by UMFHA and proposed that methyl syringate is a good marker for kānuka honey. However, as already mentioned, mānuka and kānuka are unable to be easily distinguished. The authors reported that the honeys were harvested from regions where only one type of tree was present. It is possible that the large range of methyl syringate in the mānuka honeys analysed in the current research and other literature may be due to the presence of kānuka honey in varying degrees; alternatively it may be due to different geographical regions and varieties of mānuka trees. The authors did not report absolute concentrations.

Methyl syringate has been reported in various floral honeys at varying concentrations, hence may not be a good marker. Methyl syringate has been detected at low levels in robinia, rape, chestnut, clover, linden blossom, dandelion,

sunflower and fir honeys (0.093 to 5.004 mg/kg, with rape having the highest). These levels are lower than those reported above for most mānuka honeys. It has also been detected in thyme honey[274] and *Mentha* ssp..[275] Furthermore, Tuberoso *et al.*[276] reported methyl syringate was in high concentration in asphodel[*] honey obtained from beekeepers (185.6-288.4 mg/kg, n = 9) and was proposed as a chemical marker due to its high abundance and stability for one year.[†] The levels reported by Tuberoso *et al.*[276] are higher than that found in the current research for most samples (9.10-61.27 mg/kg, with the exception of sample 946, 197.93 mg/kg). Kato *et al.*[246] also reported methyl syringate in high levels of Italian asphodel honey (n = 3). Tuberoso *et al.*[276] reported that methyl syringate predominately originates from the nectar (127.7-203.6 mg/kg). This concurs with Jerkovic *et al.*[275] who reported the presence of methyl syringate in the bee stomach and proposed that it may originate from the nectar.

Methyl syringate would not be a proton donor from 4-OH because the pKa is too high (8.7) and it will not be an acid in a honey matrix. Therefore it is not expected to influence the conversion of DHA to MGO

Leptosperin ranged from 99.27 to 735.83 mg/kg in mānuka honeys analysed in this research, but only 2.13 to 11.52 mg/kg in clover honey. 107.2 to 643.2 mg/kg leptosperin has been reported in *L. scoparium* (n = 12, purchased commercially) and *L. polygalifolium* (n = 1, purchased commercially);[246] a more recent study reported 168.30 to 679.65 mg/kg in mānuka honey (n = 20, purchased commercially)[249] both reports are similar to the concentrations reported in the current research. Leptosperin cannot act as an acid because there are no available groups. Therefore it is not likely to influence the conversion of DHA to MGO in any way. This may account for its reported stability and potential as a chemical marker.[248]

Leptosperin (methyl syringate 4-*o*-*β*-D-gentiobioside) is a novel glycoside of methyl syringate. Currently its origin is unknown; there is a structural relationship between syringic acid, methyl syringate and leptosperin; hence one possible pathway to form leptosperin may be from syringic acid. Methylation and

[*] Asphodel refers to any of the several flowering plants belonging to the Asphodeleaceae family.[277]
[†] It is unclear at which temperature the honey was stored.

transglycolation of syringic acid (in either order) could yield leptosperin. Addition of the sugar units occurs at C4-OH on syringic acid (due to its reactivity) in a 1→6 bond due to less steric hindrance on the sugar at this position. It is more probable that successive glycosidation occurs, rather than gentiobiose adding to methyl syringate, since the concentration of gentiobiose in honey is low (0.4% of oligosaccharide content[278]). An overproduction of syringic acid or methyl syringate would be required to form large amounts of leptosperin, which was not observed in the mānuka honeys analysed. The honeys examined in the current research were obtained for research shortly after harvest and stored at −18-4 °C until required and show high levels of leptosperin and low levels of both syringate and methyl syringate, suggesting that either conversion is rapid, or leptosperin comes from another source. Furthermore, there was no correlation in changes of concentration of these three compounds over time (see below).

There is no literature to support the theory that syringic acid or methyl syringate converts to leptosperin; the monoglycoside of methyl syringate (methyl syringate 4-*o*-*β*-D-glycopyranoside) has been identified in the fruit of anise,[246, 279] but leptosperin has not been observed.[246] Secondly, if leptosperin is formed from methyl syringate it would be expected in asphodel honey due to its high level of methyl syringate, but Kato *et al.*[246] did not detect it, suggesting the reaction is not concentration dependent. Thirdly, methyl syringate has been reported to be stable over the course of one year in aspodel honey[276] suggesting that in this honey type it does not convert to leptosperin.

The formation of leptosperin in mānuka honey may be related to an enzyme that is unique to mānuka honey, which in turn may be related to the origin of DHA. Alternatively, leptosperin may arise from the nectar of the mānuka flowers. There is currently little information on this in the literature and is an area that should be addressed. Tan *et al.*[31] reported that there was little similarity between the profile of mānuka nectar and honey. Additionally, other phenolic compounds in mānuka honey may be transglycolated, and work could be carried out to look for these.

Phenyllactic acid is in high abundance in the mānuka honeys (455.98-967.83 mg/kg) compared to clover honey (5.77-36.59 mg/kg). It has been suggested as a good marker for identifying mānuka honey by Oelschlaegel *et al.*[114] Furthermore,

it has been reported in mānuka nectar at higher concentrations than in the honey.[5] 4-methoxyphenyllactic acid has the largest range of the compounds analysed in mānuka honey (1.63-875.00 mg/kg), but is in low abundance in clover honey (3.12-24.07 mg/kg). Mānuka honeys harvested from the same regions had varying results, but a larger number of samples needs to be tested which contains geographical and species information to draw any conclusions. As mentioned above, Oelschlaegel *et al.*[114] tested 40 mānuka honeys and divided them into 3 groups according to their chemical profile; one group (n = 20) contained high 4-methyoxyphenyllactic acid. Stephens *et al.*[5] reported elevated levels of 4-methoxyphenyllactic acid in kānuka honey (n = 4) compared to mānuka honey (n = 19) and suggested the compound may arise from kānuka honey. Alternatively, 4-methoxyphenyllactic acid may arise from methylation of phenyllactic acid over time; however, corresponding changes in concentrations of these two compounds was not observed (see below). Phenyllactic acid and 4-methoxyphenyllactic may act as proton donors because a −I effect from 2-OH would stabilise the acid anion (COO^-); the group is too far from the ring for any resonance stabilisation to occur. The pKa of the acid group on phenyllactic acid is 3.72, which would act as an acid in the honey matrix. It is expected that the pKa for the acid on 4-methyoxyphenyllatic acid would be similar.

2-Methoxybenzoic acid was in low abundance in the mānuka honeys (1.88-18.27 mg/kg) and lower in clover honey (0.20-0.94 mg/kg). Beitlich *et al.*[248] reported high levels in jelly bush honey (n = 1) but low levels in mānuka and kanuka honey. Stephens and Schlothauer[151] reported *Leptospermum scoparium* varieties incanum and linifolium have significantly higher methoxylated benzoic acids than varieties myrtifolium and triketone. The pKa of the acid group is 4.09, hence it may act as a proton donor in the honey matrix; the acid anion would be resonance stabilised due to its proximity of the ring. However at the level that it is present, it may not contribute a lot of catalytic activity by itself, but there may be an accumulative effect with other phenolic acids.

Luteolin was found at 1.35 ± 0.40 mg/kg in mānuka honey and 0.13 ± 0.07 mg/kg in clover honey. Yao *et al.*[243] reported that it was one of the most abundant flavonoids in jelly bush honey (2.6 ± 0.17 mg/kg, n = 12, collected from apiarists) and comprised 12.6% of analysed flavonoids in mānuka honey (3.8 ± 0.07, n = 2);

the reported levels are higher than in the current research. Chan *et al.*[92] reported 1.4 ± 0.02 mg/kg luteolin in mānuka honey (n = 31 supplied by Comvita), which is similar to the current research. Resonance stabilisation of the extended conjugated system would make this a potential H donor.

Changes in concentrations over time

Sample 1404 was analysed on day 0 and 164 days after storage at 20, 27 and 37 °C and samples 25 and 41 were analysed on days 0, 21 and 314 days after storage at 37 °C. Samples are expressed in units of mmol/kg to investigate whether a loss of one compound was due to formation of another.

The change in concentration of syringic acid, methyl syringate and leptosperin was investigated to examine if there is a trend or a possible equilibrium between the three compounds. A summary of the change of each compound over time is summarised in Table 4.18. Syringic acid was in low concentration in the samples, and no change in concentration was observed at 20 or 27 °C; a very small increase was seen for all three samples over time at 37 °C (0.001 mmol/kg, 0.12 mg/kg), but is most likely from experimental error. An increase in methyl syringate was observed for all three honeys; 0.04-0.23 mmol/kg (8.60-47.61 mg/kg) at 37 °C and 0.02 mmol/kg (4.10 mg/kg) at 20 and 27 °C was gained. However, this increase is not from methylation of syringic acid. In comparison, Kato *et al.*[249] reported that methyl syringate decreased over 30 days when stored at 37 °C (30% lost) or 50 °C (~50% lost); however, this is only a short time frame compared to the current research. The time of sampling in relation to harvest of the honey may also contribute to differences in results.

Table 4.18 Change in concentration of syringic acid, methyl syringate and leptosperin over time (mmol/kg).

Sample	Days of storage	Temperature (°C)	Syringic acid (mmol/kg)	Methyl syringate (mmol/kg)	Leptosperin (mmol/kg)
25	314	37	0.002	0.225	–0.457
41	314	37	0.001	0.106	–0.087
1404	164	20	0.000	0.019	0.013
1404	164	27	0.000	0.020	–0.020
1404	164	37	0.001	0.041	–0.003

Varied results in the stability of leptosperin over time were observed; the concentration of leptosperin did not change by more than ± 0.02 mmol/kg (10 mg/kg) in sample 1404 at 20, 27 or 37 °C when stored for 164 days, which is likely to be measurement error. Kato *et al.*[249] reported that leptosperin did not vary in concentration over 100 days when stored at 37 °C and proposed that this was due to the compound having less reactive groups than phenolic compounds, such as methyl syringate. However, samples 25 and 41 showed a decrease in leptosperin over time when stored at 37 °C; leptosperin decreased from 735.83 to 491.00 mg/kg over 314 days (244.83 mg/kg, 0.46 mmol/kg lost) in sample 25, compared to sample 41 which decreased from 304.69 to 257.92 mg/kg (46.77 mg/kg, 0.09 mmol/kg lost). Both samples had approximately the same amount of initial DHA and MGO. It does not appear that an equilibrium between syringate, methyl syringate and leptosperin is occurring. A very limited number of samples and data points have been taken; hence more data is needed to establish whether or not there is a trend.

Table 4.19 summarises the change in concentration over time for the remaining four phenolic compounds analysed. Phenyllactic acid declined with time. However, the decline did not appear to be temperature dependent in sample 1404. Sample 25 also showed a loss of phenyllactic acid, but sample 41 had an increase. There was no corresponding gain in 4-methyoxyphenyllactic acid, which would be expected if methylation of phenyllactic acid was occurring. Stephens *et al.* (2010)[5] reported that phenyllactic acid declined over time in honey from 90% of reported phenolic compounds in fresh honeys to approximately 70% in 5-year-old honeys and the proportion of methoxylated phenolic components increased throughout maturation. Wilkins *et al.*[242] noted an inverse relationship between phenyllactic acid and 4-methyoxyphenyllactic acid, which was not observed in this current research or by Stephens *et al.*.[5] There was no large change in 2-methyoxybenzoic acid (−1.31 to 2.5 mg/kg) or luteolin (−0.66 to −0.17 mg/kg over time.

Table 4.19 Change in concentration of some phenolic compounds over time (mmol/kg).

Sample	Days of storage	Temp (°C)	Phenyllactic acid	4-Methoxy phenyllactic acid	2-Methoxy benzoic acid	Luteolin
25	314	37	–0.401	-0.350	0.004	–0.002
41	314	37	0.347	0.003	0.016	–0.001
1404	164	20	–0.445	0.000	–0.009	–0.001
1404	164	27	–0.688	0.000	–0.012	–0.001
1404	164	37	–0.252	0.001	0.000	–0.002

Correlations

DHA had a weak positive correlation with leptosperin (R^2 = 34%); a much stronger positive correlation between MGO and leptosperin (R^2 = 65%) was observed (Figure 4.19). Oelschaegel *et al.*[114] reported a correlation between leptosperin and MGO (R^2 = 85%) and Kato *et al.*[249] reported a correlation between leptosperin and NPA (R^2 = 73%); however, the correlation from Kato *et al.*[249] depends on whether MGO or NPA is plotted against leptosperin detected by HPLC, electrospray ionisation-MS or atmospheric-pressure chemical ionisation-MS. It is currently unknown why MGO and leptosperin have a strong correlation. A large number of authentic mānuka honeys need to be tested to determine whether or not this correlation is common; if there is a high correlation between leptosperin and MGO, leptosperin may be an important compound to detect if honey has been adulterated since leptosperin is not commercially available to dope into honey. This would remove the complication arising from not being able to distinguish naturally occurring DHA and DHA that has been doped into honey. Kato *et al.*[249] proposed that leptosperin is a good compound to identify authentic mānuka honey.

No correlation was observed in the current study between DHA and methyl syringate. Furthermore, no correlation was seen with MGO and methyl syringate; this was also reported by Kato *et al.*.[249] But there is a correlation between the total concentration of syringate, methyl syringate and leptosperin with MGO in the mānuka honey samples (R^2 = 57%).

A correlation between 2-methoxybenzoic acid and MGO in ten fresh mānuka honeys was reported by Stephens *et al.*[5] (R^2 = 80%). However, there was no correlation between these two compounds in the current study; sample 1404 was an outlier; with this sample removed the correlation increased (R^2 = 51%).

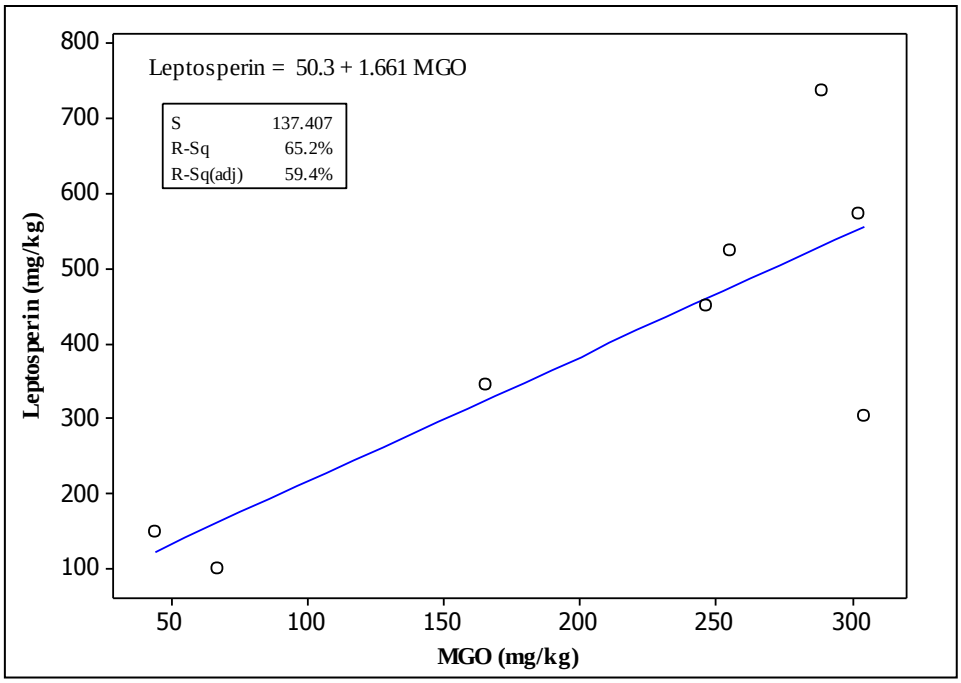


Figure 4.19 Leptosperin vs. MGO. There is a positive linear correlation between the two compounds (R^2 = 65.2%).

There was no correlation between phenyllactic acid or 4-methoxyphenyllactic acid with either DHA or MGO. Sample 946 was far from the other samples on the graphs. Furthermore, there was no correlation between the sum of phenyllactic acid and 4-methyoxyphenyllactic acid with either DHA or MGO. However, there was a strong correlation (R^2 = 78%) between 4-methoxyphenyllactic acid and methyl syringate in the current research, suggesting that they both might come from the same source, but this is a small sample size and correlations must be treated with caution.

4.3.9 Analysis of trace elements

4.3.9.1 Method development for trace element analysis

The literature reveals no common digestion method for the analysis of trace elements in honey. Most articles report the use of nitric acid and hydrogen peroxide for digestion, but the ratio and amount added vary extensively.

Four digestion methods were compared based on Fredes and Montenegro[221] and Vanhanen *et al.*.[207] Fredes and Montenegro digested honey (2 g) with 75% HNO_3

(5 mL). This was evaporated on a heated metal sheet at 100-120 ºC and diluted to 10 mL. For this experiment, the final dilution was made up to 20 mL. Vanhanen *et al.* took honey (1 g) and digested it with 69% HNO_3 (5 mL) and 30% H_2O_2 (2 mL); samples were left over night then digested in a microwave oven. As a microwave digestion oven was not available for the current project, heating was carried out for 1.5 hours in a digestion heating block and the acid ratio was change to a 2:1 ratio, which is more common in the literature. Previous work by Caroli *et al.*[209] indicated that taking a portion of honey for analysis did not give reproducible results and suggested homogenising the honey by diluting it 2:1 with water and using the resulting honey solution. This practice was adopted in this research. The four methods are summarised in Table 4.20.

Table 4.20 Summary of digestions methods.

Method	Weight of honey solution (g)	Amount of HNO_3 and H_2O_2	Heating	Dilution factor
1	1.8	4 mL HNO_3 + 2 mL H_2O_2	Digestion block	200 x
2	1.8	5 mL HNO_3	Digestion block	200 x
3	1.8	4 mL HNO_3 + 2 mL H_2O_2	Hot plate	20 x
4	1.8	5 mL HNO_3	Hot plate	20 x

A digestion block was used as the heat source for methods 1 and 2 which allowed the samples to be kept at a constant heat. The samples were digested in plastic falcon tubes, which prevented leaching of elements from the tubes and allowed easy transfer to volumetric flasks for dilution. These samples required a 200 fold dilution to bring the nitric acid content to 2% before ICP-MS analysis because the samples were not evaporated to dryness. Methods 3 and 4 were digested in beakers on a hot plate and it is possible that some elements may leach out of the glass due to the acidic environment and high temperature. This method is not as good as the digestion block at regulating the temperature because the plate turns on and off in an attempt to hold the temperature steady and airflow from the fumehood also made this difficult, therefore a precise temperature could not be obtained with this method. In addition, there is more chance that metals will be volatilised during digestion on the hot plate because the samples are heated at a higher temperature and are not in a closed vessel. The samples took a long time to

evaporate to dryness and in method 4 (HNO_3 only) samples did not completely digest (there were burnt pieces in the bottom of the beakers).

Spike recovery

A spiked honey (n = 3) was analysed by all four methods. A blank was analysed with each method and subtracted from the average of each triplicate. Each honey was spiked with a 20 ppm solution of Merck IV (Ag, Al, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Sr, Tl and Zn). Na, Mg, K and Ca could not be compared in the spike recovery test because the endogenous levels in honey exceeded the concentration of the spike. The recovery of the spiked elements should lie within 80 to 120%. The results are seen in Table 4.21.

Table 4.21 Percentage recovery of spike for methods 1 to 4.

	Method 1	Method 2	Method 3	Method 4
^{7}Li	92	**77**	92	93
^{10}B	108	108	69	91
^{27}Al	124	84	93	85
^{53}Cr	98	70	83	84
^{54}Fe	100	58	99	108
^{55}Mn	96	84	92	94
^{59}Co	94	78	65	66
^{60}Ni	95	66	64	63
^{63}Cu	97	73	55	55
^{68}Zn	150	118	54	20
^{88}Sr	95	78	97	97
^{109}Ag	106	80	87	87
^{111}Cd	100	82	95	94
^{115}In	98	80	96	95
^{137}Ba	100	78	91	92
^{205}Tl	92	76	93	91
^{207}Pb	93	84	91	89
^{209}Bi	113	96	105	97

Method 1 had the best recovery of the four methods examined. Eighty nine percent (16/18) of elements had a recovery of 80 to 120%. Only Al (124%) and Zn (150%) exceeded the limit. In comparison, only 72% (13/18) of elements meet the limits in method 3. B, Co, Ni and Cu did not meet the limits; this may be due to the loss of elements through fuming. Method 4 had similar recovery to method 3 for most elements; 78% (14/18) of the elements had recoveries between 80 to 120%. Method 4 recovered 91% of B compared to method 3 which only

recovered 69% B. However method 3 recovered more Zn (54 compared to 20%). In comparison, methods 1 and 2 which used the digestion block had high Zn recovery (150 and 118% respectively). Method 2 had the lowest recovery of elements. Only 44% (8/18) of elements met the limits; Li, Cr, Fe, Co, Ni, Cu, Sr, Ag, Ba and Tl did not have recoveries between 80 to 120%.

The spike recovery results show that the digestion using nitric acid and hydrogen peroxide, with heating in the digestion block gives the best recovery. The thoroughness of the digestion was indicated by the colour; digestion overnight produced a clear solution compared to the samples with only nitric acid. In addition, this method allows easier transfer of the samples from the digestion tube into a volumetric flask, compared to the digestions on the hotplate, which only used a 20x dilution; this small volume may have been inefficient to transfer the sample from the beaker into a volumetric flask.

Recovery of elements using method 1

Ten replicates of honey were spiked with 20 ppm of Merck IV and the recovery percentage was worked out. Fe had one outlier which was removed from analysis. All elements had a recovery between 90 – 110% (Table 4.22).These results are acceptable. The %CV for the 10 replicates was less than 10% for Mg, Cr, Mn, Co, Ni, Cd and Ba and between 10-20% for Ba, Al, Fe, Cu, Sr, Ag, Tl, Pb. Only Zn exceeded this (27.76%). Vanhanen *et al.*[207] reported a similar recovery for all elements analysed (92.2 to 123.4%)

Table 4.22 Recovery of 20 ppm multi-element standard in a clover honey matrix (n=10).

Element	Recovery (%)	Element	Recovery (%)
Ba	92	**Cu**	91
Mg	93	**Sr**	99
Al	103	**Ag**	102
Cr	94	**Cd**	97
Fe	92	**Ba**	95
Mn	93	**Tl**	95
Co	90	**Pb**	92
Ni	92		

Precision

The results from the clover honey (n = 10) doped with 20 ppm Merck IV for the recovery analysis (above) were also used to assess precision of the method (Table 4.23). The RSD (%) is lower than 10% for most elements. Na, Al, P and Zn had the highest RSDs (>15%). The high RSD for Na this may be due to contamination from glassware during the dilution step after digestion. These results are acceptable and show the precision of the method.

Table 4.23 Average concentrations of trace elements of a clover honey spiked with 20 ppm Merck IV (n = 10).

	Average (ppm)	Standard deviation	%RSD		Average (ppm)	Standard deviation	%RSD
B	24749.05	13.18	10.65	**Co**	18217.86	7.86	8.63
Na	114848.18	94.81	16.93	**Ni**	18595.34	8.76	9.42
Mg	40928.14	19.82	9.68	**Cu**	20553.84	10.68	10.39
Al	27626.30	27.05	19.58	**Zn**	26834.06	21.45	17.26
P	67750.52	51.06	15.07	**Sr**	19967.84	11.01	11.03
K	686458.89	485.04	14.13	**Ag**	20248.68	10.60	10.47
Ca	89902.16	43.94	9.78	**Cd**	19383.40	9.09	9.38
Cr	20068.18	9.49	9.46	**Ba**	19303.28	8.55	8.86
Mn	20126.58	9.30	9.24	**Pb**	19531.10	10.87	11.14

4.3.9.2 Analysis of the database

Honey samples were analysed in triplicate using method 1 for ^{10}B, ^{23}Na, ^{24}Mg, ^{27}Al, ^{31}P, ^{34}S, ^{39}K, ^{43}Ca, ^{51}V, ^{52}Cr, ^{54}Fe, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{68}Zn, ^{75}As, ^{82}Se, ^{88}Sr, ^{109}Ag, ^{111}Cd, ^{137}Ba, ^{202}Hg and ^{207}Pb. ^{56}Fe, ^{55}Mn and ^{75}As isotopes were also determined using the dynamic reaction cell (DRC) with NH_3 as the reacting gas. ^{34}S, ^{59}Co, ^{75}As, ^{82}Se, ^{111}Cd, ^{137}Ba and ^{202}Hg were not detected in the samples analysed or were below the limit of quantitation; therefore these elements have been excluded from the discussion. The 17 isotopes that were used for statistical analysis are as follows: ^{10}B, ^{23}Na, ^{24}Mg, ^{27}Al, ^{31}P, ^{39}K, ^{43}Ca, ^{51}V, ^{52}Cr, ^{56}Fe-NH_3 , ^{55}Mn-NH_3, ^{60}Ni, ^{65}Cu, ^{68}Zn, ^{88}Sr, ^{109}Ag and ^{207}Pb. The isotope number will be omitted from the remainder of the discussion.

The mean concentrations (ppm) for each element in each sample are presented in Table 4.24 A and Table 4.24 B. Outliers were detected and removed using Grubbs

outlier test. For ease of comparing the data to literature values, the average, minimum and maximum of the 23 samples were used (Table 4.25).

There are limited articles containing trace element data for mānuka honey and the number of samples analysed is small which means that there is not a good representation of trace elements in mānuka honey in the literature. For this reason the database was compared not only to mānuka honey samples, but other New Zealand honeys as well as honeys from around the world. Limited elemental analysis of mānuka honey includes one mānuka honey from Airborne Honey Ltd., Leeston (NPA rating was not stated) studied in triplicate for a full suite of elements,[207] one mānuka honey from Watson and Son for seven elements (K, Ca, Fe, Zn, Cr and Pb)[280] and two mānuka honeys with five replicates analysed by Saitoh *et al.*[239] for a full suite of elements.[239] Since there are a low number of samples, it is not expected that these values are a representation of New Zealand mānuka honey. Furthermore, trace elements in honey have been suggested as a geographical marker since the elemental composition depends of absorption from the soil and environment. Therefore the composition will differ to that of honeys produced in other countries, and possibly in different locations of New Zealand. However, most elements detected in the literature above fell within the range reported in this research.

The most abundant element in this database was K (976.79 ± 181.44); mānuka honey samples analysed by Saitoh *et al.*[239] also reported similar levels of K. This is also reported throughout the literature for other honeys.[207, 210, 212] High K levels most likely arise from the rapid secretion of K by the plant because it is high in plant tissue.[280-281] K accounted for 87.08% of the minerals analysed in this study; this is higher than 73% reported from Vanhanen *et al*., even though the concentration was higher in their study (1,290 mg/kg).[207]

Table 4.24 A Mean concentrations (ppm) of some elements for database honeys.*

Sample	B			Na			Mg			Al			P			K			Ca			V			Cr		
268	2.84	±	1.10	10.12	±	4.19	14.05	±	0.04	0.33			15.86	±	0.67	926.10	±	12.24	35.60	±	2.58	0.35	±	0.01	0.86	±	0.00
291	2.90	±	0.15	2.71	±	2.00	20.19	±	0.92	12.17	±	0.07	28.94	±	2.14	956.51	±	34.91	39.76	±	10.55	0.26	±	0.02	0.55	±	0.06
296	2.14	±	0.38		ND		14.22	±	0.00	8.93	±	7.04	20.67	±	0.78	901.47	±	8.05	34.80	±	5.85	0.26	±	0.00	0.50	±	0.03
392	2.48	±	0.10	0.35			15.50	±	0.69	21.99	±	8.74	34.02	±	0.78	879.73	±	15.30	43.35	±	5.66	0.28	±	0.01	0.56	±	0.06
449	2.42	±	0.32		ND		11.46	±	0.47	5.62	±	0.86	26.39	±	1.87	857.82	±	35.22	35.23	±	3.84	0.26	±	0.02	0.48	±	0.05
460	1.77	±	0.09		ND		10.20	±	0.84	6.60	±	5.80	21.65	±	1.84	808.70	±	22.25	22.23	±	0.04	0.26	±	0.01	0.47	±	0.05
491	1.97	±	0.07	29.09	±	37.48	11.23	±	0.02	1.48	±	0.33	20.70	±	0.38	873.84	±	5.69	35.98	±	9.12	0.27	±	0.01	0.48	±	0.04
498	3.40	±	0.06	2.50			12.14	±	0.37	0.18			24.23	±	1.19	831.54	±	17.47	37.87	±	4.92	0.37	±	0.01	0.85	±	0.03
565	1.53	±	0.63		ND		22.43	±	0.91	9.73	±	4.10	22.92	±	1.95	1146.69	±	35.24	38.70	±	0.31	0.27	±	0.02	0.48	±	0.06
597	1.86	±	0.43	13.59	±	0.63	24.13	±	0.43	5.13	±	1.03	25.21	±	2.40	991.51	±	54.67	43.50	±	15.87	0.27	±	0.02	0.47	±	0.05
608	3.65	±	1.16	32.66	±		17.79	±	2.70	2.59			14.63	±	1.85	941.95	±	54.69	46.66	±	24.24	0.33	±	0.01	0.72	±	0.00
610	2.63	±	0.79	40.29	±	1.42	15.50	±	0.18	0.91			14.98	±	0.39	1056.18	±	1.55	40.23	±	0.18	0.35	±	0.01	0.73	±	0.03
622	2.82	±	0.53	29.42			13.49	±	0.34	0.64	±	0.04	15.85	±	0.54	1029.28	±	1.04	23.47	±	1.10	0.40	±	0.01	0.91	±	0.03
637	2.59	±	0.66	27.69			12.85	±	1.26	6.28	±	7.81	24.17	±	0.01	652.76	±	20.45	52.24	±	15.86	0.36	±	0.02	0.71	±	0.01
654	2.15	±	0.48	5.94	±	8.06	10.75	±	0.27	5.04	±	1.76	19.10	±	0.13	696.74	±	1.03	52.80	±	5.22	0.27	±	0.02	0.47	±	0.05
673	2.36	±	0.15	1.94	±	2.10	26.08	±	1.14	16.34	±	13.38	30.47	±	0.10	861.94	±	35.27	67.72	±	12.52	0.25	±	0.01	0.48	±	0.02
755	3.54	±	0.89	13.31	±	10.33	19.18	±	0.41		ND		37.50	±	2.18	906.58	±	22.44	40.72	±	0.15	0.41	±	0.02	0.96	±	0.05
784	3.00	±	0.32	2.42			12.06	±	0.00	0.71			29.24	±	0.66	916.21	±	5.28	48.71	±	12.38	0.36	±	0.02	0.82	±	0.08
796	2.58	±	0.41	20.90	±	8.19	18.45	±	0.44	2.52	±	2.40	34.39	±	0.67	1122.89	±	7.07	58.76	±	1.00	0.39	±	0.01	0.92	±	0.00
802	4.48	±	1.15	11.97	±	11.21	26.11	±	1.23	0.26			45.73	±	1.35	1017.92	±	2.23	39.68	±	0.63	0.38	±	0.02	0.89	±	0.06
946	2.65	±	0.01	20.30	±	2.62	36.72	±	0.76	16.07	±	14.86	100.21	±	2.61	1097.35	±	1.15	70.95	±	1.56	0.25	±	0.00	0.45	±	0.03
953	2.11	±	0.04	12.68	±	0.03	21.95	±	0.55	20.50	±	8.41	42.85	±	1.00	1093.81	±	12.29	58.94	±	3.56	0.25	±	0.00	0.48	±	0.02
1349	2.47	±	0.02	51.68			14.83	±	1.02	33.07	±	5.74	16.77	±	1.45	824.95	±	31.94	44.58	±	11.38	0.27	±	0.00	0.59	±	0.00
66	3.62	±	0.72	188.11	±	69.74	23.54	±	2.73	1.27	±	0.54	42.69	±	5.59	1067.08	±	70.46	100.33	±	14.62	0.30	±	0.03	0.17	±	0.19

* Triplicate measurements

Sample	B			Na			Mg			Al			P			K			Ca			V			Cr		
78	4.01	±	0.20	91.39	±	6.15	25.61	±	0.11	0.63			37.33	±	1.33	1575.91	±	45.84	93.77	±	1.95	0.34	±	0.03	0.09	±	0.02
84	3.89	±	0.01	44.65	±	0.60	18.76	±	0.05	42.09			39.74	±	0.55	1124.46	±	13.47	74.19	±	15.66	0.38	±	0.00	0.19	±	0.04
14	2.99	±	0.83	27.61	±	11.21	28.43	±	0.82	7.57	±	0.03	24.15	±	0.02	1213.45	±	57.23	37.56	±	6.92	0.30	±	0.03	0.02	±	0.01

Table 4.24 B Mean concentrations (ppm) for some elements in database honeys.

Sample	Ni			Cu			Zn			Sr			Ag			Pb			Fe			Mn		
268	0.09	±	0.11	0.02			0.21	±	0.15	ND			ND			ND			0.32	±	0.22	0.71	±	0.03
291	0.03	±	0.02	0.65	±	0.47	2.44	±	1.13	0.01			0.02	±	0.01				0.90	±	0.08	1.52	±	0.14
296	0.02			1.03	±	0.68	1.67	±	0.96	ND			ND			0.47	±	0.41	0.37	±	0.16	0.64	±	0.01
392	0.03	±	0.02	0.61	±	0.43	2.74	±	1.81	0.07	±	0.03	0.09	±	0.03	0.02	±	0.02	0.70	±	0.35	0.39	±	0.08
449	0.00			0.04			1.70	±	0.04	0.02			ND			0.11			0.74	±	0.43	0.03	±	0.02
460	0.02	±	0.03	0.19	±	0.04	2.02	±	2.71	ND			ND			0.19			0.50	±	0.28	0.06		
491	0.15			0.24			0.93	±	0.69	0.06	±	0.05	ND			0.02			0.62	±	0.29	0.62	±	0.08
498	0.08	±	0.08	0.17			1.30	±	0.24	ND			ND			0.04			0.22	±	0.03	0.08	±	0.00
565	0.02	±	0.02	0.40	±	0.02	0.95	±	0.84	0.01	±	0.01	0.01			0.08			1.29	±	0.26	4.84	±	0.18
597	0.02			0.07			1.04	±	0.55	0.01			ND			ND			0.64	±	0.26	3.69	±	0.42
608	0.09	±	0.12	0.34	±	0.36	1.16	±	1.02	0.07	±	0.07	ND			0.08			0.69	±	0.45	2.16	±	0.05
610	0.02			0.09			1.80	±	0.82	ND			ND			ND			0.35	±	0.12	1.55	±	0.08
622	0.07			0.03			0.38	±	0.48	ND			0.01			0.01			0.41	±	0.19	1.98	±	0.04
637	0.08	±	0.11	0.35	±	0.35	2.75	±	1.82	0.04	±	0.03	ND			ND			0.62	±	0.00	0.15	±	0.06
654	0.08	±	0.06		ND		1.50	±	0.78	0.15	±	0.00	0.18	±	0.23	0.01			0.95	±	0.00	0.37	±	0.03
673	0.05	±	0.05	0.49	±	0.39	4.55	±	2.41	0.13	±	0.05	0.04	±	0.02	0.37	±	0.01	1.71	±	0.95	5.12	±	0.10
755	0.00	±	0.00	0.26	±		1.64	±	0.44	0.08	±	0.00	0.02						ND			0.15	±	0.06	1.99	±	0.04
784		ND					0.82	±	0.03	0.01	±	0.00	0.04	±	0.05	0.12	±	0.01	0.18	±	0.08	0.08	±	0.01
796	0.03	±	0.02	2.10			2.28	±	0.51	0.19	±	0.00		ND			ND		0.47	±	0.02	1.20	±	0.01
802	0.10	±	0.09	0.54			2.15	±	0.34	0.06	±	0.02		ND		0.03	±	0.01	0.30	±	0.23	5.53	±	0.36

Sample	Ni			Cu			Zn			Sr			Ag			Pb			Fe			Mn		
946	0.05	±	0.00	0.97	±	0.90	4.16	±	1.45	0.12	±	0.01	0.01	±	0.00	0.46	±	0.42	1.99	±	0.39	4.23	±	0.10
953	0.01	±	0.01	0.10	±	0.00	5.68	±	0.03	0.09	±	0.00		ND		0.05	±	0.06	1.16	±	0.24	1.59	±	0.02
1349	0.01			0.23	±	0.15	7.18	±	0.44	0.01	±	0.00	0.01	±	0.00	0.19	±	0.11	1.23	±	0.08	0.41	±	0.01
66	0.16	±	0.13	0.73	±	0.79	1.94			0.84	±	0.10	0.05	±		0.80	±		7.70*	±	4.69	2.49*	±	0.23
78	0.09	±	0.09	0.23	±	0.16		ND		0.80	±	0.02	0.21	±	0.00	0.19	±	0.03	3.94*	±	4.17	3.07*	±	0.11
84	0.01	±	0.01	0.80			0.23			0.41	±	0.04	0.06	±	0.24		ND		1.10*			1.65*	±	0.08
14	0.02	±		0.02	±	0.01	0.29			0.03	±	0.10	0.11	±		ND	±		1.19	±	0.01	7.82	±	0.01

Table 4.25 Summary of trace elements for database of mānuka honeys (n = 27).

Element	Average (ppm)	Standard Deviation	Minimum	Maximum	% of total elements
B	2.77	0.74	1.53	4.48	0.25
Na	29.62	40.35	ND	188.11	2.64
Mg	18.43	6.58	10.20	36.72	1.64
Al	8.79	10.67	ND	42.09	0.78
P	30.01	16.79	14.63	100.21	2.68
K	976.79	181.44	652.76	1575.91	87.08
Ca	48.83	18.81	22.23	100.33	4.35
V	0.31	0.05	0.25	0.41	0.03
Cr	0.57	0.26	0.02	0.96	0.05
Ni	0.05	0.04	ND	0.16	0.00
Cu	0.43	0.46	ND	2.10	0.04
Zn	2.06	1.69	0.21	7.18	0.18
Sr	0.15	0.24	ND	0.84	0.01
Ag	0.06	0.06	ND	0.21	0.01
Pb	0.18	0.21	ND	0.80	0.02
Fe	0.74	0.48	0.15	1.99	0.07
Mn	1.95	2.13	0.03	7.82	0.17

Ca and P are the next most abundant elements found in the honeys analysed (48.83 ± 18.81 and 30.01 ± 16.79 ppm respectively). They make up 4.35 and 2.68% of the trace elements respectively. These elements are also reported as abundant in the literature. Saitoh *et al.*[239] reported similar levels for Ca and P in two mānuka honeys. Terrab *et al*.[211] reported a much higher concentration of Ca (181 ± 35 ppm) but a similar concentration of P (51 ± 17.39 ppm). A study of Spanish honeys[210] had wide ranges of both Ca and P (42.59-341.00 and 51.17-154.3 ppm respectively).

Na (29.62 ± 40.35 ppm, 2.64%), Mg (18.43 ± 6.58 ppm, 1.64%) and Al (8.79 ± 10.67 ppm, 0.78%) were the next most abundant elements in the mānuka honeys. All remaining elements were found lower than 3 ppm. Spanish thyme honeys had a much larger concentration of Na (389 ± 69.5 ppm) than the mānuka honeys.[211]

There is very little Fe found in any of the database samples (0.72 ± 0.48 ppm). Low levels of Fe were also reported in other publications of mānuka honey[207, 239] and other honeys from New Zealand.[207] Therefore high levels of Fe may indicate

that the honey has been doped, in an attempt to hasten the conversion of DHA to MGO.

Cd was not detected in the samples analysed during this research. In comparison, Vanhanen *et al.*[207] reported 0.149 mg/kg Cd. New Zealand uses a large amount of phosphate-based fertiliser, which has elevated concentrations on Cd. However, not all forms of Cd are equally bioavailable[282] and it is unlikely that mānuka trees are grown on fertilised land because it is typically planted on land unsuitable for grazing or crops. This may be the reason that Cd is either not detected or is very low in the honey samples. In comparison, 22% of French honeys (n = 86, including lavender, chestnut and orange tree) analysed contained Cd (0.152 ppm).[217]

Correlations of elements were not seen when honeys from different honey producers were plotted on the same graph. However, honeys from the same producer had correlations. Steens honeys had a very strong trend between V and Cr (R^2 = 96%, Figure 4.20) indicating that they probably come from the same source. This most likely arises from interaction of the honey with stainless steel equipment during the extraction process. It should be noted that the concentration of both V and Cr are low and are not of concern. Other trends were also observed; however, these were not as strong. The correlations are summarised in Table 4.26. The Al correlation with Zn most likely also arises from the stainless steel in the extraction process. McLellan[281] analysed Ca, Mg, P and Na in honey and noted a relationship between K and Mg. A weak correlation (35%) was also seen in the database samples between K and Mg. In addition, Mg and Ca were also weakly correlated, which was not observed by McLellan.

No trends were observed between any elements with MGO, DHA, HMF when samples were divided into the number of years of storage. Also no trend of total mineral count and pH was observed, although Vanhanen *et al.*[207] reported a trend.

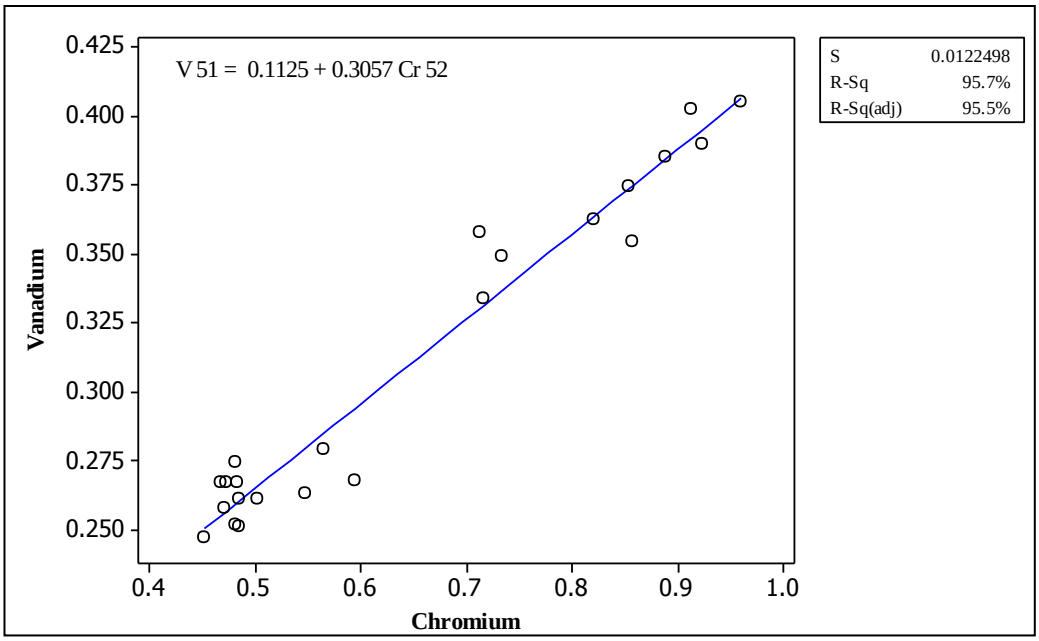


Figure 4.20 Correlation between V and Cr in honey samples from Steens honey Ltd..

Table 4.26 Elemental trends in honey from Steens Honey Ltd..

Element	Correlated with	R^2 (%)
V	Cr	96
Al	Zn	76
Mg	P	60
Cr	B	53
Ca	Sr	47
Mg	K	35
Mg	Ca	33

The mean total mineral count is expressed as the sum of all elements found in the honey; the results for individual honeys are shown in Table 4.27. The average of the samples in the database (1,116.51 ± 223.07 ppm) is similar to that reported by Vanhannen (1,470 ± 79.7 mg/kg).[207] The results are comparable because most elements tested were the same; the results shown here also included Sr and Ag, while Vanhanen *et al.* included As, Cd, Mo and S. The authors reported a large range of total mineral content between different types of honeys. Agbagwa[280] suggested that the elemental composition of honey depends on multiple factors, including plant type, season, environmental conditions and the soil composition.

Table 4.27 Total mean mineral content (ppm) for database honeys.

Sample	Total mineral content (ppm)	Sample	Total mineral content (ppm)
268	1007.47	**755**	1026.34
291	1069.54	**784**	1014.78
296	987.21	**796**	1268.09
392	1002.92	**802**	1156.14
449	942.34	**946**	1356.93
460	874.87	**953**	1262.27
491	977.68	**1349**	998.50
498	914.98	**66**	1431.63
565	1250.36	**78**	1830.60
597	1111.14	**84**	1349.86
608	1065.58	**1404**	1351.56
610	1175.61	**Average**	1116.51
622	1119.17	**SD**	223.07
637	783.64	**Minimum**	783.64
654	796.49		
673	1020.03	**Maximum**	1830.6

4.4 Conclusions

This chapter has discussed the concentration of a range of compounds (DHA, MGO, HMF, sugars, proline, primary amino acids, trace elements and phenolics) as well as physical properties (moisture content, pH and acidity) in a database of mānuka honeys. This database gives an estimation of the values that may be typical for mānuka honeys. However, the database is small (<30 samples) and analysis of a large number of samples is required to see if the results in the current research are typical of mānuka honeys from different regions and varieties of mānuka tree.

Proline was found in various concentrations in honey and declined with storage time. Iron was found in low levels in the honeys tested, suggesting that doping of iron in an attempt to increase the MGO concentration can be identified. The section on phenolic compounds has identified a high abundance of phenyllactic acid and 4-methoxyphenyllactic acid in mānuka honey. These compounds have the ability to act as proton donors and may influence the conversion of DHA to MGO, by altering the equilibrium between the DHA dimer and monomer (a postulated mechanism is shown in Figure 5.27).

Chapter Five

5 Storage trials of model systems – conversion of DHA to MGO

This chapter discusses the non-enzymatic conversion of DHA to MGO in model systems (artificial honey) with respect to time and temperature. Perturbants were added to the matrix to investigate their influence on the conversion of DHA to MGO, their ability to bind to DHA or MGO, or catalyse side reactions. Data was plotted as mmol/kg because this allowed a direct comparison between DHA and MGO and also perturbants.

The knowledge outlined in this chapter was required to build the prediction model for the conversion of DHA to MGO. Influences of various compounds in the model systems helped to predict what is occurring in real honey.

5.1 Literature review of the conversion of DHA to MGO and possible side reactions

5.1.1 Honey matrix – a unique situation

The non-enzymatic, irreversible conversion of DHA to MGO by loss of water is reported to occur readily in aqueous solutions.[14, 283-284] However, the honey matrix is a unique environment which behaves differently to aqueous solutions. A number of reaction conditions are affected by the viscous, dehydrating, acidic (~pH 4) matrix of honey. The viscosity of honey may influence the speed at which molecules can move and is likely to be one cause of slower reactions in honey compared to reactions in aqueous solution. The low water activity (0.56-0.62)[285] means that most water is bound to sugars and unable to react. The pH of honey influences the state in which molecules are present, for example, the amino group of amino acids may exist as NH_2 or NH_3^+ depending on the pH of the matrix. The type of reactions that can occur may also depend on pH. Furthermore, the pH and water content will influence the state of DHA; some will be present as the dimer and thus unable to react. The whole concept of pH becomes problematic in such a dehydrating environment; honey pH is measured in diluted solutions which may affect the true measurement of pH.

Competing reactions prevent all DHA converting to MGO. Side reactions will be influenced by factors in the environment, such as the pH. Hence reactions that may occur in some environments may not play a large role in a honey matrix. Side reactions decrease the efficiency of the conversion of DHA to MGO. DHA is known to bind readily to amino acids, and MGO is known to be an intermediate in the Maillard reaction, hence diminishing the conversion of DHA to MGO and the product yield.

There are few direct comparisons in the literature for the way DHA and MGO behave in a honey environment. In an attempt to convert DHA to MGO faster, mānuka honey is stored at room temperature or mildly warmed for an extended time, which are not common reaction temperatures examined in the literature (expect for physiological systems). Furthermore there is difficulty in measuring parameters in honey because most analyses require the matrix to be altered, usually to an aqueous solution; hence the state of compounds and their

equilibrium constants will be altered and cannot be related back to what is observed in the honey. Many experiments in the literature have been carried out in aqueous solution, and others are at much higher temperatures so cannot be directly related to the dehydrating environment of honey under mild warming.

Much of the literature focusing on the conversion of DHA to MGO is from the 1920s to 1970s. More recently, there are many studies on MGO in physiological systems due to its role in the formation of harmful advanced glycation end-products (AGE's). The review in this chapter gives an overview of possible reactions in which both DHA and MGO may be involved. It is expected that some reactions seen in the literature may still occur in the honey but it is also possible that reactions not seen in aqueous solution are occurring within the honey matrix.

5.1.2 Kinetics of inter-conversion of dihydroxyacetone and glyceraldehyde and their dehydration to MGO

The non-enzymatic conversion of trioses to MGO was first studied in the mid twentieth century.[14] The inter-conversion of DHA and glyceraldehyde and their irreversible conversion to MGO (Figure 5.1) has been studied in a number of systems (including different solvents, pH and temperatures). The dehydration of DHA or glyceraldehyde to MGO is reported as an acid-base catalysed reaction.[284] Most kinetic studies of the conversion of trioses to MGO are set up to model physiological systems or in model aqueous systems. Various catalytic species have also been examined. As previously mentioned, many of the studies are not directly comparable to the acidic, dehydrating environment of honey.

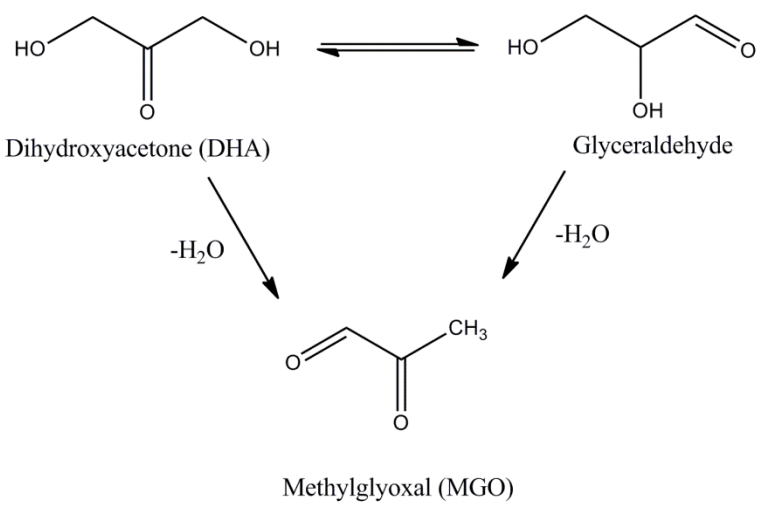


Figure 5.1 Inter-conversion of DHA and glyceraldehyde and their irreversible conversion to MGO.

Reaction mechanism and order of reaction

Strain and Spoehr (1930)[286] reported that DHA was slowly and incompletely converted to MGO in dilute acetic acid solution without the presence of a catalyst. Even though the reaction is irreversible, and hence should act as a sink, it did not go to completion in the 15 days during which it was monitored. In 1969, Fedoroňko and Königstein[283] reported that in acidic aqueous solution, only dehydration of DHA to MGO occurs; there is no isomerisation to glyceraldehyde. However, in acetate, phosphate and carbonate buffers and NaOH solutions, both isomerisation and dehydration occurred. They suggested that dehydration exhibited general acid-base catalysis, whereas the mutual isomerisation was only subject to general base catalysis, but noted that further work must be done to prove this. Hence in honey (pH 4) it can be assumed that only dehydration will occur.

Strain and Spoehr (1930)[286] suggested that formation of an intermediate enediol common to both DHA and glyceraldehyde is essential for their dehydration to MGO; this has now become the generally accepted mechanism (Figure 5.2). Bonsignore *et al.*[287] noted that increase of MGO is very slow at the start of the reaction, suggesting that the final product is formed via an intermediate compound, but did not suggest a mechanism. Lookhart and Feather (1978)[288] studied tritium incorporation into MGO formed from DHA and reported the data was consistent

with an enediol intermediate. In 1980, Fedoroňko *et al*. (1980)[289] examined the kinetics and mechanism of acid-catalysed reactions of methylated DHA and glyceraldehyde to MGO in aqueous HCl and reported almost identical rates (1.37 x 10^5 L/mol/s compared to 1.33 x 10^5 L/mol/s) for the two compounds. From a deuterated system, they deduced a mechanism that also included an enediol. Furthermore, in 1997, an enediol intermediate was also proposed by Kabyemela *et al.*[290] in a study of DHA and glyceraldehyde in sub-critical and super-critical water (273-673 K, 25-40 MPa, 0.06-1.7 seconds).

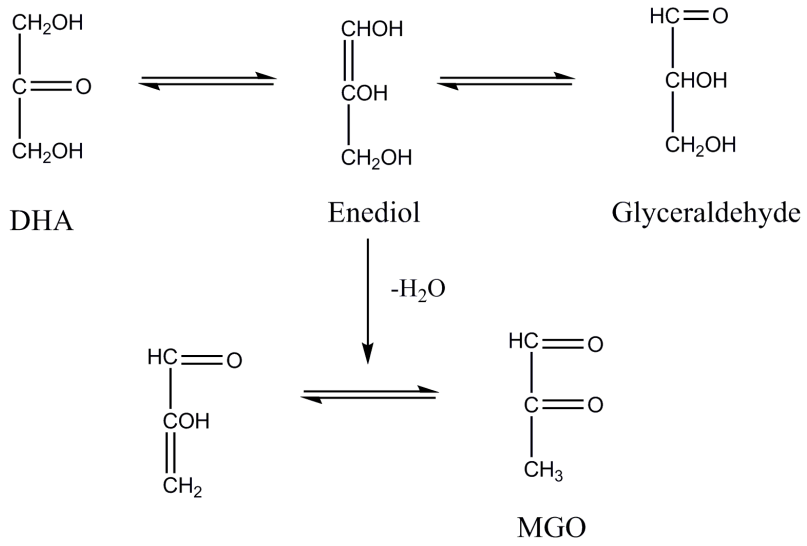


Figure 5.2 Generally accepted mechanism for inter-conversion of DHA and glyceraldehyde and their mutual dehydration to MGO through the enediol. Reproduced from Lookhart and Feather.[288]

The conversion of DHA to MGO is first-order; this order has been reported for reactions up to 240 °C, with no reverse reaction detected in acidic conditions.[291] Fedoroňko and Königstein[283] measured first-order rate constants for DHA dehydration to MGO and noted that at 50 °C it was larger in 1M aqueous solutions of acetic acid ($1.5x10^{-1}$ min^{-1}) compared to in water ($6x10^{-6}$ min^{-1}). The authors suggested this was due to undissociated acetic acid playing a role in conversion because the rate constants are much higher than those corresponding to the catalysis of the H_3O^+ ions present and by water. Activation energy (E_a) was calculated for the conversion between 30 and 60 °C using rate constants from dehydration in 4.5M HCl. This was 81 kJ/mol. When E_a was calculated using rate constants related to the unit of mean molal activity of 4.5 HCl, the E_a was 88 kJ/mol. Fedoronko *et al.*[289] reported the activation energy for the conversion of

glyceraldehyde to MGO in the same conditions was 113.6 kJ/mol (27.15 kcal/mol), which is slightly higher.

There are also reports of pseudo first-order reactions when catalysts are added to the reaction medium. Riddle and Lorenz (1968)[105] reported that the conversion was second-order for trioses and phosphate (pH 7.4, 40 °C). The second-order reaction may be due to the basic conditions; it is possible that the reaction relies on H^+ ions which would be limited in the conditions applied in this particular experiment. Bonsignore *et al.* (1972)[287] reported that the conversion of glyceraldehyde to MGO obeys first-order kinetics when lysine, phosphate or both catalysts are present (pH 7.7, 37 °C). However, the rate constant depends on the concentration of catalyst added (pseudo-first-order). Fedoroňko *et al*. (1980)[289] also reported a pseudo first-order reaction; the higher the concentration of HCl, the faster the conversion of DHA to MGO occurred.

Homoki-Farkas *et al*. (1997)[118] examined the formation of MGO in caramelisation and Maillard reactions. In the caramelisation reaction MGO is formed from DHA. They noted that MGO was formed at a slower rate under dry conditions than it was in the presence of water. In dry conditions, maximum MGO was reached later than in aqueous solution which was suggested to be due to the occurrence of side reactions. This experiment required a precursor to convert to DHA first which may have influenced the rate of conversion of DHA to MGO.

Stoichiometry

Stoichiometry of less than 1:1 conversion of DHA and glyceraldehyde to MGO has been noted throughout the literature. It is proposed that MGO reacts further in the matrix so that 100% MGO is not obtained; either due to polymerisation, reaction with other compounds in the matrix or conversion to another compound due to catalysis from a species present in the matrix. DHA may also undergo side reactions. These ideas are discussed further in section 5.1.4.

Strain and Spoehr (1930)[286] reported that glyceraldehyde was stable in dilute acetic acid, but when aniline was added the newly formed MGO underwent further reaction so that there was never more than 50% MGO in the solution. In addition, at the maximum MGO yield, no glyceraldehyde was left. Riddle and

Lorenz (1968)[105] reported loss of MGO between pH 6-11 was dependent on pH and independent of ions in the solution; the higher the pH, the more MGO was lost (1% at pH 6 through to 200% (*sic*) loss per hour at pH 11). At high pH, MGO converts irreversibly to lactic acid, due to the benzilic acid rearrangement. Fedoroňko and Königstein (1969)[283] studied the conversion of DHA to MGO in acetate buffer and reported an increase of unidentified compounds, presumed to be from side reactions of MGO. Bonsignore *et al.* (1972)[287] suggested loss of MGO at pH 7.7 (37 °C) was partly due to MGO polymerisation and further reaction of MGO with other compounds in the matrix. The extent of MGO loss depended on the catalyst used.

Sugar models (pH 5.5, 50 °C) examined by Weber (2001)[292] saw an initial increase in MGO followed by a decline, indicating further conversion. Furthermore, a hydrothermal reaction of DHA to MGO (180-240 °C) does not produce a 1:1 conversion. The maximum yield of MGO was only 40%.[291]

Catalysts

Various catalysts have been reported to affect the conversion of DHA or glyceraldehyde to MGO. There are different reports of the effect of amino acids in the literature, possibly due to the matrix in which the reaction was carried out.

Organic amines, including *p*-aminoacetanilide, aniline, α-naphthylamine and p-toluidine catalyse the conversion of glyceraldehyde to MGO in dilute acetic acid. In contrast to glyceraldehyde, amines had little effect on DHA in solution.[286] However, this reaction was undertaken in an aqueous acidic environment, compared to the dehydrating environment found in honey and amino acids are reported to catalyse the conversion of DHA to MGO under such conditions.[14] Weber (2001)[292] examined the effect that ammonia and amines (including amino acids) had on the conversion of formaldehyde and glycolaldehyde to sugars and their successive conversion to carbonyl-containing products (pH 5.5 and 50 ºC). With no catalyst, glyceraldehyde, DHA, erythrose, threose and erythrulose were the main products, but only a small quantity of MGO was formed during this uncatalysed reaction. Amine-catalysed reactions gave glyceraldehyde, dihydroxyacetone, methylglyoxal, erythrose, threose, acetaldehyde, glyoxal, pyruvate, glyoxylate and several unidentified carbonyl products. Sterically

unhindered primary amines were the better catalysts (~1000-fold) than sterically hindered primary amines, such as α-aminoisobutyric acid (Figure 5.3), secondary amines and ammonia.

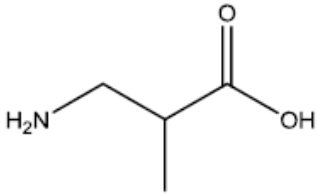


Figure 5.3 α-aminoisobutyric acid

The rate enhancements obtained by Weber[293] for the following catalysts are given in parentheses: glycine (1500, Figure 5.4, a), methylamine (1200), alanine (1170, Figure 5.4, b), alanyl-alanine (1080, Figure 5.5, a) sarcosine (410, Figure 5.5, b), ammonia (47), α-aminoisobutyric acid (17). These values are steady-state values, due to the further reaction of MGO. Sarcosine (Figure 5.5, b), a secondary amine, was reported to sustain MGO synthesis longer than a primary amine. Weber suggests that this is due to primary amino acids being able to react with trioses and MGO to form the side products pyrazine and imidazole respectively, whereas this cannot occur with secondary amines.[292]

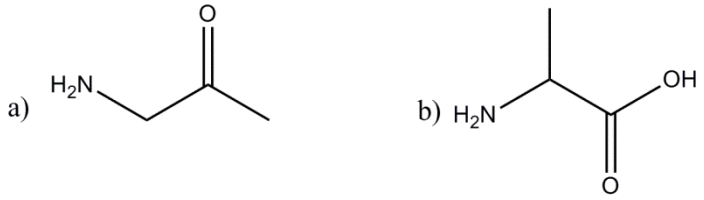


Figure 5.4 a) glycine; b) alanine

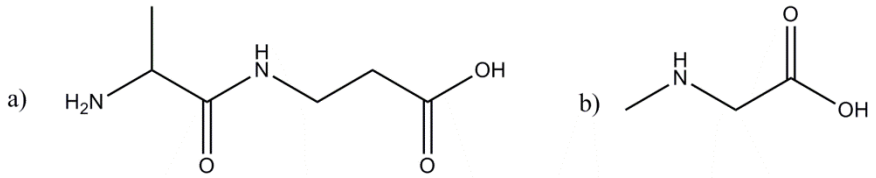


Figure 5.5 a) alanyl-alanine; b) sarcosine

Much work has been carried out on catalysis of the dehydration reaction under physiological condition. Riddle and Lorenz (1968)[105] reported that glycine had no effect on the conversion of DHA or glyceraldehyde to MGO under physiological

conditions (pH 7.4, 40 °C). The initial stage of the Maillard reaction occurs in acid, hence would have been unavailable. Furthermore, glutamate, acetate, succinate, oxalate, and manganese, barium and cadmium ions were among the compounds that had no catalytic effect. They reported twelve compounds, which successfully catalysed the conversion of DHA to MGO; this included phosphate, arsenate, fructose 1,6-diphosphate, fructose 6-phosphate, glucose 6-phosphate, bicarbonate and phytate.

Bonsignore *et al.* (1972)[287] reported that lysine had a higher catalytic rate than phosphate when converting glyceraldehyde to MGO (pH 7.7, 37 °C, Table 5.1). In addition, a cooperative effect was seen when both catalytic species were added; the rate constant was higher than the simple addition of rate constants for the two species.

Table 5.1 Rate constants for glyceraldehyde conversion to MGO extracted from Bonsignore *et al.* (1972).[287]

Catalyst	Catalyst concentration (mol L^{-1})	Rate constant ($x10^{-2}$ h^{-1})	Rate constant (day^{-1})
Uncatalysed	-	1.01	0.0242
Phosphate ion	3.0	1.70	0.0408
Lysine	3.0	23.85	5.7240
Phosphate ion + lysine	3.0 of each	88.29	21.190

Bonsignore *et al.*[294] reported no correlation between the pK of amino acids and their catalytic effect on conversion of glyceraldehyde to MGO (pH 7.7), but noted some relationships due to functional groups; OH groups in proximity to the NH_3 slowed the reaction down. Hydroxylysine was 50% less active than lysine. while serine was 50% less active than alanine; structures of the four amino acids are shown in Figure 5.6. This may have been due to an interaction between the NH_3 and OH as hydroxyproline did not show decreased activity compared to proline. The authors also proposed that the COOH group has no effect on catalysis because amino acids and the corresponding amine had the same rate enhancement. They also studied di-amino groups and suggested that two amine groups spaced by at least 5 carbon atoms (e.g. 1,5-diamino pentane) were necessary for complete

catalysis. The larger the space, the better the rate enhancement, possibly due to the molecule being able to fold to bring the two groups close to each other.

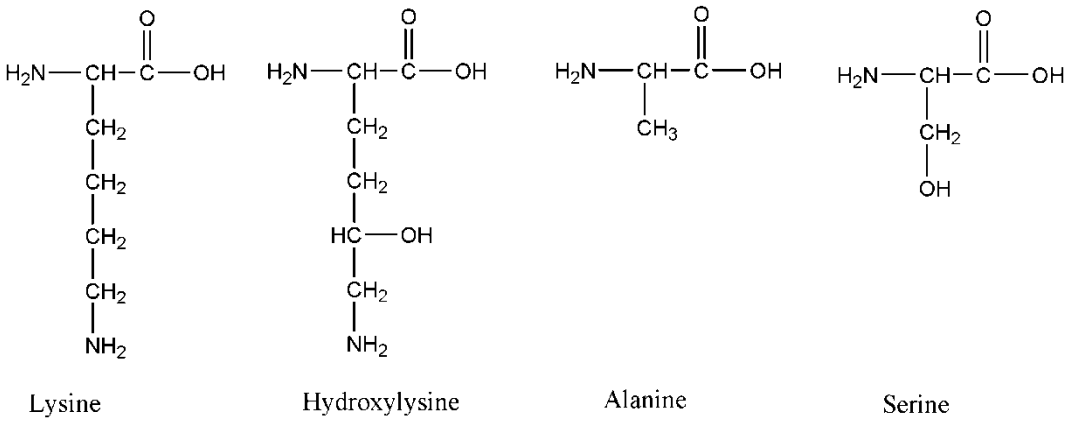


Figure 5.6 Lysine is 50% more reactive than hydroxylysine and alanine is 50% more reactive than serine due to the proximity of OH and NH_3 on hydroxylysine and serine.

5.1.3 Kinetics of conversion of DHA to MGO in mānuka honey

While there are many papers detailing the conversion of DHA to MGO in physiological and model systems, very little is known about the conversion of DHA to MGO in honey. For example, rate constants, compounds that catalyse or hinder the reaction and temperature influence are largely unknown. Section 5.1.2 discussed the dehydration of DHA to MGO in a range of media; however, the environments described are not the same as honey, moreover many of the compounds used as catalysts do not naturally occur in honey. Chapter 4 outlined components that have been found in honey. Any of these components could be a possible catalyst or inhibitor in the conversion of DHA to MGO.

Adams *et al.* (2009)[14] carried out preliminary work on the conversion of DHA to MGO in mānuka honey as well as in clover and artificial honey (both doped with DHA). Conversion of DHA to MGO occurred in all three matrices, but was slowest in artificial honey, suggesting that compounds in honey enhance the rate of conversion. They also noted that there was a lack of mass balance between the disappearance of DHA and appearance of MGO. This reflects the findings by other studies as discussed in section 5.1.2. The irreversibility of the conversion of DHA to MGO was shown in honey by adding MGO to clover honey; little change in MGO concentration and no DHA formation was observed.[14] A decrease in

MGO from side reactions may have been swamped if the MGO concentration was high (it was not stated), or because the reaction was not carried out long enough to see such reactions.

Adams *et al.* (2009)[14] also considered the possibility that DL-glyceraldehyde is the precursor to MGO. Honey doped with glyceraldehyde formed MGO with results comparable to that for DHA. However, they reported that freshly produced honey does not contain DL-glyceraldehyde, hence this cannot be the starting compound for the reaction. In addition, DHA was not formed, confirming that mutual isomerism does not occur between the two trioses in a honey matrix.

Addition of lysine or arginine to clover honey doped with DHA showed that amino acids had a catalytic effect on the conversion, but rate constants were not given. Both DHA and amino acids were added in large quantities. When the amino acid concentration was increased from 0.62 to 2.5%, MGO reached a maximum then began to decline. This may be from MGO reacting further due to a catalytic effect of the amino acid, or binding to the amino acid. The protein content of honey samples has been reported to range from 0.058% to 0.786%, therefore the minimal MGO loss due to reaction with R groups of proteins in mānuka honey would occur at a slow rate.[14]

It was hypothesised that heating honey may accelerate the non-enzymatic reactions. However, increasing the temperature from 37 °C to 50 °C resulted in loss of both DHA and MGO, possibly due to enhancing the rate of various side reactions of DHA or further reactions of MGO. Furthermore a large increase in HMF was observed.[14]

5.1.4 Side reactions of DHA and MGO

Non-enzymatic browning reactions (Maillard reaction and caramelisation) and polymerisation are side reactions that can compete with DHA conversion to MGO in honey and also remove both compounds of interest from the system. Furthermore, compounds in honey may act as catalysts to convert DHA or MGO to other compounds. The different reactions may occur simultaneously. For example, primary and secondary amines can add to aldehydes and ketones to give

different products. Primary amines give rise to imines (C=NR) which are stable enough for isolation. Imines with simple R groups will rapidly decompose or polymerise unless at least one aryl is on the nitrogen or carbon. In contrast, secondary amines reacting with aldehydes or ketones initially form N,N-disubstituted hemiaminals (HO-C-NR_2) which are unable to lose water and can be isolated. However, they are generally unstable and react further. If no α-hydrogen is present, it is converted to the more stable aminal (R_2N-C-NR_2) but if an α-hydrogen is present water (from the hemiaminal) or RNR_2 (from the aminal) can be lost to give an enamine (C=C-NR_2).[295]

The following sub-sections give a brief overview on some of the types of side reactions that could potentially occur; however, browning reactions are very complex and the literature correspondingly extensive; therefore it would be impossible to review all types of reactions and all compounds that might possibly form.

5.1.4.1 Maillard reaction

The Maillard reaction, also known as non-enzymatic glycation or browning, was characterised over 100 years ago in 1912 by the French scientist Louis Camille Maillard who observed a yellow-brown colour when reducing sugars were heated with amino acids.[296] The food industry uses the Maillard reaction to control food texture and flavour. However, Maillard products have been noted as having an involvement in the pathogenesis of various diseases, including diabetes mellitus and Alzheimer's disease.[296] 1,2-dicarbonyl compounds are reactive due to their increased electrophilicity, reacting relatively fast with R groups on proteins; crosslinked proteins are formed from subsequent glycoxidative modifications.[296]

The chemistry underlying the Maillard reaction is very complex because it refers to a network of reactions with many products formed. For example, in a reaction between xylose and glycine about 100 reaction products have been detected.[297] It was not until 1953 that a comprehensive reaction scheme of the Maillard reaction was published by Hodge.[298] He made a successful attempt, which is still referenced today, at understanding the reactions involved. Since then, this scheme has been developed and expanded to provide more information on this complex

set of reactions. There are many papers published on the Maillard reaction, which over the years have added more information to these complex reactions. Temperature, time, moisture content, the degree of reactivity of amino acids and sugars and various structures of products are among the research topics that have been undertaken. The reader is directed to several reviews which cover the topic much more extensively than this brief review.[299-304]

To gain an understanding of such a complicated and complex system, simplification is required. Hodge subdivided the Maillard reaction into three stages (initial, intermediate and final), which have also been called early, advanced and final Maillard reactions. The reactions which occur at each stage at pH <7 are:

1) Initial stage: colourless products

i. sugar-amine condensation

$$Aldose\ sugar + amino\ compound \leftrightarrow N - substituted\ glycosylamine + H_2O \quad (5\text{-}1)$$

ii. Amadori rearrangement

$$N - substituted\ glycosylamine \rightarrow 1 - amino - 1 - deoxy - 2 - ketose\ (ARP)^{*} \quad (5\text{-}2)$$

2) Intermediate stage: Colourless or yellow products

iii. sugar dehydration

$$1 - amino - 1 - deoxy - 2 - ketose\ (ARP) \rightarrow Schiff\ base\ of\ HMF\ or\ furfural + 3H_2O \quad (5\text{-}3)$$

$$Schiff\ base\ of\ HMF\ or\ furfural + H_2O \rightarrow HMF\ or\ furfural + amino\ compound \quad (5\text{-}4)$$

iv. sugar fragmentation

v. amino acid degradation (Strecker degradation)

[*] Amadori rearrangement product

3) Final stage: highly coloured products

vi. aldol condensation (to give polymers)

vii. aldehyde-amine condensation and formation of heterocyclic nitrogen compounds

$$HMF\ or\ furfural + amino\ compound \rightarrow Melanoidins \quad (5\text{-}5)$$

An adapted scheme of the Maillard reaction, reflecting the above three stages, is shown in Figure 5.7. DHA is not an aldose, but is known to react by condensing with amino acids. MGO is an aldose, so can undergo the initial pathway. Furthermore MGO is classified as a fission product in this figure – in honey it is not formed by the pathway depicted, but it may be able to react further by the pathways shown. The scheme shows that there are many products that DHA, MGO and HMF may form in the acidic environment of honey, such as aldimines and melanodins.

The products formed from the Maillard reaction or caramelisation depend on the matrix and are influenced by temperature and water activity.[305] The Maillard reaction is favoured in food with high protein and reducing carbohydrate content, pH between 4 and 7, an intermediate water content and temperatures above 50 °C.[305] The initial stages in the Maillard reaction are reversible; whereas further steps are irreversible. The Maillard reaction is influenced by pH, which alters the percentage of sugars that are in the open chain form and also the protonation state of the amino group present. At low pH there are more protonated amino groups present in the equilibrium; these are less reactive with sugars.[302] While the Maillard reaction is often carried out at high temperature it also occurs at 37 °C.[301]

The glycosylamine formed in the first step exists in equilibrium with a Schiff base. The reaction of formation of Schiff bases is reversible, but they will slowly convert to a more stable product. An Amadori rearrangement is the acid or base catalysed rearrangement reaction of the *N*-glycoside of an aldose or glycosylamine to the corresponding 1-amino-2-deoxy-1-ketose (Figure 5.8). This reaction can occur at 25 °C and is irreversible.[297] Nursten (2002)[297] gives a good overview of each step occurring in the Maillard reaction and the reader is directed to this paper for further information.

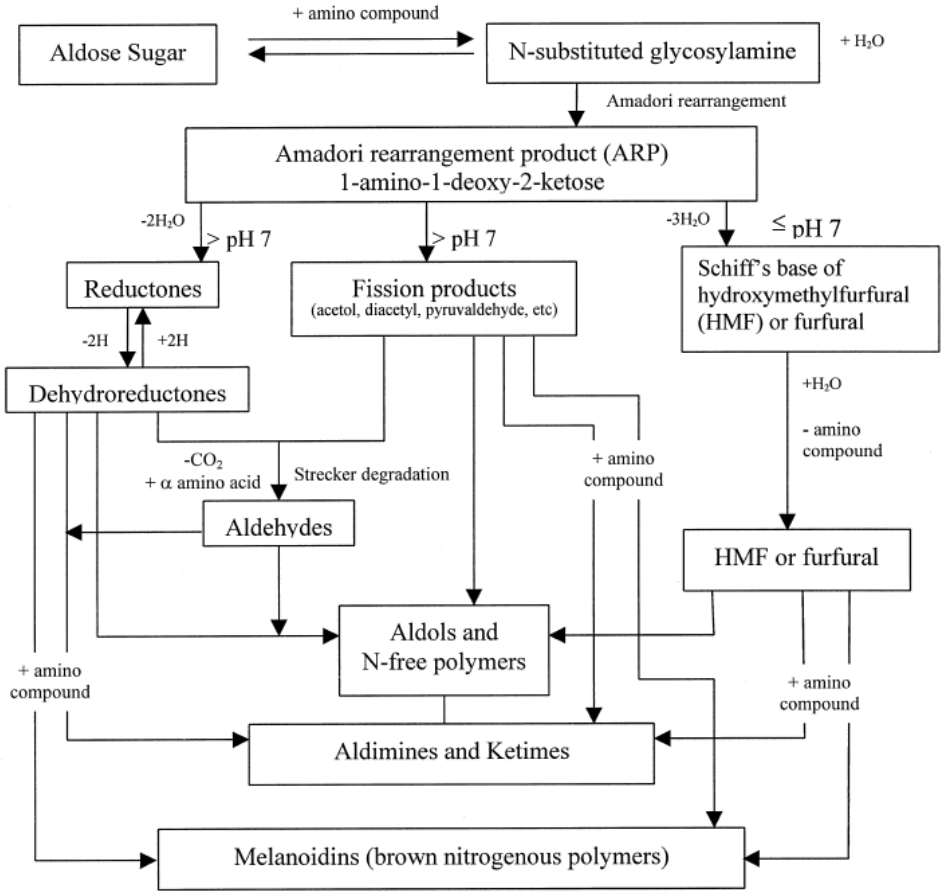


Figure 5.7 Maillard reaction scheme extracted from Martins *et al.* with permission.[302]

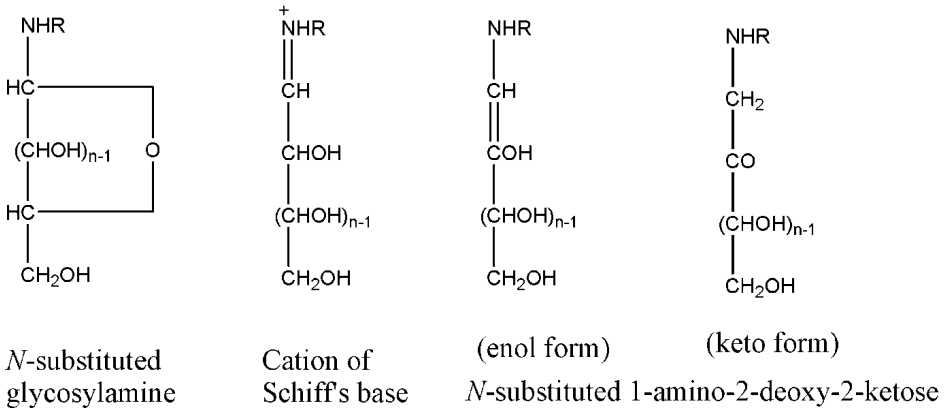


Figure 5.8 Amadori rearrangement to produce 1-amino-2-deoxy-2-ketose. Reproduced from Nursten.[297]

Maillard reactions produce water, hence a high water activity will decrease the reaction, and often water must be removed to shift the equilibrium.[306] However, mobility of compounds is reduced if the matrix is too concentrated. The reactions reach a maximum at a water activity of 0.6 to 0.7, which is the water activity of

honey. Water content influences the browning rate of DHA and amino acids.[307-308] A water content of 11% has been reported to give a maximum reaction,[309] which is close to the water content of some honeys, but other honeys may have a water content which is too high (13.4-22.9% as reported by Sanford[155]) hence the equilibrium would favour the left.

DHA is used as the active ingredient in sunless tanners due to its ability to bind to the amino terminal of skin surface proteins to give a melanoid and tanned appearance to the skin.[310-311] Hence there are many articles published on the reaction of DHA with amino acids carried out in conditions similar to those found on skin. DHA condenses with the free unprotonated amino group (Figure 5.9) to form an *N*-substituted glycosyl amine; dehydration gives a Schiff base, then rearrangement produces a Heynes product which is colourless (Figure 5.10). A series of condensations with amino acids, dehydrations and rearrangements produces the coloured melanoidin compounds.[312]

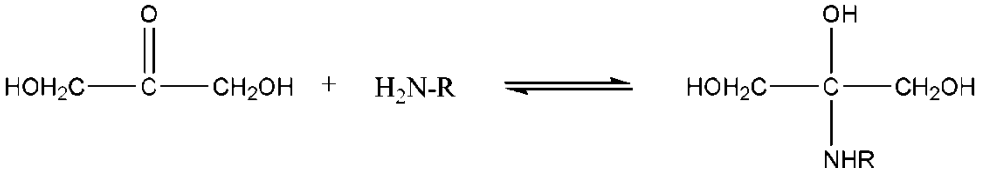


Figure 5.9 Initial step in Maillard reaction between DHA and amine group to produce *N*-substituted glycosyl amine. Reproduced from Carnali *et al.*.[312]

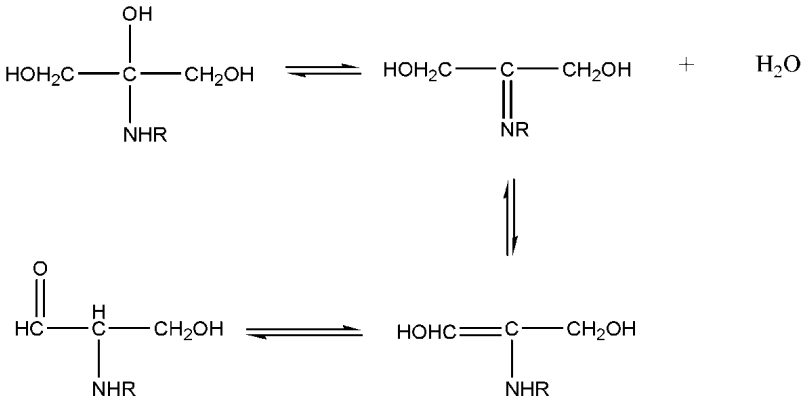


Figure 5.10 Further reaction of DHA to form a Heynes product. Reproduced from Carnali *et al.*.[312]

Reactions on the skin are visible within an hour, but take from 24 to 72 hours to reach maximum colour.[313-314] The developed colour depends on the amino acid

and ranges from yellow to brown.[314] In addition, higher concentrations of DHA give darker results.[313] Generally ketones react more slowly with amines than aldehydes do and consequently higher temperatures and longer reaction times are required.[306] MGO is more reactive than glyceraldehyde in Maillard reactions, due to the greater reactivity of the aldehyde carbonyl.[315]

Many studies have been carried out on DHA and amino acid reactions. Glycine and lysine were reported to produced significant pigmentation compared to alanine, serine and arginine when reacted with DHA (37 °C, 24 hours). However, the author[316] acknowledged that alanine and serine were not completely soluble in the media used. At 100 °C (4 hours) the reaction occurred more rapidly but the same products were formed. Meybeck[316] suggested that DHA initially condenses with free amino acids at the skin's surface which is followed by polymerisation and linking to proteins in the stratum corneum, probably through lysine side chains. A study in phosphate buffer (pH 7) at 32 °C showed that lysine reacted with DHA as did methionine sulfoxide, but cystine and cysteine did not react well with DHA.[311] Reactions carried out at room temperature between DHA and amino acids buffered at pH 5 and 7 showed that lysine, glycine and histidine reacted with DHA most rapidly at pH 7.[309]

There is little literature on the main cause of darkening in honey over time. Fructose has been proposed as an important component in the browning of honey, whereas it was suggested that amino acid-sugar condensation only had a secondary effect.[270] Gonzalez (1999)[270] proposed that honey changes colour during storage due to Maillard reactions at 37 °C. The authors reported a 10-30 day induction period where no browning occurred, followed by an increase in colour over time. This was assigned a pseudo zero-order rate. Non-mānuka honeys were tested, so there was no effect from reactions of DHA or MGO on the colour. Reactions of the DHA and MGO with amines can produce coloured products which may have a significant influence on the dark colour of mānuka honey. However, there have been no studies on this topic.

DHA was noted to be an important compound in the Maillard reaction in 1963 when Wiseblatt and Zoumut[317] boiled aqueous solutions of DHA and amino acids (17 in total, 1:1 mol) at pH 7 for 15 minutes. DHA and proline (equimolar)

produced a much stronger cracker/ bread-crust aroma than any other amino acid. The same aroma was produced with glyceraldehyde, but occurred more slowly. When the compound was isolated and boiled at pH 3 it lost its aroma, but it returned when the solution was neutralised, whereas in a basic medium there was no affect on the aroma. In contrast, when MGO was heated with proline an over powering burnt-sugar smell with rapid browning was reported. Reactions of DHA with alanine produced a weak caramel aroma, while a baked potato aroma was given from the reaction with glycine. Compounds prepared by the reaction of DHA with valine and phenylalanine have been patented as a flavour enhancer[318] and a honey-like flavourant[319] respectively.

The compound responsible for the bread-crust aroma was of great interest and various papers have been published on the subject. In 1969, Hunter *et al.*[320] isolated the compound responsible and reported it to be 6-acetyl-1,2,3,4-tetrahydropyridine (ATHP). ATHP is an unstable molecule that undergoes chemical and physical changes, including colour, aroma and solubility, when exposed to air. They reported that only 1 part in 100 000 gives an odour whereas, in 2004, Adams *et al.*[321] reported that the odour threshold is 1 ppb in water.

Figure 5.11 shows the condensations of DHA and proline to give ATHP, which exists in two stable tautomeric forms; Figure 5.12 shows side products produced.[321] The reaction was carried out at temperatures between 100 and 200 °C; increased temperature had no effect on the yield of ATHP but increased the yield of side reactions. Hunter *et al.*[320] also noted side products from a reaction of proline and MGO were coloured with unpleasant odour.

ATHP and other Maillard flavour compounds have been extensively reviewed by Adams and De Kimpe (2006).[322] The review discusses the sources of the compounds and their mechanisms of formation.

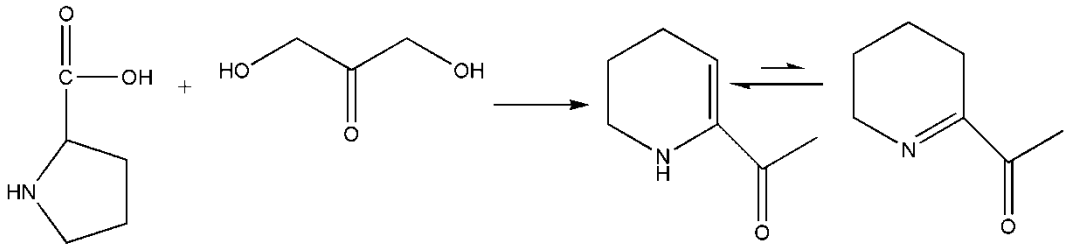


Figure 5.11 Reaction of DHA and proline to form 6-acetyl-1,2,3,4-tetrahydropyridine (ATHP). Reproduced from Adams *et al.*[321]

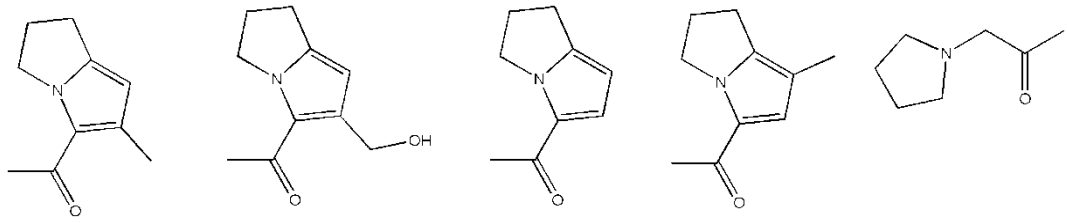


Figure 5.12 Other products of the condensation of proline and DHA. Reproduced from Adams *et al.*.[322]

Polymeric species can be formed from products of the final stage of the Maillard reaction. At low temperature, aldehydes and amines react readily to produce polymeric high molecular mass coloured products.[297] The reaction of MGO with glycine gives high enolic hydroxyl and low ether content products. Further condensations produce higher molecular weight compounds.

MGO is often referred to as an important intermediate, indicating that it readily participates in further reactions. MGO has two carbonyls and an H group on the methyl. It can easily condense with itself or other compounds and form low and high molecular weight colour compounds.[118] α-Oxoaldehydes, such as MGO, are 20,000-fold more reactive than glucose in glycation processes. Therefore they bypass the requirement for a fructosamine precursor in the formation of advanced glycation end products (AGEs).[323] The reader is directed to a review by Singh *et al.*[324] which includes the chemistry of AGE formation.

Under physiological conditions, AGEs form relatively slowly; hence, they are prominent in long-lived proteins, such as tissue collagens. Biologically active

proteins and enzymes are deactivated through inter- and intra- molecular crosslinks causing formation of AGEs. MGO has been reported to modify MRJP1, a dominant protein in honey, as well as defensin1, a peptide with antibacterial properties.[71] Furthermore MGO also cross-links with the enzyme glucose oxidase, *in vitro*, inhibiting it, as determined by electrophoresis gels.[57]

Lo *et al.*[113] examined the binding of MGO to amino acids under physiological conditions. They reported that MGO reacts with *N*-α-acetylarginine to reversibly form glycosylamine and 4,5-dihydroxy-5-methylimidazolidine derivatives and over time it irreversibly converted to an imidazolone, *N*-α-acetyl-*Nδ*-(5-methyl-4-imidazolon-2-yl)ornithine. Figure 5.13 shows the reaction of MGO with arginine, lysine and cysteine residues, as described by Lo *et al.* The reaction is considered to proceed via reaction with the unhydrated form of MGO. This is applicable to honey due to its dehydrating environment; however, the pH is different. The apparent rate constant for the formation of imidazolone at pH 7.0 and 37 ºC was reported as 2.07×10^{-4} $mmol^{-1}$ day^{-1}.

MGO reacts reversibly with cysteine residues, forming hemithioacetal adducts, and with lysine and arginine residues, forming glycosylamine residues. In addition, MGO reacts irreversibly with lysine residues, forming N_{ε}-(1-carboxyethyl)lysine (CEL) and 1,3-di(N_{ε}-lysino)-4-methyl-imidazolium (MOLD), and with arginine, forming N_{δ}-(4-carboxy-4,6-dimethyl-5,6-di-hydroxy-1,4,5,6-tetra-hydropyrimidine-2-yl)ornithine (THP) and argpyrimidine.[150] Pentosidine is an AGE which can be synthesised *in vitro* by incubating a mixture of arginine, lysine and a reducing sugar, such as glucose.[296] Reports also show that it can be formed by incubating short chain carbohydrates, such as MGO and glyoxal, with a mixture of N_{α}-acetylarginine and N_{α}-acetlysine.[325] Furthermore, MGO reacts with amino-containing lipids to form lipoxidation end products (ALEs) and with nucleic acids. These reactions change the chemical and biochemical properties of the compounds.

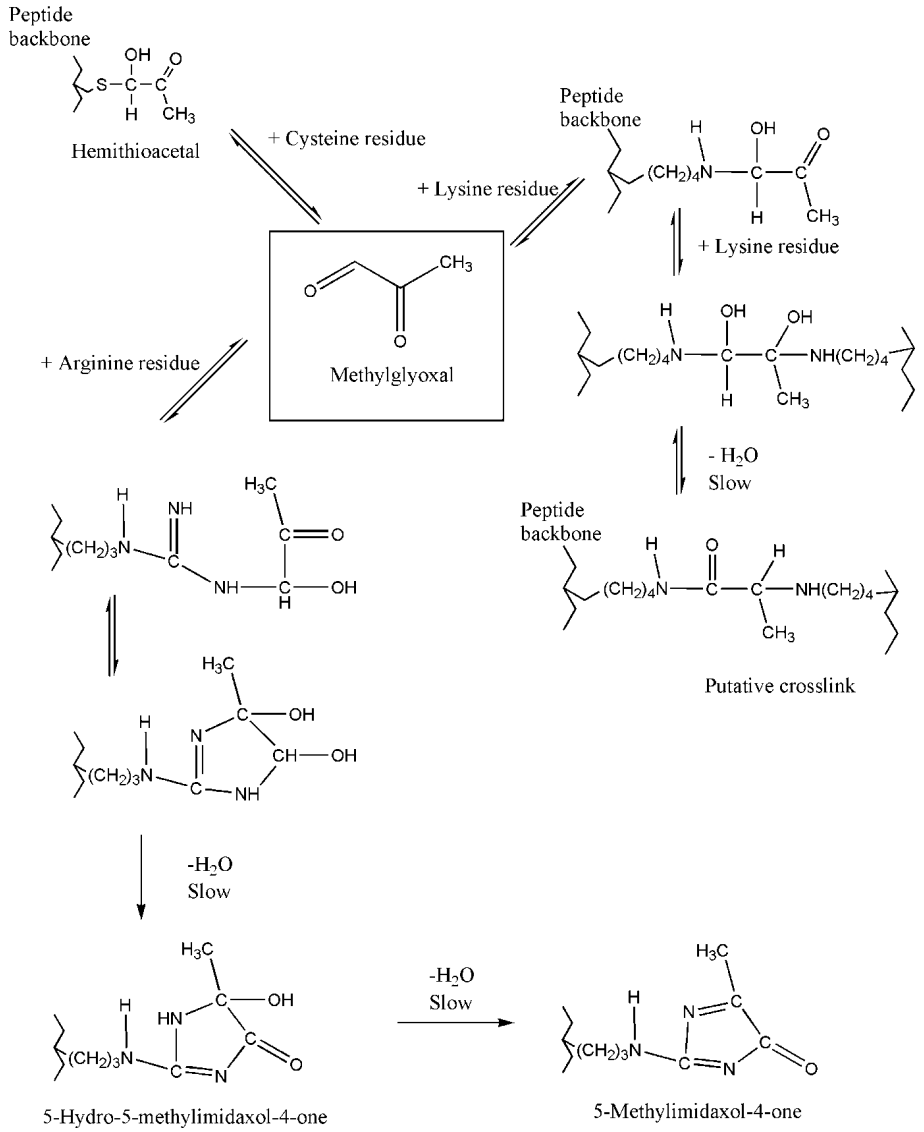


Figure 5.13 Reactions of MGO with arginine, lysine and cysteine residues, reproduced from Lo *et al.*.[113]

5.1.4.2 Caramelisation

Caramelisation belongs to the group of browning reactions. It commonly occurs when food is baked, roasted, heated or cooked, but can also occur during prolonged storage in food with a high sugar content. Caramelisation can occur not only from sugars but other compounds, including α-dicarbonyl compounds. In contrast to the Maillard reaction, caramelisation occurs without amino acids. The reaction occurs at high temperatures without the need of a catalyst. For example, fructose will caramelise at 110 °C and both glucose and sucrose will caramelise at 180 °C.[326] Catalysts are required when the reaction is carried out at lower

temperatures. Catalysts include carboxylic acids and their salts, phosphates and metallic ions and reactions can occur under both acidic and basic conditions. Even with the catalysts, the reactions are much slower than the Maillard reaction.[297] It is noted that caramels can be made with the use of reagents or catalysts (such as ammonia for use in cola[327]); however, these have been specifically excluded from this section as they are not relevant to the reactions that will occur in honey.

The initial steps in caramelisation are the opening of the hemiacetal ring followed by 1,2 enolisation by the Lobry de Bruyn-Alberda van Ekenstein transformation (compared to an Amadori rearrangement in the Maillard reaction) to produce isomeric carbohydrates. Dehydration reactions occur and are favoured in an acidic medium with only low levels of isomeric carbohydrates formed. Volatile and non-volatile low and high molecular weight caramelisation products are formed depending on the temperature, pH, time of heating and the matrix of the sample. Most literature describes caramelisation experiments at high temperatures, but it can also be an important reaction in samples at lower temperatures under appropriate conditions. The reader is directed to two reviews and the references within for further discussion on caramelisation.[327-328]

5.1.4.3 Dimerisation and polymerisation

Dimerisation of α-hydroxy aldehydes and ketones has been known since the late 1890s. In 1937, Bell and Baughan[329] stated that the dimeric form of DHA is due to semi-acetal (sic.)[*] formation between the two molecules. They investigated the depolymerisation of DHA in aqueous solution and reported that the reaction is first-order and catalysed by both acids and bases.

Many articles in the literature mention that DHA exists as a dimer (cyclic hemi-ketal) in the solid state, but dissociates into monomeric form upon dissolution or heating. In water this dissociation occurs relatively fast, but is much slower in dimethyl sulfoxide (DMSO). Davis[330] reported that in DMSO only 50% of the dimer had converted to the monomer after 64 hours. The conversion was first-order with a rate constant of 0.034 min^{-1} which corresponds to a half life of 20.4 minutes. In contrast, a separate study reported that glyceraldehyde converted to

[*] The dimeric form of DHA is a hemi-ketal, similar to cyclic fructose.

the monomer in 1-3 hours in DMSO. Yaylayan *et al.*[331] reported that the rate of dimer to monomer conversion depended on the solvent and temperature. In dimethyl sulfoxide and dioxane at ambient temperature the conversion is very slow. DHA is able to dissociate more easily than glyceraldehyde under the same conditions. This is due to the instability of the hemiketal bond compared to the hemiacetal bond and the extensive hydration of the aldehyde groups relative to the ketone. In D_2O, the DHA dimer generates 45-fold more monomers than the glyceraldehyde dimer under similar conditions. Davis suggested that in solution, DHA dimer exists in trans-isomer of chair conformation with the hydroxymethyl groups in an equatorial position and the hemiketal OH groups axial. The proportion of DHA existing as the dimer and monomer in honey is unknown, but is more likely to be similar to DMSO than water. A separate study is currently being carried out to examine this.

Popoff *et al.* (1978)[332] studied reactions of DHA in aqueous solutions at 96 °C in pH 4.5. They reported twenty compounds from the ethyl acetate-soluble part of the mixture including reaction of 2 DHA or DHA with MGO. Amongst the products are a series of 6-carbon straight chain products, 6-carbon cyclic products – some with O in the ring, and products with two rings. The major seven products arise without carbon chain cleavage through dehydration and cyclisation reactions. They proposed mechanisms for some of the products (Figure 5.14) which included partial dehydration of DHA to MGO before condensation to dimers and trimers. They also suggested condensation of DHA as its enol form with MGO (aldol condensation) followed by cyclisation and dehydration through β-eliminations which leads to other compounds. Aldol condensation can occur in both base and acid with aldehydes and ketones. An enol or enolate reacts with a carbonyl compound to form a β-hydroxyketone or β-hydroxyaldehyde. March[306] noted that the reaction between two ketones is seldom attempted. The product of an aldol condensation is still an aldehyde or ketone, hence further condensation is possible.

Aldol condendation of MGO can produce dimers and trimers; further condensation leads to polymers, which are coloured, conjugated systems. Furthermore the dimers can react with MGO. Reactions occurring due to thermal treatment of MGO in a mild acidic matrix (pH 5, 100-130 °C) are shown in Figure

5.15).[333] After 300 minutes 20% of MGO was still present. At pH 8, different products were formed and the degradation was faster. When alanine was added there was not much effect at pH 5, but its presence influenced the products at pH 8.[333]

In water, MGO spontaneously converts to methylglyoxal monohydrate and methylglyoxal dihydrate over several hours; when dehydrated it exists as small polymeric structures. Studies show that the equilibria of the different forms of MGO are strongly affected by the solvent, temperature and amount of water available. Freeze drying of MGO in aqueous solutions causes it to polymerise into small polymeric structures. However, when it is re-exposed to water, the polymers are converted into monomeric mono- and di-hydrate forms of MGO.[334]

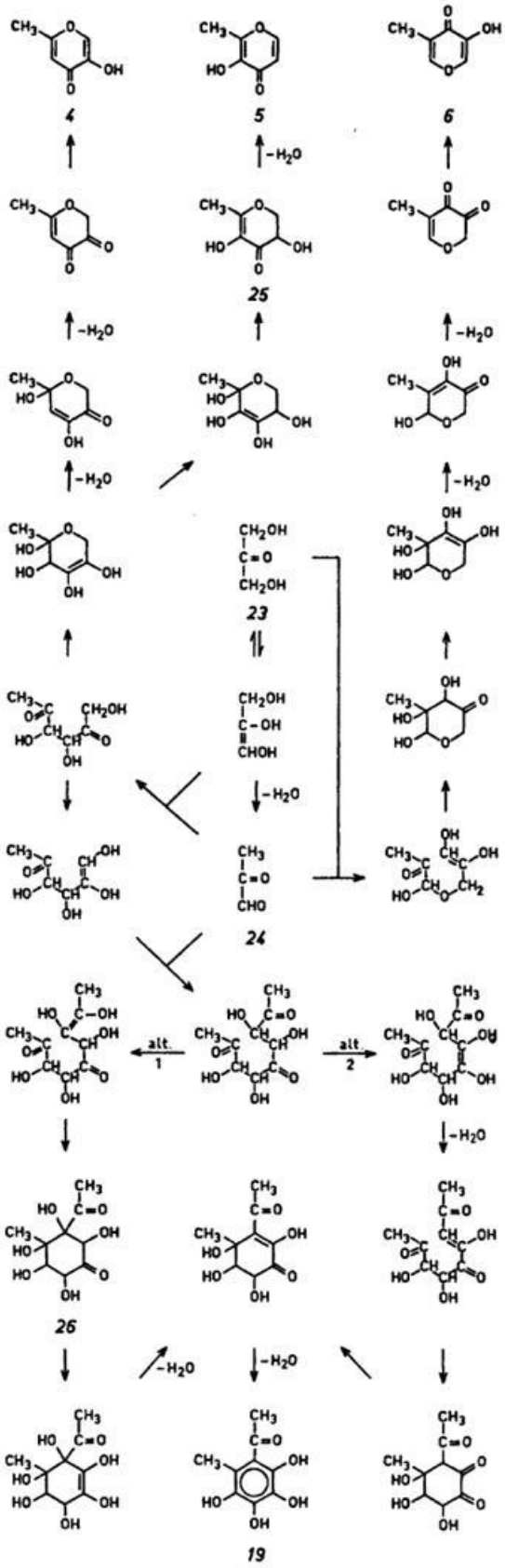


Figure 5.14 Proposed pathways for reactions of DHA (23) and MGO (24) in acidic conditions. Extracted from Popoff *et al.*[332]

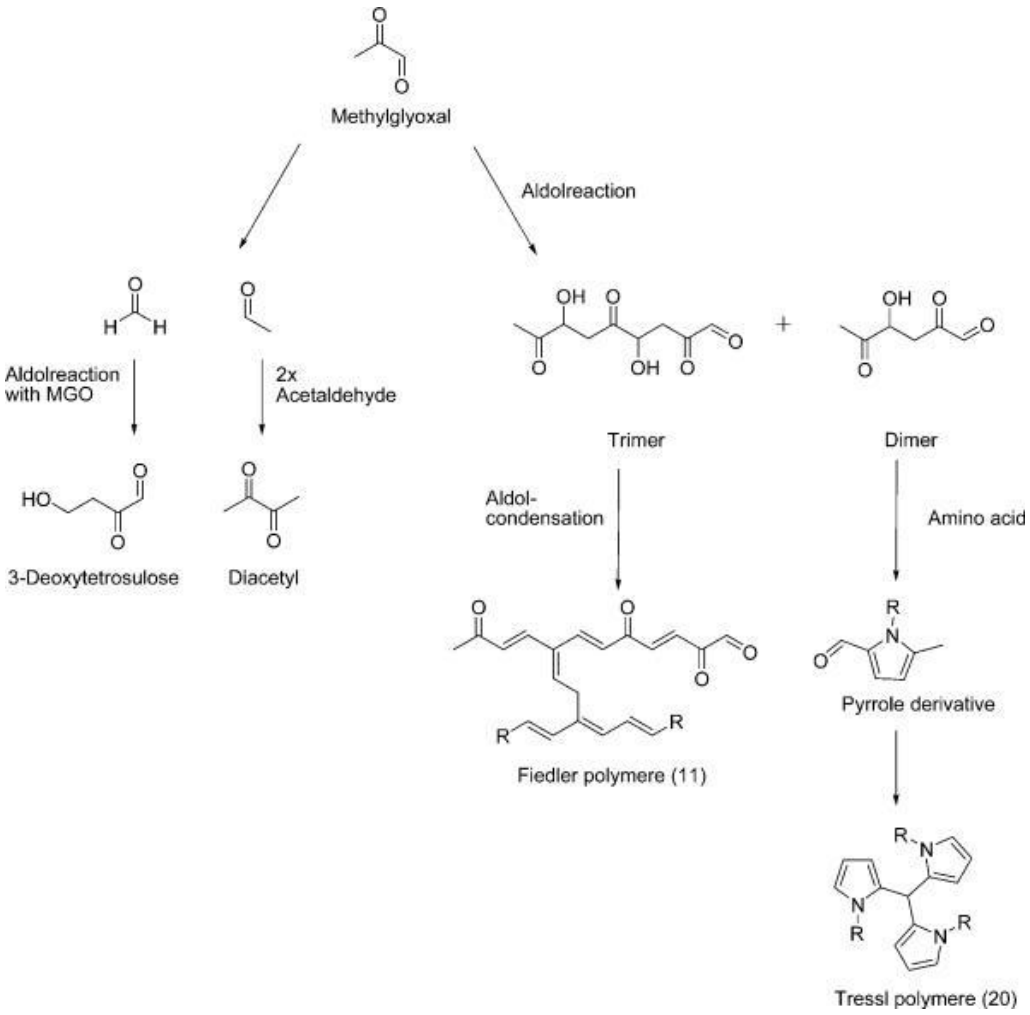


Figure 5.15 Degradation pathways of MGO (100 and 130 °C, pH 5). Short chain alpha-dicarbonyl compounds and high molecular weight melanoidins were produced. Reprinted with permission from Pfeifer *et al.*. Copyright 2013 American Chemical Society.[333]

5.1.4.4 Catalysis of DHA and MGO to form side products

Compounds present in honey may act as a catalyst in side reactions of DHA or MGO which will lower the efficiency of the dehydration reaction. During the current research, proline catalysis has been identified as a possible cause of the lack of 1:1 stoichiometry in honey.

Proline catalysis

Proline is able to act as both an acid and base catalyst due to the amino and carboxylic acid functionalities. While this is true of all amino acids, proline, a secondary amino acid, differs in reactivity compared to primary amino acids. It has Lewis-base-type catalysis that facilitates iminium- and enamine- based

transformations. Proline is able to form iminium ions and enamines with carbonyl compounds more readily than most other amines. In addition, proline also acts as a ligand in asymmetric transition metal catalysis. It is an effective organocatalyst of transformations such as aldol, Mannich and Michael reactions.[335] Figure 5.16 shows the four modes of proline catalysis. The reader is directed to the reviews by List[335] and Jarvo[336] and the references therein.

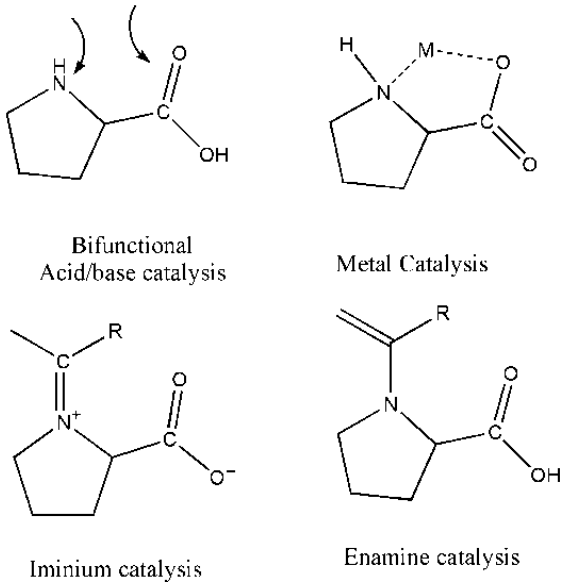


Figure 5.16 Modes of action in proline catalysis. Reproduced from List.[335]

Proline is used as a catalyst in the aldol reaction. It was initially shown to be unreactive with aldehydes. However, the intermolecular reaction between a ketone and aldehyde is possible if a large excess of ketone is present. This is the situation present in mānuka honey, where initially, a large excess of DHA (ketone) is present with a small amount of MGO (aldehyde). List reported acetone (20 vol.%, ca. 27 equiv.) reacts with isobutyraldehyde in DMSO to give the corresponding aldol (96%, Figure 5.17).

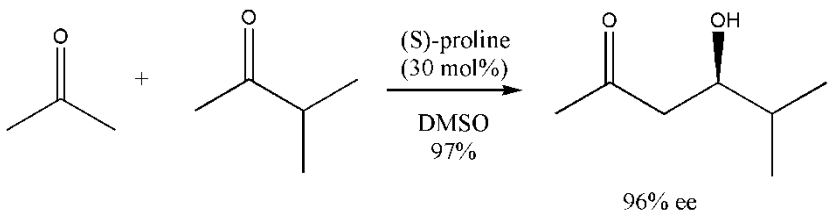


Figure 5.17 Reaction of acetone with isobutyraldehyde to give a very high proportion of the corresponding aldol. Reproduced from List.[335]

The three-component Mannich reaction (between a ketone, aldehyde and amine is also catalysed by proline. The product, a β-amino ketone, is produced in high yields (Figure 5.18).[335]

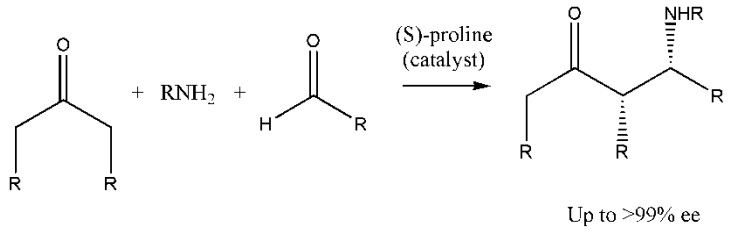


Figure 5.18 Mannich reaction between a ketone, aldehyde and amine, catalysed by proline as proposed by List.[335]

5.1.5 High pressure processing (HPP)

High pressure processing (HPP) is used as a way to preserve the quality of food by destroying and inactivating microorganisms. This is a non-thermal technique and the nutritional properties of the product are mostly unaffected.[337] In 2012, Al-Habsi and Niranjan[338] reported the use of HPP and temperature as a means of increasing the MGO content of mānuka honey. If this method worked, it would replace the need to store mānuka honey for long periods of time to increase the MGO content. Furthermore, high pressure can kill yeast spores and prevent honey from spoiling. However, while HPP is known for its effect on microbial cells and large molecules, it is not generally known for affecting small molecules such as DHA and MGO.

Al Habsi and Niranjan[338] subjected mānuka honey to pressures of 100-800 MPa for between 15 and 120 minutes. They reported that the MGO concentration increased, while HMF and diatase stayed the same. Higher temperatures caused a loss of MGO. However, the authors did not measure the DHA concentration at any time throughout the reaction; therefore it is unknown whether DHA was converting to MGO or whether MGO was liberated from a possibly reversible binding (e.g. sugar-bound MGO) caused by the increase in pressure. During the study, the authors noted that after 15 minutes at 800 MPa the MGO concentration levelled off. Further experiments at lower pressures did not show a levelling off at 120 minutes, indicating that the reaction did not go to completion.

Al Habsi and Niranjan[338] only analysed one commercial mānuka honey. This honey would have been stored before being sold so that the MGO level attained a maximum, and hence the majority of DHA would have already been converted to MGO. Therefore the rate of MGO formation from this honey may be very different from a freshly collected mānuka honey, which has a high DHA starting concentration.

Grainger *et al.*[120] attempted to reproduce the findings by Al Habsi and Niranjan.[338] Fresh mānuka honeys and clover and artificial honeys doped with DHA were examined. However, the authors reported no consistent trends in the decrease of DHA or increase in MGO or HMF for any of the samples at any combination of time (15, 45 or 90 minutes) and pressure (100, 400 or 600 MPa) examined. They concluded that HPP does not affect the conversion of DHA to MGO.

HPP has also been reported in the literature as a way to increase the concentration of other properties or compounds in mānuka honey, including antioxidant activity and total phenolic content. Fauzi *et al.*(2013)[339] studied the effect that HPP has on the quality of honey (antioxidant activity, colour and viscosity). Samples were subjected to different pressures (200-600 MPa) and held between 10-30 minutes. Various temperatures from ambient up to 70 °C were compared. The authors reported that samples subjected to 600 MPa for 10 minutes at ambient temperature had a 30% increase in the antioxidant activity and no colour change. Samples that were also thermally treated during HPP did not show any extra antioxidant activity. Furthermore the colour was significantly degraded when samples were processed at 70 °C for longer than 15 minutes.

Akhmazillah *et al.* (2013)[340] analysed the influence that HPP treatment (200, 400 and 600 MPa and 5, 10 and 15 minutes at ambient and moderate temperature) had on the total phenolic content of mānuka honey. The authors reported that honey subjected to 600 MPa for 10 minutes at ambient temperature showed the greatest increase in total phenolic content (47% increase compared to untreated honey). Heating (50-70 °C) while subjecting the honey to pressure did not show any significant different in the total phenolic content. They suggested the increase may be due to the pollen, but acknowledge that research needs to be carried out to

confirm this. The authors also suggested that the high pressure may cause conformational changes and denature the proteins which make the phenolics more available or some enzymes may be deactivated which could account for a higher antioxidant activity.

5.2 Experimental

HPLC with PFBHA derivatisation was used for analysis of DHA, MGO and HMF in the storage trials. Details on the method and the matrices can be found in chapter 2.

5.3 Results and discussion

5.3.1 Setting the scene – reaction order, state of DHA, initial and secondary reactions, ratio and efficiency of the reaction

This section addresses ideas that are used during analysis and interpretation of the storage trials of artificial honeys in this chapter, storage trials of real honey matrices (chapter 6) and also the formation of a prediction tool (chapter 8). Rather than repeating the explanation in each section in which it is required, an overview has been given here. Information on the state of DHA (monomer or dimer), reaction order for the conversion of DHA to MGO, initial and secondary reactions, ratio of DHA:MGO and efficiency of reaction are given here.

5.3.1.1 State of DHA in the honey matrix

The dehydrating environment of honey may influence the state in which compounds exist. DHA is reported to exist as a dimer in the solid state but converts to a monomer in aqueous and other solutions.[329-330, 341] Since honey is neither solid nor aqueous, it is difficult to estimate the amount of DHA that exists as the dimer and monomer at equilibrium. DHA tied up as the dimer is unable to react. Therefore the dissociation of the dimer to the monomeric form may be the rate determining step in the conversion of DHA to MGO and side reactions

The equilibrium constant between the active (monomeric DHA) and inactive forms (e.g. dimeric DHA) of DHAP has been investigated.[342] A change of the

solvent can alter the equilibria of free DHAP and inactive DHAP. Moreover, other inactive species of DHA may be formed depending on the solvent used.

If DHA predominately exists as the dimer in the honey matrix, it is expected that this would be reflected in the first-order reaction calculation. The concentration of DHA would depend on the square root of the equilibrium constant multiplied by the concentration of the dimer, as shown in equations 5-6 to 5-8.

$$K = \frac{[DHA]^2}{[dimer]} \quad (5\text{-}6)$$

$$[DHA]^2 = K \times [dimer] \quad (5\text{-}7)$$

$$[DHA] = \sqrt{K \times [dimer]} \quad (5\text{-}8)$$

However, this is not seen in the data, which is likely to be due to the analytical technique used. Sample preparation for HPLC analysis requires samples to be in aqueous solution for derivatisation; therefore any dimer would be converted to the monomer during preparation and would be detected as DHA in the method. The derivatising agent may enhance this conversion because it is removing free DHA from the system (as the derivative) therefore the equilibrium will shift from the dimer towards the monomer.

An estimation of the concentration of dimer present in the system is unachievable; a dehydrating solvent, such as DMSO could potentially be used to detect the dimer. However, once the honey is put in this solvent, the equilibrium would lie well to the left and possibly detect all the DHA as a dimer. Work is currently underway in another project to investigate the equilibrium between monomeric and dimeric DHA.

5.3.1.2 Reaction order

DHA

The literature reports that the conversion of DHA to MGO is first-order in DHA in aqueous solution.[283-284] Due to the uniqueness of the honey matrix, the

disappearance of DHA and appearance of MGO were examined to confirm their reaction orders in honey.

First- and second-order reactions are difficult to distinguish using the integrated formulae unless the reaction has been carried to at least 80% conversion.* Completion of the reaction to this level in honey is a challenge to achieve due to side and secondary reactions that occur with the species of interest. In the case of the honey matrix, both DHA and MGO are involved in multiple pathways. A short explanation of why reactions should be 80% completed to assign a rate order can be found in Appendix C.

To determine the reaction order of DHA disappearance, first-order (ln[DHA] vs. t) and second-order (1/[DHA] vs. t) plots were created. A plot that describes a particular order properly will be linear when the data is fitted. Only samples that had achieved 80% or higher loss of DHA were examined for identifying the reaction order to avoid misinterpretation. All samples showed linearity for first-order plots, and significant deviation from linearity for second-order plots. An example is shown in Figure 5.19 (sample 946 with 10,000 mg/kg DHA starting concentration) which had lost 93% of the starting DHA concentration.

As mentioned, the rate constant for a first-order reaction is independent of the initial concentration. The storage trials only considered two different starting concentrations (2,000 and 10,000 mg/kg); the rate of disappearance for sample 946 with 10,000 mg/kg DHA was $k = 0.0087\ day^{-1}$ compared to $0.0084\ day^{-1}$ for the same honey matrix with 2,000 mg/kg DHA. The difference in the rate constants are within experimental error. If the reaction was second-order there would be a factor of 5 increase in the rate constant in the 10,000 mg/kg sample compared to the 2,000 mg/kg sample because the second-order reaction depends on the initial concentration.

* Three half lives can also be used as a measure of reaction completion.

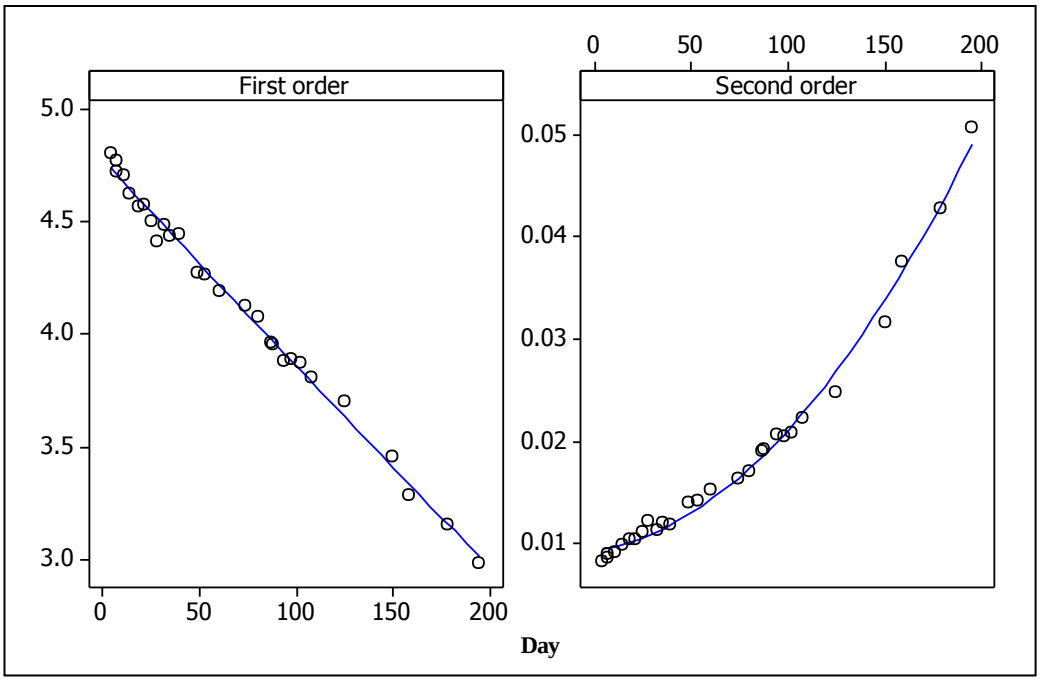


Figure 5.19 Mānuka honey 946 doped with DHA (10,000 mg/kg, 120 mmol/kg) and incubated at 37 °C. This sample had lost 93% of DHA. The first-order plot (ln[DHA] vs. time, left) shows linearity compared to the second-order plot (1/[DHA] vs. time, right) which shows deviation from linearity and is more suited to a quadratic fit.

An experiment was conducted with a clover honey sample that was divided into portions and doped with various levels of DHA (250, 500, 1,000, 2,000, 4,000 and 8,000 mg/kg). The results were also used to check the order of the reaction, although only 50% of the starting material had been removed. The samples were incubated at 37 °C and tested at day 0, 35 and 83; Only three data points were used to create the first-order plots, so there may be significant error associated with them, but most cases fitted a linear trend well. Furthermore, other storage trials were conducted with a higher density of data points and had a linear trend. The rate constant for DHA disappearance was not the same for all samples of differing concentration. There was an anomaly at the lowest concentration (250 mg/kg DHA); this sample had the fastest rate constant for DHA disappearance (0.0141 day^{-1}) whereas samples with higher DHA concentrations (500-8,000 mg/kg DHA) had similar rate constants (0.0084 ± 0.0004 day^{-1}). There appears to be a saturation effect occurring after 500 mg/kg DHA because the rate constant does not change significantly with increasing concentration. This may be due to some influence from an equilibrium of the DHA monomer and dimer at lower

concentrations. Another option is that the detection of DHA is not as reliable at this low concentration (although it is still above MDL).

Data supplied by C.J Adams* for clover honey doped with five starting concentrations of DHA (680, 1,500, 2,490, 6,190, 10, 400 mg/kg) and incubated at 37 °C was analysed. The samples had also only removed 50% of the starting DHA. The five samples had the same rate constant for DHA disappearance. However, they did not have a low concentration comparable to the 250 mg/kg DHA sample reported above. The agreement among the three sets of samples at concentrations higher than 500 mg/kg suggests that the disappearance of DHA in a honey matrix is first-order.

The disappearance of DHA in a honey matrix was treated as a first-order reaction and calculated as follows for all data:

$$\frac{d[DHA]}{dt} = -k[DHA] \quad (5\text{-}9)$$

The equation was integrated to give:

$$\ln[DHA] = -kt + \ln[DHA]_0 \quad (5\text{-}10)$$

A plot of ln [DHA] vs. time gave a slope equal to $-$k, where k is the first-order rate constant for disappearance of DHA and the intercept is equal to $[DHA]_0$.

MGO

As previously mentioned, DHA may react in multiple pathways, so not all of the DHA will form MGO. The rate treatment of MGO needs to reflect this. Equations 5-11 to 5-14 were used to interpret the rate data for conversion of DHA to MGO and other products. The goal is to calculate the rate of MGO appearance in the honey.

$$DHA \rightarrow xMGO + (1-x)other\ products \quad (5\text{-}11)$$

* This data was background material to the Adams *et al.*[14] (2009) article.

The following equation (5-12) was used to describe the rate of MGO production.

$$\frac{d[MGO]}{dt} = k'[DHA] \quad (5\text{-}12)$$

where

k' = rate constant for appearance of MGO

k ≠ k' because the conversion is not stoichiometric

The integrated rate equation for the disappearance of DHA is rearranged to:

$$[DHA] = [DHA]_0 e^{-kt} \quad (5\text{-}13)$$

Equation (5-13) is substituted into equation (5-12) and the result integrated to give:

$$k\frac{[MGO]}{[DHA]_0} = k'[1 - e^{-kt}] \quad (5\text{-}14)$$

A plot of $k\frac{[MGO]}{[DHA]_0}$ vs. $[1 - e^{-kt}]$ gives slope k', which is the rate constant for the appearance of MGO.

As established above, the dehydration of DHA to MGO is treated as a first-order reaction, which means that irrespective of the initial concentration the rate of DHA disappearance will be the same. The average rate constant for MGO appearance in the clover samples was 0.0033 ± 0.0001 day^{-1}. Despite the larger rate constant for DHA disappearance for the sample that had 250 mg/kg DHA, there was no difference in the rate constant of MGO appearance.

5.3.1.3 Initial and secondary reactions in the presence of perturbing species

DHA

At the beginning of analysis of some systems, perturbants either reacted rapidly with DHA in an irreversible manner or catalysed the removal of DHA in a side reaction. This was followed by a slower loss of DHA, indicated by a break in the [DHA] vs. t plot. These regions were assigned as initial (k_1) and secondary (k_2) reactions and rate constants were calculated for both reactions. In some cases the initial DHA lost was equivalent on a molar basis to the perturbing species added and the initial reaction was initially thought to occur due to DHA binding to/ reacting in an unspecified manner with the perturbant. However, a more plausible

explanation is a limited amount of monomeric DHA is available for reaction. In the artificial honey there are limited perturbants to dissociate dimeric DHA, hence this would be the rate limiting step for all further reactions. Real honeys have multiple compounds which could dissociate dimeric DHA, hence two rates are not observed. The secondary reaction was used for comparison with other artificial honey samples and real honey samples.

MGO

MGO formation is dependent on the conversion of dimeric DHA to monomeric DHA, therefore, an initial fast reaction was not seen in MGO formation comparable to that seen in the disappearance of DHA. MGO rate constants were calculated corresponding to the regions of k_1 and k_2 for DHA disappearance and were labelled k'_1 and k'_2 respectively.

At later sampling times, the formation of MGO began to level out and decline. This may occur due to side reactions of MGO becoming more prominent at later times because the concentration of DHA is lower. This is discussed in more detail in chapter 6.

5.3.1.4 Error in the rate constants

An error estimate is required for the rate constants. All samples were carried out in duplicate, which does not give enough data points to calculate a reliable standard deviation. In this case, the standard deviation is the square of the difference between the two points. Work carried out with clover doped at various concentrations was used to assess the error in the rate constants for DHA and MGO. This was also compared with data supplied by C.J. Adams.[*]

Clover honeys doped with various levels of DHA (500, 1,000, 2,000, 4,000 and 8,000 mg/kg) and stored at 37 °C in the current research had an average rate constant for DHA loss of 0.0084 ± 0.0004 day^{-1}; an error of 5%. Furthermore data supplied by C.J Adams contained five experiments of clover honey doped with different starting concentrations and stored at 37 °C. The average and standard deviation of the rate constant for DHA disappearance was 0.0041 ± 0.0003 day^{-1};

[*] This data was background material to the Adams *et al.*[14] (2009) article.

a 7% error. From these results, we can assume that the error is in the fourth decimal place. Consequently all rates that have been calculated here are to four decimal places. These results will have error in the last decimal place. Rate constants for DHA loss can be classified as the same if they fall within ± 0.0004 day^{-1} of each other.

The clover honeys had an average rate constant for MGO gain of 0.0034 ± 0.0002 day^{-1}; an error of 6%. The samples supplied by C.J Adams had a rate constant for MGO appearance of 0.0017 ± 0.0001 day^{-1}, also a 6% error. From these results, we can assume that the error for the rate constant for formation of MGO is in the fourth decimal place. Consequently all rates that have been calculated here are to four decimal places. These results will have error in the last decimal place. Rate constants for MGO gain can be classified as the same if they fall within ± 0.0002 day^{-1} of each other. The error in the rate constant of MGO is half the error reported for the rate constant for the disappearance of DHA. This arises because the analysis of MGO concentration does not have as much scatter in the data compared to the analysis of the DHA concentration.

Samples stored at 20 and 27 °C were carried out in duplicate; no experiment was carried out that analysed a single honey with various DHA starting concentrations at these lower temperatures, hence an error calculation could not be carried out. There is more scatter in the disappearance of DHA in samples stored at the lower temperatures compared to samples stored at 37 °C. However, this random scatter is expected to be minimised when the slope is calculated, hence it is assumed that the error in the rate constants for loss of DHA and gain of MGO will be the same as for the samples stored at 37 °C.

5.3.1.5 Efficiency of the conversion

Theoretically, one DHA should convert to one MGO. However, the honey matrix is complex and there are side reactions of DHA and MGO occurring, so that the conversion is not 1:1. Therefore the efficiency of the conversion of DHA to MGO was monitored. Efficiency of the reaction was expressed as the percentage of DHA that converted to and was analysed as MGO at a given time (i.e. $\frac{\text{MGO gained}}{\text{DHA lost}} \times 100$). This is an empirical way to record the efficiency at any point

in time and can show the change of efficiency in the reaction over time. It must be emphasised that MGO reacts further so the efficiency is not how much DHA is converted to MGO, but the overall efficiency of the reaction. The average percentage efficiency was taken for the region where MGO was increasing and was compared to the stoichiometric factor (see below). For some samples, the efficiency of the reaction fluctuated over time; measurement error in the analysis of the concentration of DHA and MGO on HPLC and initial reversible reactions of both DHA and MGO are likely to account for the majority of this error. In the initial stages of the reaction while the system equilibrated, an average was not reported due to excessive fluctuation.

A stoichiometric factor, x, was also used to report the efficiency of the reaction. This was calculated as $x = \frac{k_n'}{k_n}$ (where n = 1 or 2, for initial or secondary reaction respectively). In comparison to the above calculation, the stoichiometric factor only takes into account the linear portion of the graph and reports the proportion of DHA that has converted to MGO, not side reactions or the loss of MGO at later times. This value will be less than 1.

It is expected that the calculated percentage efficiency and the stoichiometric factor will give similar results in the region where MGO is increasing (i.e. before it is consumed at later times). Samples that consumed a lot of DHA in the initial reaction showed a large difference between the stoichiometric factor and percentage efficiency for the secondary reaction if the concentration of DHA at time 0 was used. Hence the efficiency was calculated using the concentration of DHA at the start of the secondary reaction. This was closer to the stoichiometric factor.

5.3.1.6 Ratio of DHA:MGO

Freshly harvested honey has a large DHA:MGO ratio, which becomes smaller over time. The ratio is a way to monitor the progress of the reaction. In some artificial honey systems MGO reached a maximum then began to decline. This occurred when the ratio was low; hence side reactions of MGO became prominent. This helped to identify perturbants that were reacting with MGO and have been discussed in each system in section 5.3.5. This is analogous to real honey matrices,

where the MGO concentration decreases if the honey is stored for an extended period of time and further information on the ratio is discussed in section 6.1.2.

5.3.2 Overview for storage trials of artificial honey

This section discusses the artificial honey matrix, rationale for the choice of potentially catalytic compounds and conditions of storage for the storage trials that were carried out. Investigation of the conversion of DHA to MGO in an artificial honey matrix (model systems) which can be approximated by the following groups:

- Artificial honey doped with 10,000 mg/kg DHA at 37 °C
- Artificial honey doped with 2,000 mg/kg DHA at 37, 27, 20 and 4 °C
- Artificial honey doped with 2,000 mg/kg MGO at 37 °C

Perturbants added to artificial honey were proline, alanine, lysine, serine, iron and potassium phosphate. A summary of the starting concentrations of DHA/MGO and perturbants added to the artificial honeys can be found in Appendices D and E. The following sections detail the effect of perturbants on the conversion of DHA to MGO at the various temperatures. Analysis of rate constants, loss of DHA and gain of MGO are reported as the average of duplicate samples.

5.3.2.1 Artificial honey matrix

The honey matrix is a very complicated system with hundreds, if not thousands, of reactions occurring. A single compound may be involved in multiple reactions. Therefore to be able to study the conversion of DHA to MGO the system needs to be simplified. Model systems containing only a subset of the species present in the real system are a useful way to investigate reactions in complex systems such as honey. An artificial honey matrix allows the reaction to take place under simplified conditions, preventing too many reactions occurring at once; the compounds present are known and their initial concentrations can be controlled. A 'building block' approach was adopted in order to gain an understanding of the conversion of DHA to MGO, influence of other species, and the effect of temperature on individual reactions. Initially single perturbants were added to the matrix followed by systems with two or more perturbants. Artificial honey was chosen as a matrix because it allows control over the composition of the system

and is similar to real honey. The basic artificial honey matrix consisted of fructose (41.60%), glucose (37.30%), sucrose (2.91%) and water (18.20%). The water was used to dissolve DHA (or MGO) and perturbants. The pH of the resulting solution was corrected to 3.8-4.2 using gluconic acid (or in the case of MGO, sodium citrate[*]) before adding it to the matrix. This ensured the pH was regulated and was the same for all samples. All reactions were carried out in duplicate. Measurements were initially taken daily and then at diminishing frequency; aliquots were removed from the container and analysed for DHA, MGO and HMF by HPLC with PFBHA derivatisation as described in chapter 3.

Perturbants were added to the matrix to assess their catalytic ability. A catalyst is defined as a compound that enhances the rate of a reaction without being consumed or undergoing any permanent change. Acid catalysis is applicable to this research because honey has a pH around 4. There are two types of acid catalysis; general acid catalysis is where a proton is transferred to the reactant during the slow step of the reaction and specific acid catalysis is when the proton is fully transferred before the slow step of the reaction. Nucleophilic catalysis (also known as covalent catalysis) is where the catalyst acts as a nucleophile and forms a covalent bond with the reactants. These three types of catalysis may be occurring in the honey matrix.

Due to the honey matrix being such a complex system, a compound that may act as a catalyst in one reaction may be consumed in another reaction. There are different scenarios that may occur with a perturbant in the honey system:

1. Rate enhancement of the conversion of DHA to MGO as a catalyst.
2. Consumption of perturbant in a separate unrelated pathway.
3. Reaction of perturbant with DHA or MGO to form a different product.
4. Catalysis of DHA or MGO side reaction

All four reactions may occur alone or concurrently. Therefore even though the compound may act as a catalyst to enhance the conversion of DHA to MGO, it may also react in other pathways and be consumed. Hence, while it is a catalytic species for the reaction of interest it is not otherwise inert in the honey environment. Therefore within the entire honey matrix it is not correct to use the

[*] MGO in water had a pH around 2 before pH adjustment, due to a pyruvic acid impurity.

term catalyst without mentioning the process it catalyses. This research has used either the term catalyst or rate enhancement when referring to a species which increases the rate of the conversion of DHA to MGO because this is the reaction of interest and side reactions of a catalyst are typically not of importance to this study (except when they remove the catalyst or reactants or products from the system).

5.3.2.2 Starting concentration of DHA

The current research used Adams *et al.* (2009)[14] study on real honeys as a starting point for the investigation of the conversion of DHA to MGO. The latter study used exceptionally high levels of DHA (up to 10,400 mg/kg), that do not naturally occur in honey. Initially, a similar starting concentration was used for this work. However, the reaction may behave differently with a large amount of DHA due to the presence of side reactions and the amount of monomeric DHA available for reaction. Furthermore, some reactions may be swamped from the large concentration of DHA and low concentration of potential catalyst. Subsequent trials used a starting concentration of 2,000 mg/kg DHA, which is closer to that seen in nature.[37] In addition, this allowed the samples to reach an end-point sooner and limited the use of derivatising agent in the analysis.

5.3.2.3 Temperature

Adams *et al.*[14] incubated samples at 37 °C. This is close to the hive temperature; brood nest temperatures in hives have been reported between 33 and 36 °C.[10, 343] The initial temperature chosen for this research was 37 °C. It is also chosen because it allowed the reaction to occur at a rate that was fast enough to see a change within the time period of this research. However, later in the research lower temperatures (4, 20 and 27 °C) were included; this allowed comparisons to be made between DHA and MGO behaviour at various temperatures. Furthermore, these temperatures encompass the range at which honey is likely to be stored in a commercial environment.

5.3.2.4 Perturbants

Adams *et al.*[14] doped clover honey with primary amino acids (arginine and lysine) and reported that they catalysed the conversion of DHA to MGO. However, addition of large concentrations of amino acids resulted in a loss of MGO. Amino acids (proline, alanine, lysine and serine, Figure 5.20) were the main focus of this current research, but iron and potassium phosphate were also used. Perturbant concentrations were either chosen to reflect the concentration found in honey, or in a few cases (aliphatic amines*, aliphatic amide and iron) were increased so that a dramatic effect was observed. An overview of each of the compounds used to perturb artificial honey during this research follows.

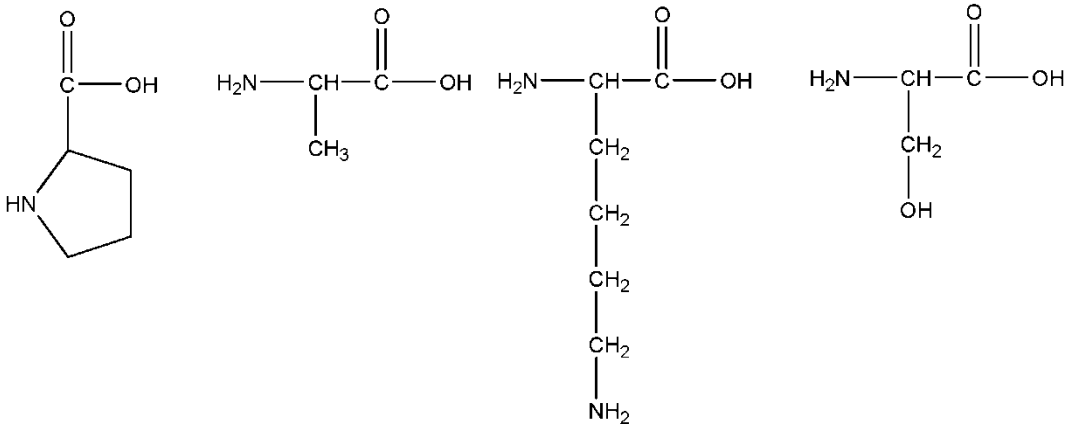


Figure 5.20 Amino acids examined for their potential catalytic role in the conversion of DHA to MGO. From left to right: proline, alanine, lysine and serine.

Primary and secondary aliphatic amines and amide (model compounds)

A primary and secondary amine were used to examine the behaviour of these functional groups without influence from the R group of amino acids. Proline, a secondary amino acid, is the most abundant amino acid in honey, comprising 50-85% of the total amino acids.[162] Therefore it is important to know if primary and secondary amino acids behave differently. Additionally, an amide was used to model the amide linkage in protein. Honey is approximately 0.3% protein; therefore amide functionalities are present (in low concentration) in honey and were examined to see if they influenced DHA conversion.

* A primary aliphatic amine, aliphatic secondary amine and aliphatic amide were added to artificial honey matrices; these compounds are not found in honey.

Propylamine was used to represent free primary amino acids and diethylamine was used to represent a secondary amino acid. *N*-methylacetamide was used to represent the amide functional group in a protein. These three compounds were chosen because they are of similar molecular weight (Figure 5.21).

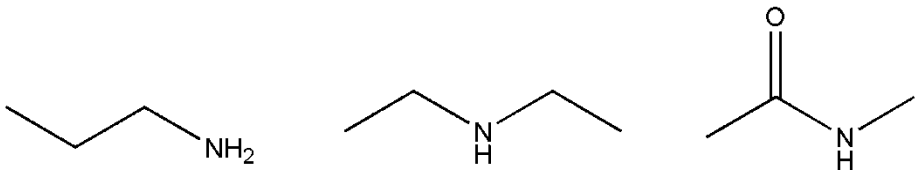


Figure 5.21 Propylamine (primary amine, left), diethylamine (secondary amine, center) and *N*-methylacetamide (amide, right).

The three experiments were conducted in artificial honey with a very large amount of DHA (10,000 mg/kg, 111 mmol/kg). The perturbing compounds were added in proportions similar to the concentration of amino acids found in real honey 800 mg/kg (14 mmol/kg) of primary amine, 1,000 mg/kg (14 mmol/kg) secondary amine, whereas the amide was added in higher concentration than protein is found in honey (1,000 mg/kg/ 14 mmol/kg amide), so that it had a similar perturbant concentration to the amines.

Proline

Proline, a secondary amino acid, was chosen as a perturbant because it is the most abundant amino acid in honey. Proline is widely known for its catalytic ability as an organocatalyst.[335-336] It has four main modes of action – bifunctional acid/base catalysis, metal complex catalysis, iminium catalysis and enamine catalysis[335] as discussed in section 5.1.4.4. Therefore proline has the potential to catalyse the conversion of DHA to MGO, but may also readily catalyse side reactions. The secondary amine and carboxylate group are reported as critical groups for catalysis. The pK_a of the NH_3^+ group is 10.60. Therefore in the honey environment it will be protonated, compared to the COOH group which will be deprotonated (pK_a = 1.99), hence proline will exist as a zwitterion.

Proline concentrations in honey are reported at varying levels (see chapter 4). This may be because as honey ages (regardless of floral origin) the proline content decreases. In mānuka honey, proline may decrease more quickly due to side

reactions with DHA or MGO. Moreover, the decrease in proline is accelerated at higher temperatures.[179, 186] While a higher temperature may speed up the conversion of DHA to MGO, it may also remove some of the DHA and/or MGO from the honey in reactions with proline or side reactions, which would lower the overall yield of MGO. A concentration of 800 mg/kg (7 mmol/kg) of proline was chosen for the storage trials because it is a naturally occurring level in honey. Two fresh mānuka honeys (946 and 953) analysed in chapter 4 had this level of proline. Czipa *et al.*[179] also reported this level of proline in mānuka honey. Other honeys tested in this current research had lower concentrations, as did other literature,[153, 180-182] whereas other non-mānuka honeys have been reported with higher concentrations.[184-185] Further information needs to be acquired on the proline concentration in mānuka honey.

Primary amino acids – alanine, lysine and serine

Amino acids can be used to determine the geographical origin of a honey.[182] Therefore it is difficult to know the individual concentration of amino acids in mānuka honey because there is currently no literature on amino acids in honey throughout New Zealand. A review of amino acids in other floral honey from other countries show widely varying concentrations; 105.5 mg/kg primary amino acids was found in fir honey, compared to 1,278.5 mg/kg in Lavender honey.[183] A concentration of 400 mg/kg (5 mmol/kg) was chosen as the concentration for the total primary amino acids in the storage trials. Mānuka honeys (n = 6) were analysed for free amino acids (section 4.3.7). They ranged from 83.0 to 275.2 mg/kg. Therefore the concentration chosen for the storage trials (400 mg/kg) is approximately 150% higher than that seen in the honeys analysed. However, this is a small sample set and until a large number of mānuka honeys are analysed for their free amino acid content it is unknown whether the choice of 400 mg/kg is high for all samples. As reported in chapter 4, the concentration of amino acids is similar to reported concentrations in other floral types. However, it cannot be ruled out that the concentration in mānuka honey is lower due to possible reaction with DHA or MGO. A high concentration of amino acid in the artificial honeys will give a more pronounced effect on the fate of DHA and MGO. Instead of creating a sample with multiple amino acids, one amino acid was used to represent all amino acids. This allowed the influence of individual amino acids to be examined.

Alanine, a primary amino acid, was initially chosen to represent all primary amino acids found in honey because it is one of the simplest amino acids (R = CH_3). It is also one of the top four most abundant free primary amino acids analysed in the mānuka honey samples (n = 6, chapter 4). Valine, isoleucine and leucine have similar R groups to alanine and are expected to behave in the same way. The R group of methionine is similar but contains sulfur; as discussed in chapter 4, it is the least abundant amino acid. Phenylalanine (the most abundant amino acids analysed in the mānuka honeys in chapter 4), tyrosine and tryptophan also have hydrophilic side groups, but include aromatic rings; they will not H bond to the amino group, but the size of them could cause steric hindrance. Furthermore, glycine also has a similar structure to alanine. In comparison to alanine, lysine has a large R group (R = $CH_2CH_2CH_2CH_2NH_3$) which contains a NH_3 group. If amines affect the conversion of DHA to MGO, lysine is expected to have more of an effect than alanine due to the extra amine group on its side chain, and is one of the top four most abundant free primary amino acids analysed in the six mānuka honeys in chapter 4. Arginine and histidine have similar R groups and are expected to behave in a similar manner. Serine has a polar, uncharged side chain (R = CH_2OH); threonine, asparagine and glutamine have similar R groups and are expected to behave in the same way. Therefore these three amino acids may give a good approximation of the effect of most amino acids. Aspartic acid and glutamic acid have COO- groups on the R group and may behave slightly differently.

In alanine, the pK_a of the NH_3^+ group is 9.69 and the pK_a of the COOH group is 2.34, suggesting that it exists as the zwitterion in the honey matrix. In lysine the R group has a pK_a of 10.53 and the NH_3^+ group has a pK_a of 8.95, so both groups will be protonated in the honey environment. Adams *et al.*[14] also used lysine and found that it enhanced the rate of conversion of DHA to MGO. The pK_a of the amine group in serine is 9.15, so it is expected to be protonated in the honey environment. Lysine and serine were examined at the same molar concentration used for alanine so that direct comparisons could be obtained.

Iron

A folklore exists that the addition of iron (oxidation state unspecified) to fresh mānuka honey accelerates the conversion of DHA to MGO. There is no science

behind this folklore nor any publications. There has been suggestion that iron use to leach out of the drums during storage. However, food grade lined drums are now required for storage of honey. Documentation is required for the lining from the drum supplier for each batch of new or reconditioned* drums.[344]

Iron commonly exists as Fe^{II} and Fe^{III}. Iron (II) sulphate is used medically for iron deficiency and to fortify foods[346-347] and is accessible for purchase for deliberate addition into honey. Iron (III) is found in rust (e.g. $Fe_2O_3.nH_2O$), so in earlier days it potentially could have enhanced the reaction if rusty drums where used.

Iron was found in low levels in the database of honeys analysed (< 2 mg/kg) so deliberate addition of $iron^{II}$ could be readily detected. In this study iron (II) sulphate was added in a high concentration (870 mg/kg, 3 mmol/kg) to see if there was any truth to the folklore.

Potassium phosphate

Potassium is the most abundant ion in honey (653-1,147 mg/kg in the honeys analysed in chapter 4). Literature[105, 287] shows that there is a co-operative catalytic effect on the conversion of glyceraldehyde to MGO when phosphate is added together with lysine. Potassium phosphate was added to an artificial honey matrix together with alanine.

5.3.3 Conversion of DHA (10,000 mg/kg) to MGO in artificial honey at 37 °C with amine and amide model compounds

Investigation of the way primary and secondary amines, as well as amides, react in a honey matrix with DHA and MGO was undertaken. This was carried out with aliphatic compounds that had primary and secondary amine groups and a compound with an amide group; the compounds used are not found in honey. The experiments were incubated at 37 °C.

DHA as starting compound

An extremely high DHA concentration (121 mmol/kg) was used for the initial starting concentration, but the added perturbants were not used in exaggerated

* Reconditioned drums have the old top cut off and a new lining, top and plastic bung are fitted.

amounts (see Appendix D for starting concentrations). Table 5.2 summarises the rate constants for the disappearance of DHA and appearance of MGO. A comparison of the samples with the control sample for the loss of DHA and gain of MGO can be seen in Figure 5.22 and Figure 5.23 respectively.

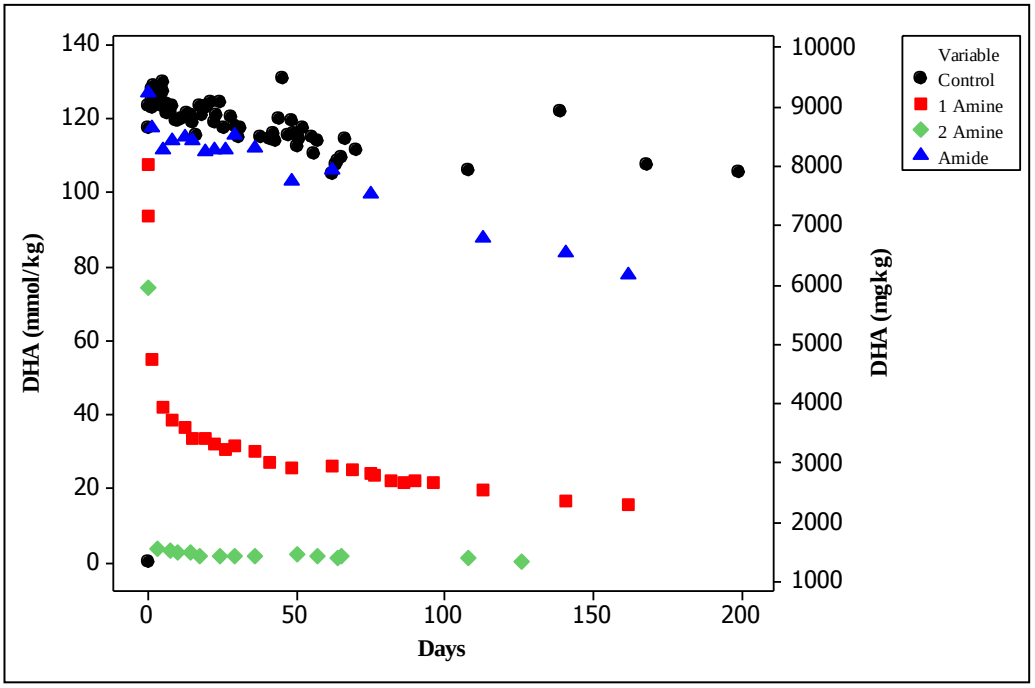


Figure 5.22 Disappearance of DHA vs. time for control (circle), primary amine (proplyamine, 14.69 mmol/kg, square), secondary amine (diethylamine, 14.42 mmol/kg, diamond) and amide (*N*-methylacetamide, 14.56 mmol/kg, triangle) systems at 37 °C. Both the primary and secondary amines caused a very fast initial loss of DHA due to either binding to DHA or reacting with DHA in a side reaction. The amide had no effect on DHA concentration.

The DHA-control sample had no added perturbants and was used to identify the basal rate of conversion of DHA to MGO and side products. The loss of DHA was linear over time. The first-order rate constant for DHA loss (k) was 0.0015 day^{-1}. The first-order rate constant of MGO gain (k') was 0.0005 day^{-1}. With no added perturbants, it is expected that all DHA would convert to MGO; however, the efficiency was only 28 ± 17%.The stoichiometric factor matched this (0.33). This indicates that side reactions are occurring; possible reactions have been discussed in section 5.3.5.1.

Table 5.2 First-order rate constants for disappearance of DHA and appearance of MGO for DHA systems (10,000 mg/kg) perturbed with amines and amide.

Sample	Initial rate constant for DHA disappearance (day^{-1}, k_1)*	Initial rate constant for MGO appearance (day^{-1}, k'_1)*	Stoichiometric factor (x_1)*‡	Secondary rate constant for DHA disappearance (day^{-1}, k_2)	Secondary rate constant for MGO appearance (day^{-1}, k'_2)	Stoichiometric factor (x_2)‡	Efficiency (%)§
Control	-	-	-	0.0015	0.0005	0.33	28 ± 17
Propylamine (1° amine)	0.1174	0.0038	0.03	0.0057	0.0050	0.88	60 ± 13
Diethylamine (2° amine) †	-	-	-	0.0122	-	-	12 ± 7
N-methylacetamide Amide	-	-	-	0.0026	0.0005	0.19	16 ± 4

*The rate constants for the first and second reaction were shown with a subscript denoting the reaction.

† Initial rate constants for the secondary amine could not be calculated as DHA was removed in one day and there were not enough data points to obtain a slope.

‡The stoichiometric factor was calculated as $x = k'_n/k_n$

§ The efficiency presented in the table was calculated as the millimoles of DHA lost that appeared as MGO. The concentration of DHA at the first point in the secondary rate was used as $[DHA]_0$.

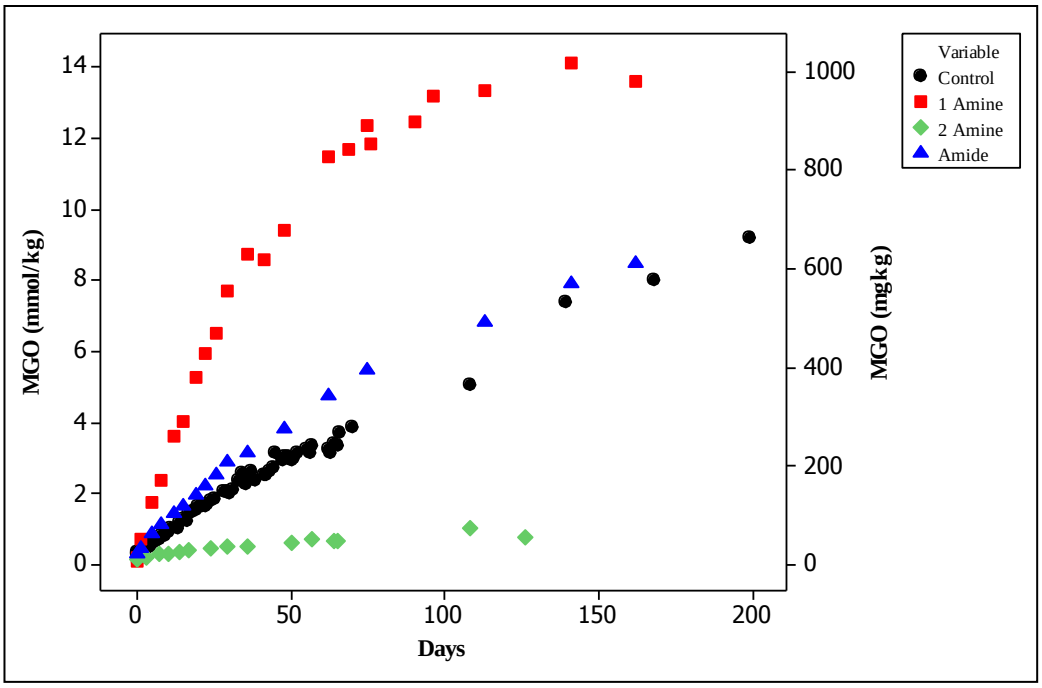


Figure 5.23 Appearance of MGO vs. time for control (circle), primary amine (proplyamine, square), secondary amine (diethylamine, diamond) and amide (*N*-methylacetamide, triangle) at 37 °C. The primary amine is the best catalyst; the system with the secondary amine is unable to form MGO as all of the DHA was consumed by side reactions. The amide had no effect on the conversion.

A secondary amine (diethylamine, 15 mmol/kg) was added to an artificial honey matrix with 121 mmol/kg DHA. There was a very fast reaction of the secondary amine with DHA; after day one most of the DHA has been consumed. By day seven 98% of DHA had been removed. The results suggest a catalytic conversion of DHA by the secondary amine because the concentration of DHA exceeded the secondary amine concentration by over eight times. An initial rate of DHA disappearance could not be calculated because the DHA was removed too fast and not enough data points were taken during this time; hence the rate constant for the initial formation of MGO was unable to be calculated because the rate constant for DHA loss is required in the calculation. The secondary rate constant for DHA loss was 0.0122 day^{-1}, which is a factor of 10 faster than any of the other reactions. A rate constant for MGO appearance was not calculated as MGO was not formed due to the rapid consumption of DHA. If any MGO was produced it would have reacted very quickly with diethylamine or been converted to a side product by diethylamine catalysis.

A primary amine (propylamine, 15 mmol/kg) was added to an artificial honey matrix with 120 mmol/kg DHA. The DHA-primary amine system also showed an initial fast reaction, but this was not as fast as for the secondary amine. The initial reaction occurred over eight days and 68% of DHA was removed; this sample also lost more moles of DHA than the initial amount of moles of amine; 82 mmol/kg of DHA had been lost after eight days which is 5.5 times more than the amine added, suggesting a catalytic conversion rather than amine binding to DHA. A plot of [DHA] vs. time shows the two rates of reaction (Figure 5.24). The initial rate constant for DHA disappearance (k_1) was 0.1174 day^{-1} and the initial rate constant for MGO appearance (k_1') was 0.0038 day^{-1}.

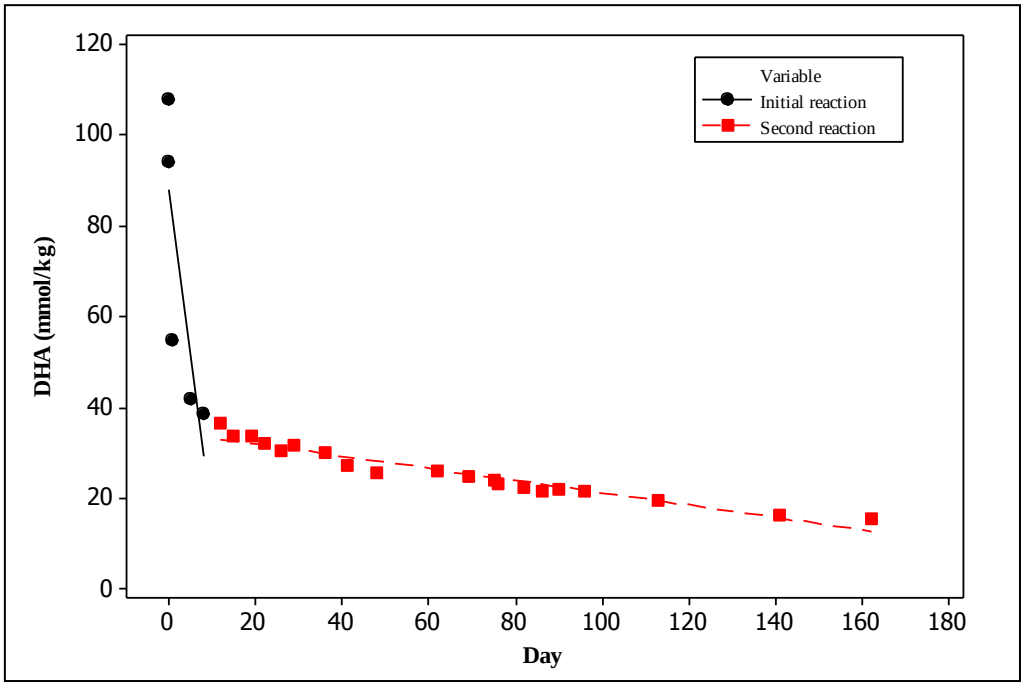


Figure 5.24 [DHA] vs. time for primary amine (121 mmol/kg DHA and 14 mmol/kg primary amine, propylamine). Two rates of reaction are observed; the initial rate (cirlces) is likely to be due to DHA binding to or reacting with the amine to form other products. The amine may be regenerated in some pathways. The second rate (squares) is predominantly (60%) the conversion of DHA to MGO.

The primary amine had a rate-enhancing effect on the conversion of DHA to MGO compared to the secondary amine. The secondary rate constant for DHA disappearance (k_2) was 0.0057 day^{-1}. The first-order plot for MGO deviated from linearity at later times due to side reactions of MGO. The secondary rate constant for the formation of MGO (k_2') was calculated in the linear region before this deviation and was 0.0050 day^{-1}, a factor of 10 larger than the control, amide and

secondary amine. At the end of data accumulation, the DHA:MGO ratio was 1:1; the [MGO] vs. t plot (Figure 5.23) shows MGO reaches a maximum before decreasing, due to side reactions of MGO. These may be catalysed by the primary amine. Strain and Spoehr[286] noted methylglyoxalamine compounds were formed from MGO and amine in an acidic aqueous medium if the amine concentration was too high. The efficiency of DHA conversion to MGO in the system perturbed by the primary amine was 11 ± 3% if $[DHA]_0$ was used for the secondary reaction. If the concentration of DHA at the time of commencement of the second reaction was used, the efficiency was increased to 60 ± 13%. This was closer to the calculated stoichiometric factor (0.88).

If amines are catalytic, then only a small amount is required for the conversion of DHA to MGO, which could still occur if there was an equilibrium between the free DHA and amine and the product formed between them. It is likely that the product formed is an imine, an initial step in the Maillard reaction, hence it would be in equilibrium with free DHA and amine.

Weber (2001)[292] reported that amines catalyse the conversion of DHA to MGO (pH 5, 50 °C). In contrast, Strain and Spoehr (1930)[286] reported that amines did not have a catalytic effect on the conversion of DHA to MGO in acidic aqueous solution, but were catalytic for the glyceraldehyde to MGO conversion. These latter results vary from the current research, however, it is unknown why they did not see an increase in MGO production.

In comparison to the amines, the amide (*N*-methylacetamide) did not react with DHA and only one rate of DHA loss was observed. By day eight only 6% of DHA had been removed and this is most likely due to the conversion of DHA to MGO at the control rate. The rate constant (k) for DHA loss was 0.0026 day^{-1}, which is slightly higher than the control sample. This may be a real effect or could arise from the large amount of scatter in the control data, which produced a larger deviation in the slope of the line. The amide system did not show any acceleration in MGO production ($k' = 0.0005\ day^{-1}$) compared to the amines. The efficiency of the reaction was 16 ± 4%, slightly lower than the control sample (28 ± 17%). The stoichiometric factor was also similar (0.19). If the disappearance of DHA in the amide experiment is in fact higher than the control, then this suggests that there is

a slight acceleration from the amide on the disappearance of DHA. However, it does not make a significant contribution. Furthermore, the protein content of honey is very low (0.058 to 0.786%[153]), so its effect can be neglected in any modelling of the system.

The reaction/binding of DHA with amines is evident by the colour changes observed; Figure 5.25 shows the primary and secondary amine and the amide samples. The primary amine (left-hand side) changed to a dark brown, the secondary amine (centre) changed to yellow and the amide (right-hand side) showed no colour change The brown colour in the primary amine sample initially developed over a few hours then slowly became darker over time. DHA is used in self-tanning cream because it readily binds to amino acids in the stratum corneum of the skin,[310] which is a Maillard-type reaction (see section 5.1.4.1 for a review on the reaction). The initial products formed in the Maillard reaction are reversible and colourless, but further reactions are irreversible and produce coloured products. The shade of colourisation (yellow through to brown) depends on the amino acids present and their concentrations.[314] It must be noted that 10,000 mg/kg DHA does not naturally occur in mānuka honey; therefore the colours were very intense and developed faster than in samples with a lower DHA starting concentration.

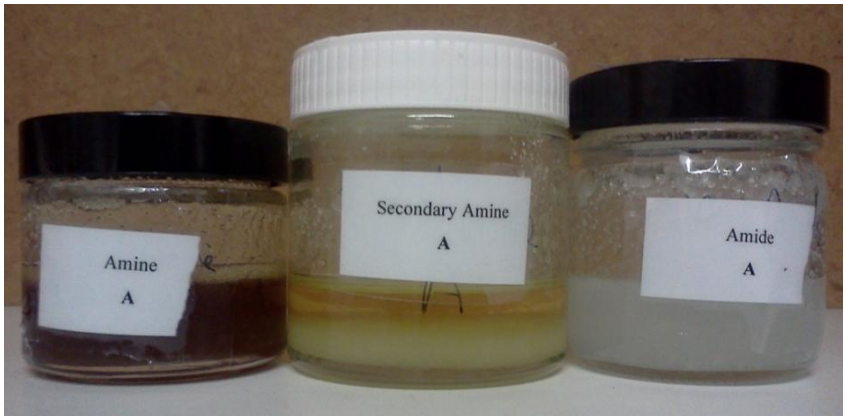


Figure 5.25 Three artificial honey storage trials showing various degrees of colouration due to the reactions/binding of DHA and amines. The primary amine (propylamine, left) is dark brown, the secondary amine (diethylamine, centre) is yellow and the amide (*N*-methylacetamide, right) did not change colour.

MGO as starting compound

Artificial honey with MGO (2,000 mg/kg, 22 mmol/kg) and one amine or amide were used to examine whether the amines or amide were reacting with/binding to MGO. A similar effect was observed as with the DHA. Figure 5.26 shows the plot of MGO vs. time; the secondary amine (diethylamine, 13 mmol/kg) reacts quickly with MGO; after two days 92% of MGO has been removed. After three days there is no MGO left in the system. The primary amine (propylamine) reacts with MGO but not as fast; after two days 77% of MGO has been removed from the system. After two days, 22 mmol/kg MGO were removed but only 16 mmol/kg primary amine had been added. The amines are able to remove more than the equal molar amount, which was also seen in the systems starting with DHA. This suggests that the amines are acting as a catalyst in a side reaction and are being regenerated.

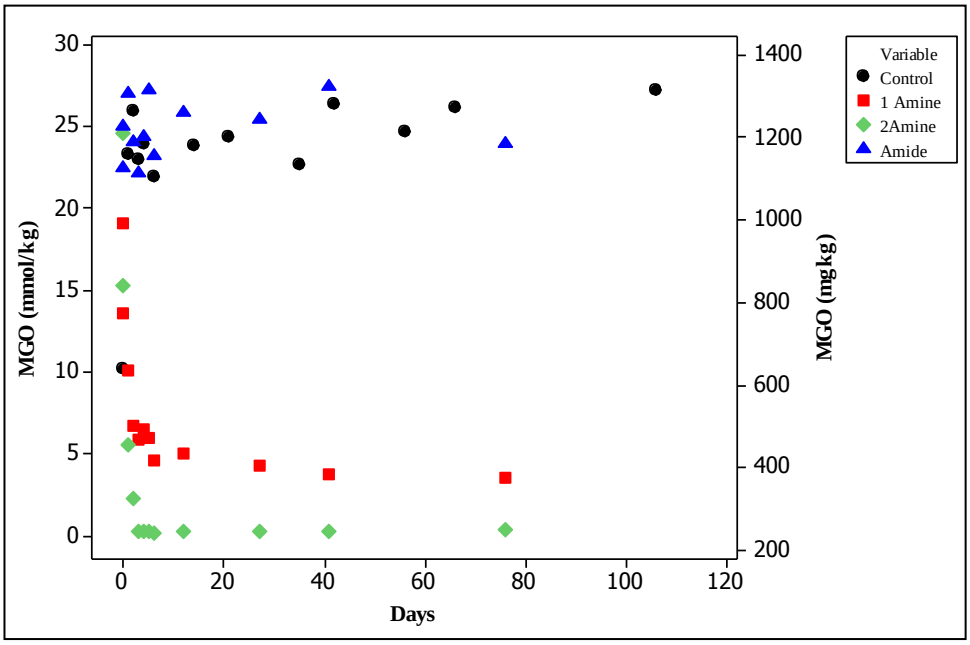


Figure 5.26 MGO vs. time for artificial honey doped with MGO as the starting compound in the perturbed systems. The secondary amine (diethylamine, 13 mmol/kg, diamond) reacts quickly with MGO; the primary amine (propylamine, 15.83 mmol/kg, square) reacts with MGO but not as fast. The amide (*N*-methylacetamide, 16.64 mmol/kg, triangle) has no effect on the concentration of MGO and the system behaves the same as the control (circle).

The amide (*N*-methylacetamide, 17 mmol/kg) had no effect on the concentration of MGO, which reinforces the decision to neglect the contribution of protein in model systems. It must be noted that while MGO does not react with the amide function of proteins it may react with the side chains of amino acids to form cross-

links. The advanced glycation end products are formed relatively slowly. These products have the potential to deactivate enzymes.[296] Recently a study[57] has shown that the MGO in mānuka honey modifies the enzyme glucose oxidase, which explains the lack of H_2O_2 production upon dilution of mānuka honey.

Similar colour changes seen for the DHA systems perturbed with the amines and amide were also seen for the MGO systems perturbed with the same compounds, visually indicating that MGO also reacts with amines to form side products (possibly Maillard products).

Various studies have also reported the reaction of MGO and amines; Strain and Spoehr (1930)[286] noted that in aqueous solution, the amine concentration must be kept low because many amines form methylglyoxalamine compounds with MGO. Adams *et al.*[14] reported that when the amine concentration was increased in a honey matrix, the yield of MGO was decreased; this may have been due to MGO and the amine reacting together in various other reactions or from the amine catalysing a side reaction.

5.3.4 Conversion of DHA (10,000 mg/kg) to MGO in artificial honey at 37 °C with naturally occurring perturbants

A set of storage trials were carried out in artificial honey with extremely high DHA as an initial starting concentration (10,000 mg/kg) in conjunction with levels of potential catalysts which were realistic for normal honey. The density of points was not high enough at the beginning of the reactions to see if two rates of reaction occurred, as observed with the amine and amide systems (section 5.3.3). The disappearance of DHA and appearance of MGO were both first-order reactions. Table 5.3 summarises the rate constants for the disappearance of DHA and appearance of MGO and the stoichiometric factor. The efficiency of these systems is not reported because there was a large amount fluctuation in some results, which made reporting an average efficiency difficult. Only a brief summary of the systems will be given here. Section 5.3.5 discusses possible reactions occurring in the honey in more detail as these artificial honey systems started with 2,000 mg/kg DHA which is closer to the concentration seen naturally in honey.

Two rates of reaction were not observed; this may be due to a large amount of monomeric DHA present for reaction (due to the high DHA starting concentration). Furthermore, it may account for differences in rate constants in systems that had the same perturbant but different starting concentration of DHA (10,000 vs. 2,000 mg/kg).

Table 5.3 Summary of rate constants for disappearance of DHA and appearance of MGO in artificial honey with 10,000 mg/kg DHA added.

Sample	Rate constant for DHA disappearance (k, day^{-1})	Rate constant for MGO appearance (k', day^{-1})	Stoichiometric factor (x)
Control	0.0015	0.0005	0.33
Proline (400 mg/kg)	0.0024	0.0007	0.29
Proline (800 mg/kg)	0.0029	0.0012	0.41
Iron (400 mg/kg)	0.0047	0.0010	0.21
Iron (800 mg/kg)	0.0043	0.0013	0.30
Proline (400 mg/kg) + Alanine (400 mg/kg)	0.0030	0.0026	0.87
Proline (800 mg/kg) + Iron (800 mg/kg)	0.0053	0.0029	0.55

Control

The control sample had a slow rate of DHA disappearance (0.0015 day^{-1}) and slow rate of MGO appearance (0.0005 day^{-1}). It is expected that because there are no other compounds for DHA to react with, the efficiency of the reaction will be high. However, the stoichiometric factor was only 0.33, indicating that only 33% of the DHA converted to MGO. DHA may have converted to side products or reacted with MGO. A system with only MGO in artificial honey showed that MGO was stable over time, indicating that MGO does not react with itself or the sugars.

Proline

Proline was added to artificial honey in two different concentrations (400 and 800 mg/kg). The rate of DHA disappearance for systems with 400 mg/kg (4 mmol/kg) and 800 mg/kg (7 mmol/kg) were 0.0024 and 0.0029 day^{-1} respectively, which are within experimental error. The results for appearance of MGO are also within experimental error (0.0007 and 0.0012 day^{-1} respectively). An effect of the concentration of proline may have been swamped due to the large amount of

DHA added. The concentration of DHA was higher than that of proline by 30 times for the samples with 400 mg/kg proline and 15 times higher for samples with 800 mg/kg proline. This is in comparison to samples with 2,000 mg/kg, where it was only 3 times higher than proline.

The rate constant for the loss of DHA (0.0024 and 0.0029 $days^{-1}$ for 400 and 800 mg/kg proline respectively) is much smaller than the rate constant for the aliphatic secondary amine (diethylamine) studied in section 5.3.3 (0.0122 $days^{-1}$). This is likely to be due to the difference in structures.

Iron

Iron was added to the artificial honey in two different concentrations (400 and 800 mg/kg iron). However, this did not affect the rate constants for DHA loss or MGO appearance. There was no difference in the rate constants, within experimental error. The DHA-iron system had the largest rate constant for DHA loss out of the systems studied (average $k = 0.0045\ day^{-1}$). There was also a slight increase in the rate constant for MGO appearance (average $k' = 0.0012\ day^{-1}$) compared to the control sample.

Proline + Alanine

Artificial honey was doped with DHA, proline (450 mg/kg) and alanine (450 mg/kg). The rate constant for the loss of DHA was the same as for the sample with only proline added. However, the rate constant for MGO appearance was larger than the sample with only proline (at both concentrations). This sample did not have the highest rate of DHA loss or MGO gain, but it had the highest stoichiometric factor, suggesting that alanine is involved in converting DHA to MGO, rather than to a side product. This was also noted in section 5.3.3 where the aliphatic primary amine (propylamine) had the largest stoichiometric factor compared to the systems perturbed by the secondary amine and amide.

Proline + Iron

The proline-iron system had the largest rate constant for DHA loss for the systems starting with 10,000 mg/kg DHA. However, this is smaller than an addition of the rate constants for the two individual reactions for the loss of DHA, possibly due to competition for monomeric DHA.

The rate constant for MGO appearance is also the largest for the six systems with 10,000 mg/kg DHA. The rate constant is a summation of the two individual systems, hence no synergy is observed, unlike for the conversion of glyceraldehyde to MGO where a synergy between lysine and phosphate was observed by Bonsignore *et al.*[287]

5.3.5 Conversion of DHA (2,000 mg/kg) to MGO in artificial honey at 37 °C with naturally occurring perturbants

A summary of all trials carried out with 2,000 mg/kg DHA at 37 °C can be found in Table 5.4; this contains the average rate constants for DHA disappearance and MGO gain, the stoichiometry factor (x) of the reaction and average efficiency for the conversion. Some of the storage trials did not obey first-order kinetics for the disappearance of DHA over the entire working range due to an initial fast loss of DHA at the beginning of the experiment, followed by a slower reaction. The two reactions were treated separately and were both linear. They were termed initial reaction (k_1) and secondary reaction (k_2). See section 5.3.1 for information on these calculations.

During the storage trials the colour of the artificial matrices changed, with all colours becoming more intense over time. The colour was influenced by the nature of the perturbant and the initial concentration of DHA; samples with 10,000 mg/kg DHA were darker than samples with only 2,000 mg/kg DHA. Due to the changes in colour it was too hard to assign a colour to the samples.

This set of storage trials (2,000 mg/kg DHA) were used to build the prediction tool of DHA conversion to MGO (chapter 8). Therefore each storage trial has been discussed separately below. Possible reactions occurring in the system have been proposed.

Table 5.4 Summary of rate constants for disappearance of DHA and appearance of MGO in artificial honey with 2,000 mg/kg DHA added and stored at 37 °C.

Perturbant	Initial rate constant for DHA disappearance (k_1, day^{-1})	Initial rate constant for MGO appearance (k'_1, day^{-1})	k_1'/k_1 for initial reaction (x_1)*	Number of days break occurs at	Secondary rate constant for DHA disappearance (k_2, day^{-1})	Secondary rate constant for MGO appearance (k'_2, day^{-1})	k'_2/k_2 for secondary reaction (x_2)*	Efficiency (%)†
Control	-	-	-	-	0.0025	0.0006	0.24	18 ± 5
Proline	0.0075	0.0006	0.08	29	0.0014	0.0006	0.43	38 ± 19
Alanine	0.0067	0.0022	0.31	24	0.0021	0.0016	0.73	52 ± 11
Lysine	0.0064	0.0006	0.09	42	0.0017	0.0007	0.41	27 ±11
Serine	0.0052	0.0007	0.13	42	0.0014	0.0007	0.50	38 ± 17
Iron	-	-	-	-	0.0047	0.0015	0.32	30 ± 15
Proline + Alanine	0.0068	0.0020	0.29	44	0.0031	0.0014	0.45	39 ± 7
Alanine + Potassium Phosphate	-	-	-	-	0.0026	0.0020	0.77	68 ± 15
Proline +Iron	-	-	-	-	0.0040	0.0017	0.43	40 ± 8
Alanine + Iron	0.0119	0.0037	0.36	10	0.0037	0.0026	0.70	86 ± 12
Proline + Alanine + Iron	-	-	-	-	0.0056	0.0024	0.43	34 ± 10

*The stoichiometric factor was calculated as $x = k'_n/k_n$.

†Efficiency is calculated as the gross percentage of MGO gained relative to the amount of DHA loss. This takes into account all side reactions.

5.3.5.1 DHA control system

The DHA-control system contained only DHA (2,000 mg/kg, 22 mmol/kg) in the artificial honey matrix. This system was used to calculate the control rate (the lowest expected rate of DHA loss and MGO formation) because there are no added perturbants in the matrix that have a potential rate enhancement other than gluconic acid which was used for pH adjustment.* As expected, this reaction occurs slowly. After 1 year, not all of the DHA had reacted. An incomplete reaction was also seen for most real honeys samples. For example, a honey harvested in 2003 and stored at ambient temperature until analysis in 2012 had not converted all of the DHA. Strain and Spoehr[286] also reported that the conversion did not go to completion in a model system. The conversion is irreversible, confirmed by the MGO-control system which did not produce any DHA, hence MGO and DHA cannot be in equilibrium. One explanation may be that the derivatising agent in the analytical method is able to remove DHA from one or more alternative stable equilibria so that it is detected as free DHA.

The first-order plot for DHA disappearance is linear over the entire sampling range. The rate constant was 0.0025 day^{-1}. The first-order rate constant for the appearance of MGO was also linear over the entire sampling range (0.0006 $days^{-1}$). This was the smallest rate constant for the formation of MGO in a system that started with 2,000 mg/kg DHA. The control sample doped with 10,000 mg/kg DHA had a smaller rate constant for DHA loss (0.0015 day^{-1}), but the same rate constant for MGO gain. The reason for this is unknown. The ratio of DHA:MGO was 2:1 at the end of data collection due to the reaction occurring slowly.

A study[309] reported that DHA is more reactive at pH 7 than at pH 4 because between pH 4 and 5 DHA is stable as the dimer. Furthermore, DHA exists as a dimer in the solid state but converts to a monomer in solution.[329-330, 341] This occurs rapidly in water (0.034 min^{-1}, 48.96 day^{-1}), but is very slow in DMSO with only 50% converted after 64 hours.[330] Therefore it is likely that in the dehydrating, acidic (pH 4) honey environment a large proportion of the DHA exists as the dimer and would need to convert to the monomer before it was reactive; however, the equilibrium constant for the dimerisation of DHA in honey is unknown and

* Gluconic acid was required to lower the pH to 4 to reflect the pH of honey. Only a very small amount was required. Gluconic acid has a pKa of 3.7.

would be difficult to obtain because it would change when the matrix was altered. Determination of the equilibrium constant might be achievable by NMR analysis. Initiation of the conversion from dimer to monomer is probably H^+ catalysed since it occurs more readily in water than in DMSO. In the honey matrix, most of the H^+ and water are likely to be H bonded to the sugars. Therefore the free H^+ concentration would be low and the conversion of the dimer to the monomer would occur slowly and be reliant on perturbants. Heating the solution may unbond some H^+ from sugar, hence the reaction may increase. Additionally, in the artificial honey matrix dissociation of the dimer is likely to be very slow due to limited species that can donate H^+. This will cause competition between the reaction of interest and side reactions.

Aqueous systems in the literature are compared to this reaction; however, emphasis must be put on the different experimental conditions (pH, temperature) and matrices (aqueous solution), which may affect the reactions that are occurring. Weber[292] reported that a sugar model (formaldehyde and glycolaldehyde,) control sample at pH 5.5 and maintained at 50 °C gained 0.004 mM MGO day^{-1}. Fedoroňko and Königstein (1969)[283] reported that the dehydration of DHA to MGO occurs in water at 50 ºC, with a first-order rate constant (k) of 6.10^{-6} min^{-1} (0.0088 day^{-1}) for both trioses. Both studies have larger rate constants than observed in the artificial honey matrix in the current research due to the reactions being carried out in aqueous solution and at elevated temperature. In 1 M aqueous acetic acid (pH 2.18) the rate constant was even faster 1.9 x 10^{-5} min^{-1} (0.027 day^{-1}) which the authors[283] suggested was due to the undissociated acetic acid playing a role in catalysis of conversion of DHA to MGO. This latter rate of DHA disappearance is an order of magnitude faster than for the control sample of artificial honey. Bonsignore *et al.*[287] reported that the rate constant for conversion of glyceraldehyde to MGO at pH 7.7 was 0.0242 day^{-1}, which is much faster than observed for DHA in acidic conditions in this research.

Figure 5.27 shows a possible mechanism for the conversion of dimeric DHA to monomeric DHA. This is highly likely to be the rate determining step in the conversion of DHA to MGO in honey. Hence compounds that can donate H^+ will catalyse the conversion. In comparison, the studies mentioned above were carried out in aqueous solution so the conversion from dimer to monomer is not required.

Therefore the rate determining step would be different, which will account for the much faster reaction.

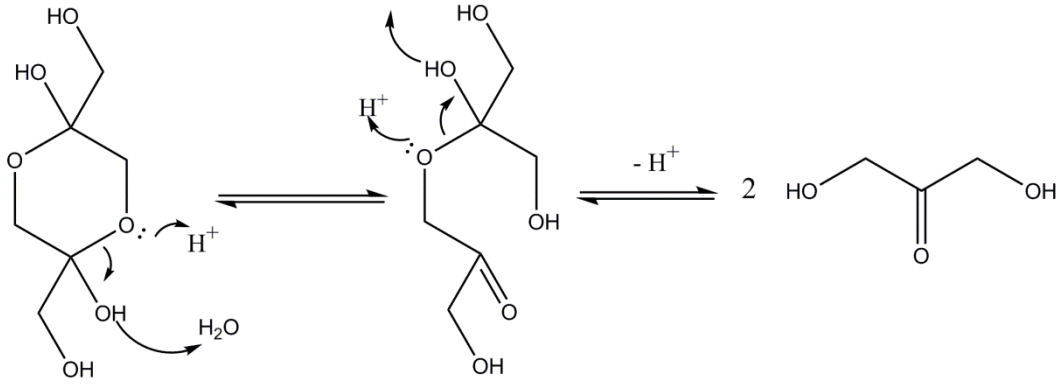


Figure 5.27 Possible mechanism for the dissociation of DHA dimer to two monomeric DHA, requiring H^+ catalysis. The direction the equilibrium lies depends on the environment.

Monomeric DHA is the source which is able to convert to MGO because it has a free carbonyl. It is generally accepted that DHA undergoes dehydration via β-elimination to the enolic form of MGO.[283, 288, 292] Strain and Spoehr[286] suggested that for DHA to rearrange to MGO an enediol modification may be essential (Figure 5.28). The DHA/enediol equilibrium most likely lies heavily towards DHA. It is unlikely that the enolic form of MGO would go back to the enediol, because addition of water would most likely give a different product. This may be a gemdiol, where the OH would join onto the carbon in the C=C bond with fewer alkyl substituents (Markovnikov's rule). The enolic form of MGO and MGO equilibrium would lie heavily towards MGO.

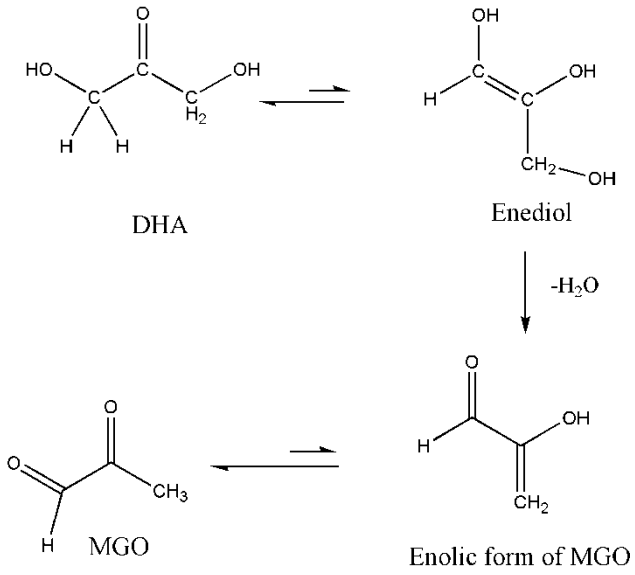


Figure 5.28 Pathway of MGO formation from DHA via an enediol intermediate. Modified from Strain and Spoehr.[286, 348]

Competing reactions prevent a 1:1 stoichiometry of DHA converting to MGO; this occurs even in artificial honey with only DHA added to the artificial honey matrix. In the current research, MGO only accounted for 18 ± 5% of the utilised DHA. The stoichiometric factor for the reaction was 0.24, which is close to the estimated efficiency of the reaction. The lack of mass balance between the loss of DHA and gain of MGO indicates that the DHA is reacting in side reactions and/or MGO is reacting further. In the control sample the compounds can react or bind with only themselves, each other or sugars. In honey, the sugars are likely to predominately exist in the cyclic form so are unavailable for reaction with DHA or MGO. Bonsignore *et al.*[294] noted that the conversion of glyceraldehyde to MGO is not stoichiometric. They suggested polymerisation of MGO because they observed increasing molecular weight compounds. However, MGO was not lost when added to the artificial honey matrix (analysed up to 100 days, section 5.3.5.2), suggesting that MGO does not polymerise irreversibly in the honey matrix. Furthermore, dimerisation of DHA can be also ruled out as the cause of

lost efficiency because the analytical method dissociates the dimer back to the monomer so that it is detected as DHA.*

DHA may react with itself to form other compounds. Aldol condensation can occur in acid and has been shown to occur as low as pH 4;[349] it also commonly occurs in base.[306] In acid, dehydration of the aldol condensation product usually occurs, followed by further condensation because the product is still an aldehyde or ketone.[306] A postulated route for the aldol reaction of two molecules of DHA is shown in Figure 5.29. All steps are reversible. However, in the honey matrix the reverse step for the dehydration would not readily occur due to the limited amount of water. Aldol condensation reactions are also used to close 5 and 6 member rings and can occur in acid.[350]

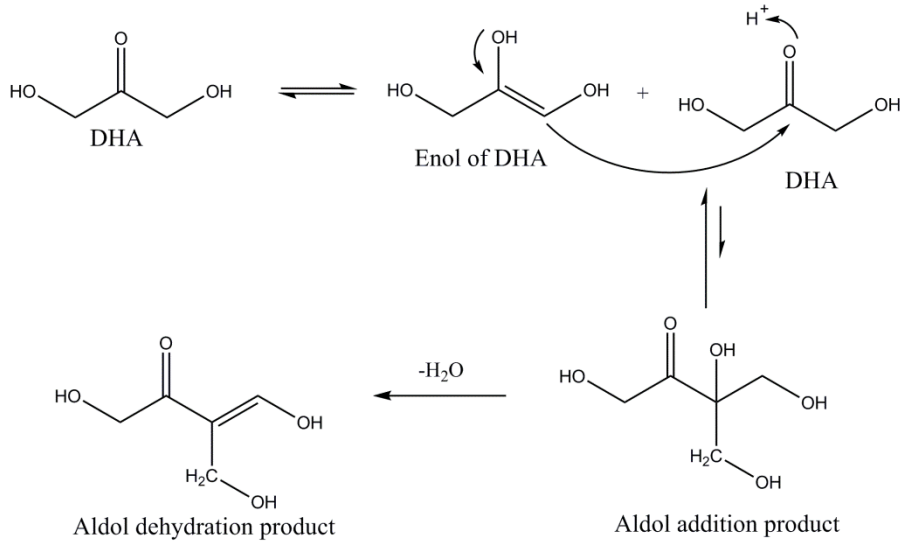


Figure 5.29 Postulated aldol condensation of DHA with itself.

Popoff *et al.*[332] reported various products formed from DHA when reacting with itself or with MGO (pH 4.5, 96 °C, 24 hours). These were not simple dehydration products and included ring compounds, confirming that aldol condensation reactions are present. Twenty compounds were identified; six are shown in Figure 5.30. The products are discussed in section 5.1.4.3. They suggested DHA may partially dehydrate before forming dimers and trimers as well as DHA and MGO

* For example, if a solution contained 100 mmol/kg DHA and 50% were found as dimers (i.e 50 monomers/25 dimers), 10 monomers converted to MGO, 20 converted to side products and 20 were unreacted. Then 70 mmol/kg DHA and 10 mmol/kg MGO would be detected by the HPLC method. Loss of DHA would be 30 mmol/kg (33% efficiency). Even though the dimer could not react in the solution it was still detected by the analysis method and hence is not classed as 'lost'.

reacting to form products. They also suggested that aldol condensation between DHA in the enol form with MGO was occurring, followed by cyclisation and dehydration through β-eliminations to form further products. The products of aldol condensation would still have carbonyl functionalities, hence further condensation is possible.[306] Further research (mass spectrometry or NMR) would need to be carried out to identify which products form in honey.

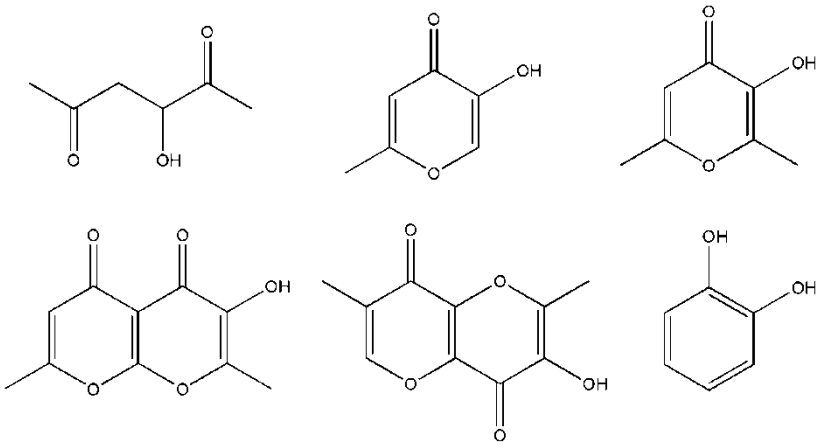


Figure 5.30 Six of the twenty products formed from DHA and MGO in acidic conditions. Extracted from Popoff *et al.*.[332]

Fedoronko and Konigstein[289] (1969) reported that under acidic conditions DHA was lost faster than glyceraldehyde during the dehydration to MGO, without a corresponding gain in MGO. The unidentified compounds may be due to side reactions of DHA with itself.

The DHA-control system gradually turned fawn colour, indicating coloured compounds were forming; however, it is unknown if this colour change is due to DHA, MGO, or the sugars reacting. Conjugated systems, such as some products of the proposed reactions of DHA with itself could be coloured. In addition, later stage Maillard type products are also coloured.

5.3.5.2 MGO control system

Artificial honey with MGO had no change in concentration over 100 days, indicating it does not react irreversibly with the matrix or itself over this time frame. The sample was tested again at 479 days and there was still no change in

the MGO concentration showing that MGO had not irreversibly reacted with itself or the matrix. Adams *et al.*[14] reported a similar result in clover honey.

The sample stayed white up to 100 days. However, at a later time, the sample changed to yellow. The reason for this colour is unknown but may be due to various reversible side reactions of MGO, or because the sugars were forming coloured compounds. Bonsignore *et al.*[287] also reported a yellow colour in reactions carried out with MGO, which was not in a sugar matrix, implying most of the colour is from a reaction of MGO. This is possibly a reversible polymerisation. MGO can dimerise, trimerise and form small polymeric structures. These are stable in dimethylsulfoxide but convert back to monomeric MGO in aqueous solution.[334] Hence they may form in the dehydrating honey environment but can still be detected using the HPLC method so do not account for the lack of efficiency of the reaction.

Samples with MGO as the starting compound were more viscous than samples with DHA as the starting compound. The MGO samples did not separate into two layers like some of the DHA samples. In addition, samples that started with DHA became more viscous over time, possibly due to the increase in MGO content. It is possible that MGO hydrogen bonds to sugars or reversibly cross-links with itself.

5.3.5.3 DHA-proline system

An artificial honey with 800 mg/kg (7 mmol/kg) proline and 2,000 mg/kg (23 mmol/kg) DHA showed an initial fast loss of 7 mmol/kg DHA in the first 29 days (Figure 5.31) which corresponds to the amount of proline added. DHA may irreversibly bind to proline; this hypothesis is supported by the use of DHA as a sunless tanner due to its ability to readily bind to amino acids. Body temperature is 37 °C, the same temperature as the storage trial suggesting that at 37 °C the side reaction of DHA with proline may be predominant over the conversion of DHA to MGO. However, the apparent 1:1 reaction may be coincidental and relate to the amount of monomeric DHA available. As a rough approximation, if 25% of DHA existed in the monomeric form, there would be 6 mmol/kg monomeric DHA in this system, similar to the amount that was removed. In comparison, systems with 10,000 mg/kg would not be constrained by limited monomeric DHA (30

mmmol/kg if approximation of 25% monomeric DHA is used). The DHA-secondary amine system lost more DHA than the amount of diethylamine added, indicating catalysis to a side product. Furthermore, two rates of reaction were not seen for the DHA-proline system with high DHA.

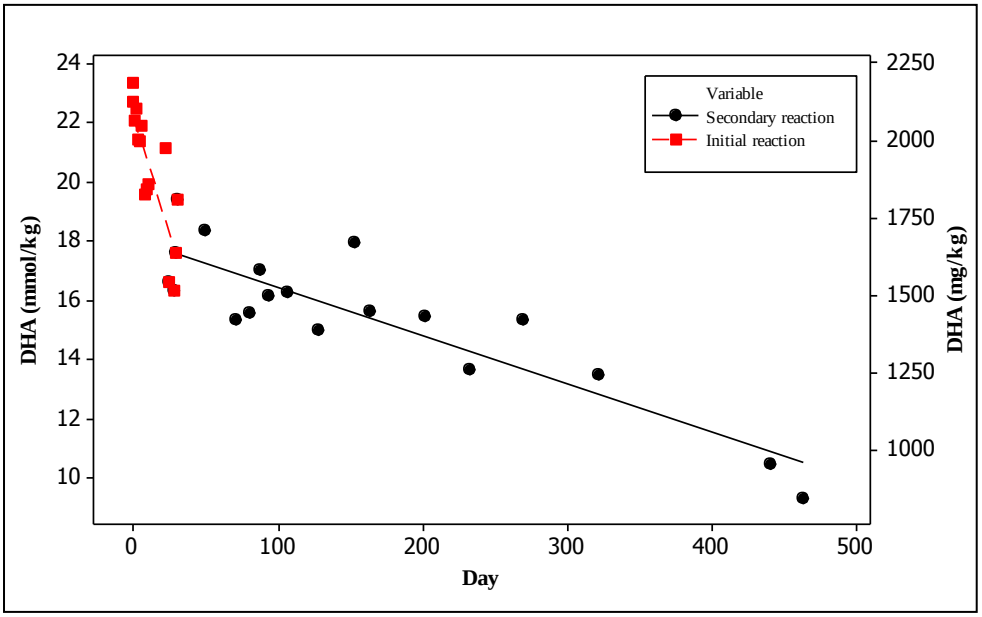


Figure 5.31 Disappearance of DHA vs. day. There is an initial rapid decrease in DHA, which corresponds to the amount of proline added. The second reaction of DHA loss is slower than the rate in the control sample.

The initial reaction for the loss of DHA, k_1, has a rate constant of 0.0075 day^{-1} and the secondary rate constant for DHA loss (k_2) was almost a factor of 2 smaller than for the control sample (0.0014 day^{-1} compared to 0.0025 day^{-1}). However, this was not seen in the samples that started with a high amount of DHA (10,000 mg/kg). The dissociation of dimeric DHA may be limited by the lack of H^+ in the system, therefore it may be the rate determining step for all subsequent reactions (conversion to MGO and side reactions). Free proline and DHA may be in an equilibrium with a proline-DHA complex (likely to be an imine), with the equilibrium favouring the complex. Therefore only a limited amount of proline is available to donate a proton to the dimer for dissociation. The DHA bound reversibly with the proline would still be detected by the HPLC method because during sample preparation it is likely to unbind.

Sugars will exist in a cyclic form in the honey matrix and only a small proportion will be in open chain form. Therefore the sugars are not readily available for reaction with amino acids, hence will not largely participate in the Maillard reaction. Therefore amino acids will not be consumed in side reactions with other sugars. In comparison, DHA has an available ketone group and MGO has two available carbonyl groups which can readily react with amino acids.

The two rates of reaction are not likely to be seen in commercial samples because there are multiple compounds available to dissociate the dimer; the secondary reaction is of more importance. However, the initial rate is also of interest in this work.

When DHA and proline solutions were added one after another to the artificial honey matrix and mixed a bread crust/popcorn aroma was produced, but faded over time. This was also reported by Wiseblatt and Zoumut (1963)[317] for proline and DHA at pH 7. They reported that when proline was added, a much stronger cracker/ bread-crust aroma was given off on equal molar basis than any other amino acid. When isolated and boiled at pH 3 the aroma was lost, but returned when it was neutralised, indicating reversibility. The current research was carried out at pH 4, which may explain the fading of the aroma. This may also explain why the loss of DHA was slower in the DHA-proline system than it was in the control system because DHA that was initially bound to proline may have been released.

Secondary amino acids can form *N*,*N*-disubstituted hemiaminals with aldehydes and ketones.[306] These are unstable, and when there is no α-hydrogen a more stable aminal (2 amine groups attached to the same carbon) compound is formed. A proposed mechanism for the reaction of DHA and proline is displayed in Figure 5.32, which could be a pathway that is irreversibly removing DHA. While this may occur, it is not possible for all 7 mmol/kg DHA that was removed in the initial fast reaction to be forming an aminal as only 7 mmol/kg proline were added to the sample and 14 mmol/kg would be required. This suggests that proline may be catalysing other reactions of DHA to form alternate products.

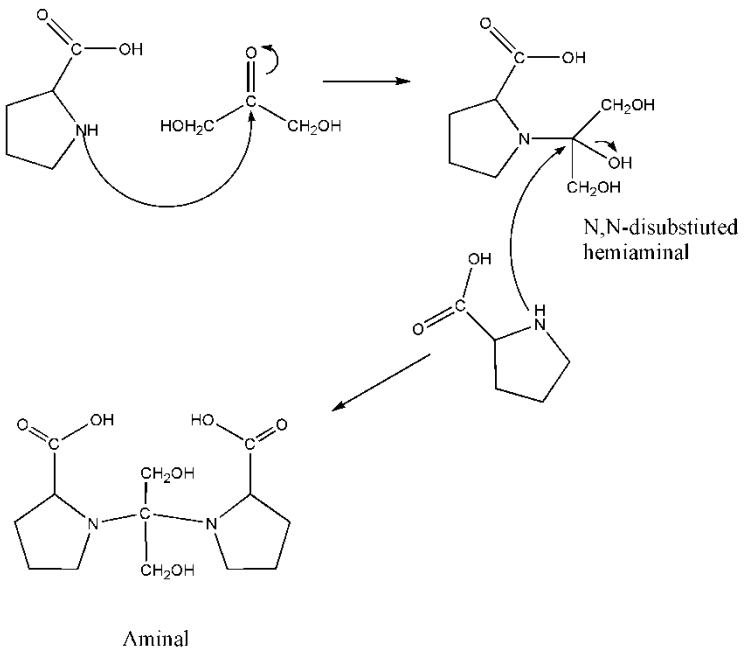


Figure 5.32 Proposed structure for the adduct of proline with DHA

As discussed in section 5.1.4.4 proline is a very good catalyst. It is known to catalyse the condensation of a ketone and aldehyde to the corresponding aldol. This can occur if the ketone is in high proportion, which is the situation that is found at early times in these trials. Therefore, instead of catalysing the conversion of DHA to MGO, proline may catalyse a side reaction to an aldol condensation product. A proposed product is shown in Figure 5.33; in acid the DHA would react in the enol form. The dehydration to the condensation product would be spontaneous because the product would favour the extended conjugation.

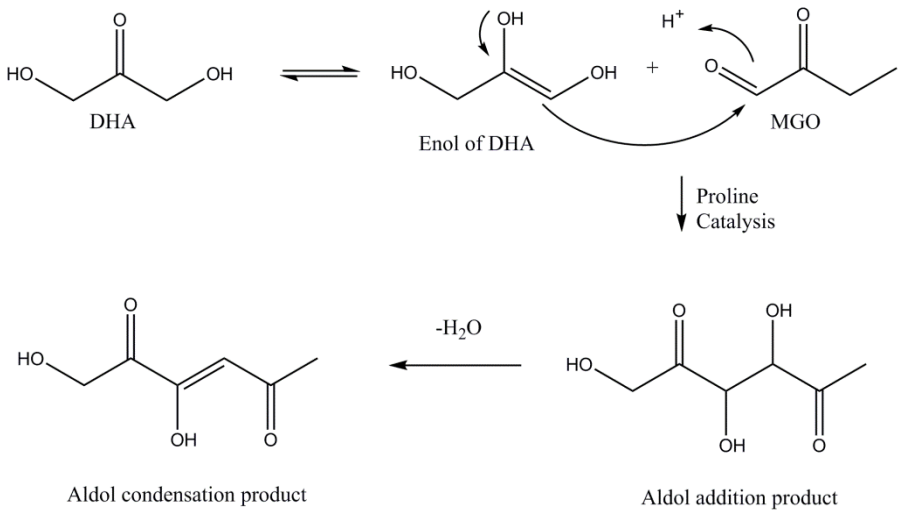


Figure 5.33 Proposed aldol product of DHA with MGO, catalysed by proline.

Weber[292] reported that secondary amines cannot react with trioses or MGO to form the side products pyrazine and imidazole, respectively, compared to primary amines which can. Hence they can sustain MGO synthesis longer than primary amines. However, rate enhancement of DHA conversion to MGO is not seen in the artificial honey system presumably because proline is reacting with DHA in side reactions, which would deactivate the catalytic ability of proline, or proline is catalysing side reactions of DHA and/or MGO hence there is no net gain of MGO. In comparison, when proline is bound, such as in a peptide, the nitrogen does not have an hydrogen bound to it, hence it cannot act as a hydrogen donor.[351] Therefore the measurement of proline in high-proline proteins is not important and only free proline detection is required. To the authors knowledge, the composition of bee saliva has not been analysed, but may be high in proline.

The rate constant for MGO appearance did not change between the initial and secondary reactions (0.0006 day^{-1} for both k_1 and k_2). Furthermore, this is the same rate for the control sample, indicating that proline does not enhance the conversion of DHA to MGO. However, a comparison of [MGO] vs. time for the control and DHA-proline system, shows that there may be a slight enhanced formation of MGO due to proline (Figure 5.34). Proline may catalyse the conversion to MGO, but due to the rate constant for disappearance of DHA being lower than expected, this would alter the calculation for the rate constant for the

formation of MGO. A reaction scheme for one possible reaction of MGO and proline was also proposed by Adams *et al.*[321] (Figure 5.35).

Sample with 10,000 mg/kg DHA and 400 or 800 mg/kg proline had slightly higher rate constants for the loss of DHA compared to the control sample, probably due to a higher concentration of monomeric DHA available to react. The system with 400 mg/kg proline had the same rate constant for appearance of MGO (0.0007 day^{-1}) as the sample presented here (0.0006 day^{-1}), but the sample with 800 mg/kg proline had a slightly higher rate constant (0.0012 day^{-1}), possibly due to a slight enhancement of the reaction from proline.

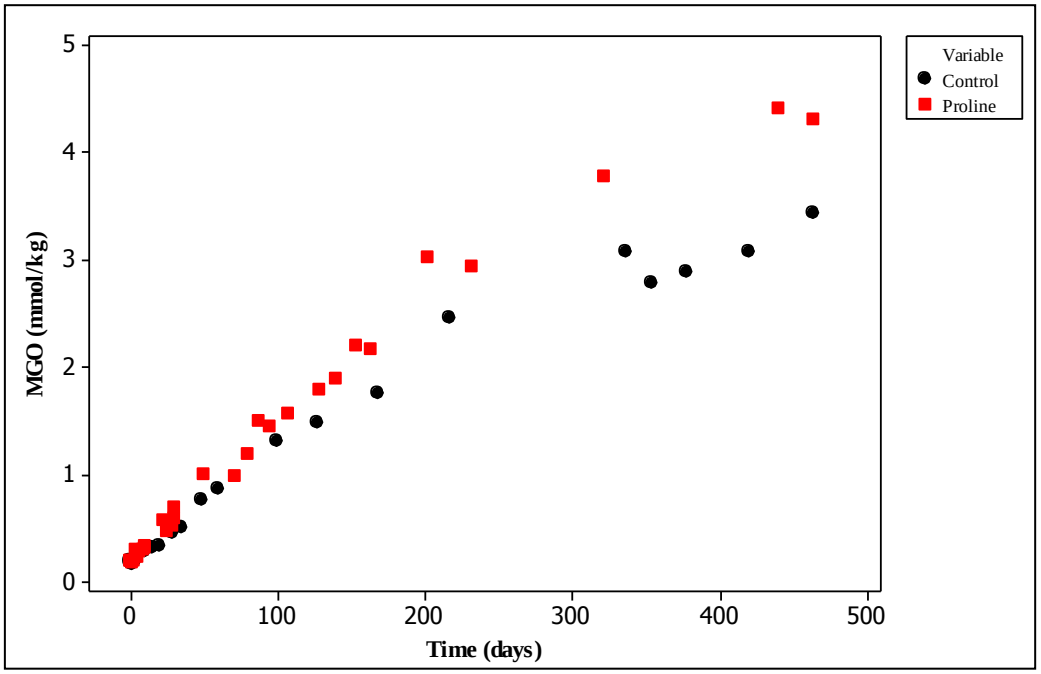


Figure 5.34 [MGO] vs. time plot for control (circles) and DHA-proline (squares) systems. A small rate enhancement can be seen in the sample perturbed by proline.

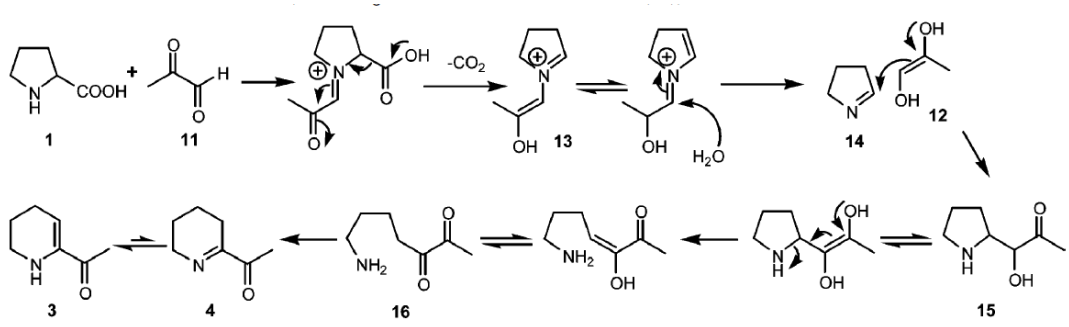


Figure 5.35 Proposed mechanism of formation of ATHP from proline and MGO (extracted from Adams *et al.* with permission.[321])

The initial reaction was only 5 ± 2 % efficient in converting DHA to MGO. The stoichiometric factor was 0.08. This suggests that the initial binding of proline to DHA and/or its conversion to side products is very fast. The reaction efficiency increased to approximately 40 ± 20% for the secondary reaction, this was the same regardless of whether [DHA] at t_0 or at the start of the second reaction was used; however there was a large standard deviation. The stoichiometric factor was similar to the estimated efficiency (0.43). Even though the rate constant for MGO appearance was no larger than the control, the stoichiometric factor was higher for DHA-proline system compared to the control (0.43 cf. 0.24), due to the rate constant for DHA loss being smaller than expected (as previously discussed). Furthermore, the stoichiometry factor is similar to that seen for the systems with 10,000 mg/kg DHA for the sample perturbed with 800 mg/kg proline (0.41).

The system reached a 2:1 DHA:MGO ratio around 460 days. A plot of [MGO] vs. t did not show MGO reach a maximum then decline. The MGO-proline system (section 5.3.5.4) shows a loss of MGO, hence it is expected that if the system had been studied for longer a maximum in MGO would have been reached, followed by a decline in MGO concentration, if some proline was available.

Samples with DHA and proline turned a light fawn colour and over time began to resemble the colour of clover honey, indicating the formation of intermediate and final stage Maillard products. Browning reactions of DHA-proline systems are affected by water content, with a 15 times higher rate of browning in absolute methanol compared to 95% methanol.[308] Absolute methanol is dehydrating, similar to the honey system, suggesting browning reactions could readily occur in honey.

The AOAC 998.12 C-4 sugar method tests for illicit addition of C-4 cane sugar. by measuring and comparing the ratio of the isotopes ^{12}C and ^{13}C in a honey sample and its associated protein. A postive shift in the $\delta^{13}C$ honey suggests addition of cane sugar. Unadulterated mānuka honey frequently fails the test due to a negative shift in the $\delta^{13}C$ protein.[161, 352] The reason for this was previously unknown, but from the current work it was thought that the concentration of DHA and/or MGO present in the honey would affect the C-4 result due to the binding of DHA and/or MGO to proline (or primary amino acids and proteins) in the honey

matrix. This was investigated as a subsidiary piece of research and the resulting publication[121] can be found in Appendix F.

5.3.5.4 MGO-proline system

A storage trial with MGO and proline was also carried out (7 mmol/kg of proline and 29 mmol/kg MGO). This showed a decrease in MGO over time. The system was monitored for 100 days, at which time the same amount of MGO (mmol/kg) had been removed as the amount of proline added. Both the aldehyde and ketone of MGO could react with the proline, but reaction with the ketone would be slower. Figure 5.36 shows proposed structures for the reaction of proline with both the ketone and aldehyde groups on MGO. A burnt-sugar aroma was produced when the sample was prepared and there was rapid browning, indicating new compounds were formed. The same aroma was also noted by Wiseblatt and Zoumut in an aqueous matrix.[317]

The system was analysed again at 519 days and MGO was still decreasing and the amount of MGO lost exceeded the amount of proline added. This suggests that the catalytic ability of proline to convert MGO into a side product has not been poisoned by MGO or one of the side products. Furthermore, there was no break in the system which would indicate that proline had been consumed.

Weber[292] reported that secondary amines cannot react with MGO to form an imidazole, like primary amines can. Therefore the browning of the solution and burnt sugar smell do not appear to be due to proline binding to MGO, but due to a side reaction catalysed by proline.

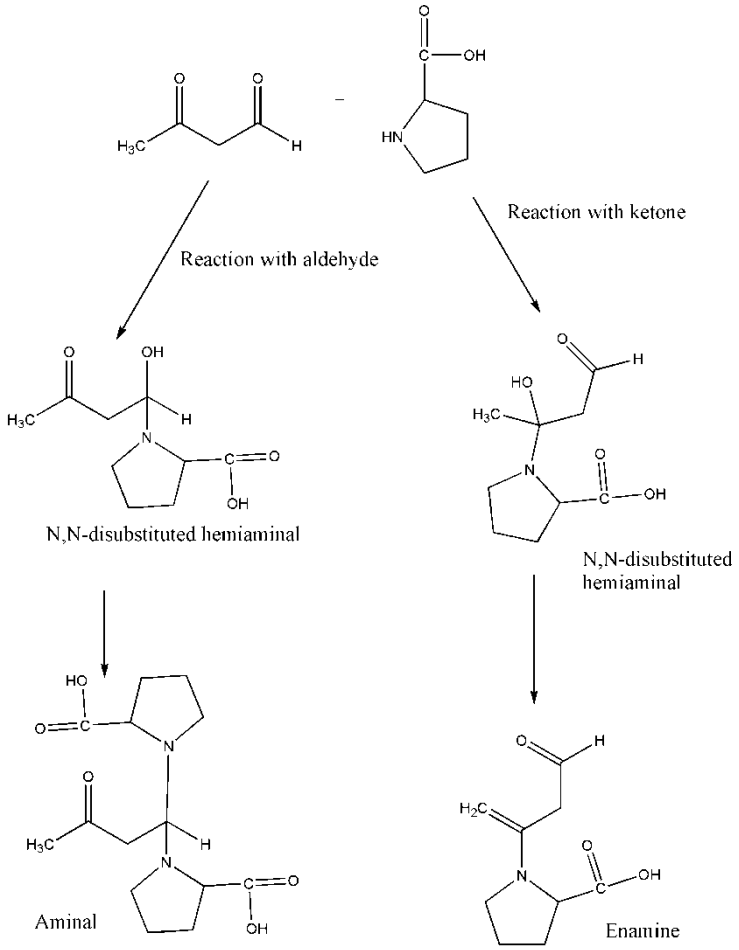


Figure 5.36 Reactions of MGO and proline via the aldehyde and ketone.

5.3.5.5 DHA-alanine system

The DHA-alanine system contained 2,000 mg/kg (22 mmol/kg) DHA and 430 mg/kg (5 mmol/kg) alanine. A [DHA] vs. t plot had a break in linearity, due to an initial fast loss of DHA (Figure 5.37). In the initial reaction (24 days), 6 mmol/kg DHA was lost, which is slightly more than the amount of alanine added. This is similar to that observed in the DHA-proline system, where the amount of DHA removed in the initial reaction was equivalent to the amount of proline added. As previously mentioned in the DHA-proline system (section 5.3.5.3) the apparent 1:1 conversion may be coincidental and relate to the amount of monomeric DHA available in the matrix (i.e. it is constrained by the rate constant for the dissociation of the dimer). Unfortunately a system with 10,000 mg/kg DHA perturbed with alanine was not carried out, so it is unknown if the amount of alanine lost was larger than the amount of alanine added. The amount of DHA removed in the DHA (10,000 mg/kg)-primary amine system (propylamine) was

more than the amount of primary amine added, which may suggest catalysis is occurring. Weber[292] reported that a sterically unhindered amine can sustain the conversion of DHA to MGO for longer than the primary amino acid. Hence further investigation is required.

The system was monitored past a 2:1 DHA:MGO ratio, but did not show deviation in the [MGO] vs. t plot until much later and the deviation was only slight. This suggests MGO is not readily lost from a reaction catalysed by alanine, which would be prominent when the concentration of DHA is low, or that the alanine had been poisoned from other reactions. Adams *et al.*[14] reported that MGO in clover honey doped with DHA and lysine or arginine reached a maximum then declined. This was also seen in real honeys in the current study (chapter 6) but the real honey matrix is much more complex.

Two rate constants were calculated for DHA disappearance; the initial rate constant (k_1) for DHA disappearance was not as large as seen for proline (0.0067 day^{-1} over 44 days compared to 0.0075 day^{-1} over 29 days); this reflects the results of the addition of the primary and secondary aliphatic amine compounds where the secondary amine reacted faster with DHA than the primary amine (section 5.3.3). The secondary rate constant for DHA loss ($k_{2,}$ 0.0021 day^{-1}) was the same as for the control system (within experimental error). As mentioned in section 5.3.5.3, the reaction may be limited by the availability of monomeric DHA. DHA and alanine are likely to be involved in the initial reversible stage of the Maillard reaction, which would prevent alanine donating H^+. However, the equilibrium appears to lie further toward the free compounds compared to proline because the secondary rate constant for DHA loss in the DHA-alanine system is not slower than the control. This suggests that alanine is more readily available to donate H^+ to the dimer for dissociate to the monomer, compared to proline.

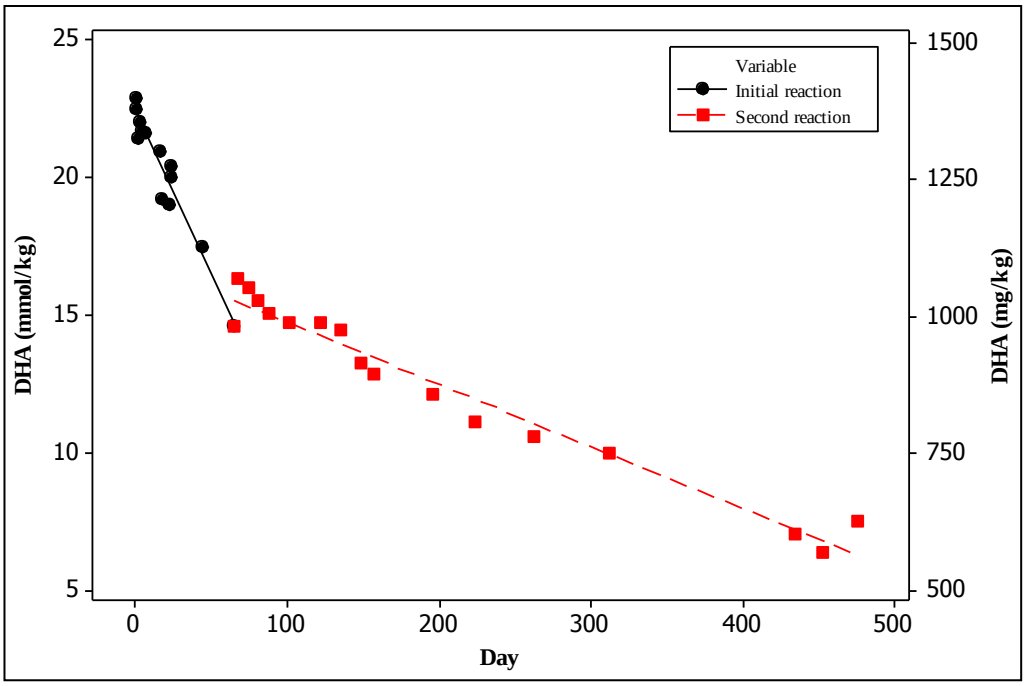


Figure 5.37 Disappearance of DHA vs. time for 2,000 mg/kg (22 mmol/kg) DHA perturbed with 400 mg/kg (5 mmol/kg) alanine. There is an initial fast rate of reaction. The second reaction rate occurs at the same rate as the control sample.

The reaction of a primary amino acid with a ketone or aldehyde initially produces an unstable compound that will lose water to form an imine (Schiff base). If the water content is too high, then the reverse reaction will become dominant and there will be more free DHA and amino acid present. On the other hand, if the water content is too low, the molecules will not be as mobile and will not be able to move together as easily to react. Work carried out on DHA as a sunless tanner proposed that 11% moisture was ideal.[309] The water content of some honeys may be considered too high and the equilibrium will favour the left. Therefore initially DHA and alanine may form an imine, but favour the left of the equilibrium allowing DHA to react and free up alanine for catalysis.

The imine is also able to react further into a non-reversible product (dead end route). However, these further steps favour neutral and alkaline conditions, so will not be prominent in the honey matrix. Schiff bases can undergo further reaction. Glycosyl-amino products undergo Amadori rearrangement to form ketosyl-amino products which further react irreversibly; these reactions include dehydrations and rearrangements. Glycosyl-amines formed from amines are more stable than those

formed from amino acids; the latter are immediately converted into the Amadori products (N-substituted 1-amino-1-deoxy-2-ketoses).[353] MGO may also react with alanine to form an imine, which is a reversible reaction or the imine can enter into a non-reversible reaction.

The formation of MGO in the sample perturbed by alanine was faster than the control sample (0.0006 day^{-1}). The initial reaction (k_1') was 0.0022 day^{-1} and the secondary reaction (k_2') was 0.0016 day^{-1}. This system had the largest rate constant for MGO appearance out of all the individual perturbants studied (Figure 5.38, the system perturbed by iron had the same rate constant as the system perturbed by alanine). Yet, as presented above, the DHA rate constant was no larger than the control; this suggests that alanine catalyses the conversion of DHA to MGO and prevents it from entering a dead end route or it is able to recover it from an otherwise dead end route. A suggested dead end route is aldol condensation where DHA has joined to another DHA, before it has condensed into a ring. Catalysis may occur by H^+ donation from alanine to the dimer which can then dissociate into two monomers.

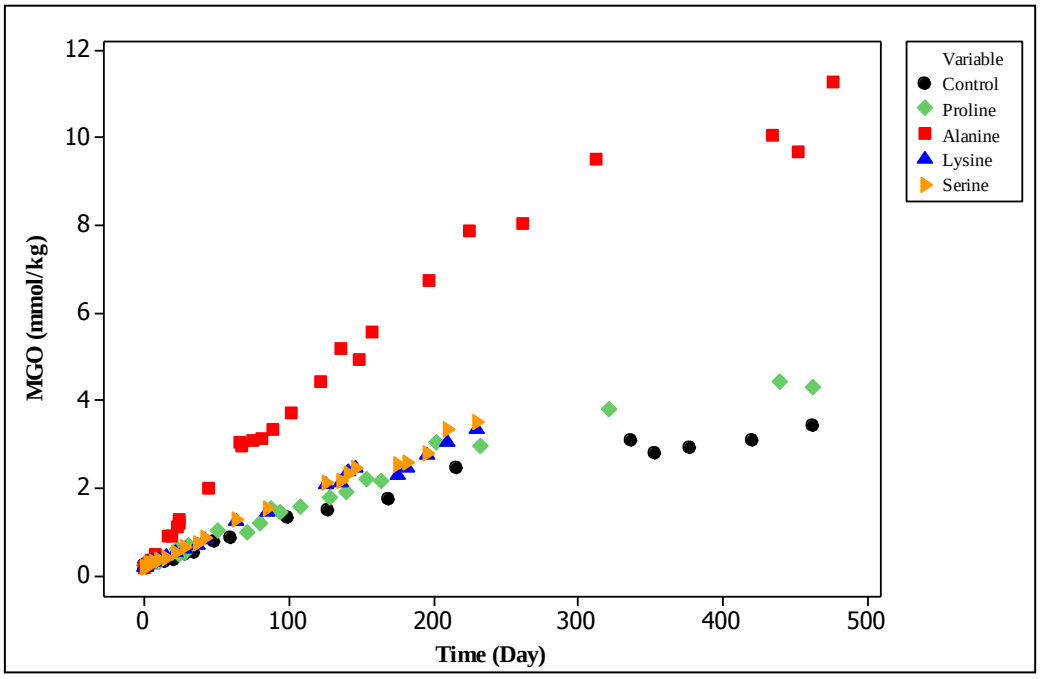


Figure 5.38 MGO (mmol/kg) vs. time for artificial honey systems perturbed with amino acids and stored at 37 °C. The system perturbed with alanine had the greatest gain of MGO.

The pK_a of the amine group in all amino acids lie between 8.80 (asparagine) and 10.60 (proline). Therefore in the acidic honey environment the amine group in all amino acids will predominately exist as NH_3^+, which is less reactive toward binding with other compounds but will readily be able to donate a H^+ for catalysis.[302] The protonated group is in equilibrium with the more reactive NH_2, hence a portion of the amino acid will be reactive and able to bind to compounds in the matrix. Furthermore the pK_a for carboxyl groups of amino acids range from 1.82 (histidine) to 2.83 (tryptophan), so will exist as COO^-. The state of the amino and carboxyl groups on the amino acids can be disregarded as a reason for the differences in DHA conversion to MGO in the various samples analysed because all amino acids have very similar pK_a values for their amine and carboxyl groups. It is reasonable to assume that the R group on amino acids influences their rate of binding to DHA or ability to convert DHA to MGO. Large R groups may sterically hinder an incoming molecule from getting close . Moreover, intra-molecular interactions between the R group and the amine in the amino acid may have an effect on the ability of the H^+ to be transferred. Bonsignore *et al.*[287] reported that in aqueous solution (pH 7.7) alanine was 50% more reactive in the conversion of glyceraldehyde to MGO than serine, due to H-bonding between NH_3^+ and OH in serine, which would prevent the H being as labile. While this is a basic solution, the amino acids will be in the same state as in honey, due to the pK_a values. Similar results were seen in this research – the DHA-alanine system had a larger rate constant than the DHA-serine system (0.0016 cf. 0.0010 day^{-1}). It also had twice the rate of the DHA-lysine system (0.0007 day^{-1}).

Alanine may be a good catalyst because it has a small R group (CH_3), that would not react with the amine or cause steric hindrance, making it easier for the amine group to donate H^+. Iglesias *et al.*[188] (2006) saw only a small change in alanine concentration over 6 to 24 months when honey was stored at ambient temperature. The authors suggested that although alanine is involved in Maillard reactions, its reactivity is lower than other amino acids. Sanz *et al.*[354] analysed free amino acids in control honey and honey stored at 25 and 35 °C. They also noted that alanine did not decrease as much as other amino acids over 12 months. This supports findings in the present research of more MGO produced in the sample with alanine than other amino acids, presumably due to less reactivity in the Maillard reaction and enhanced catalytic ability. It must be noted that the decline of amino

acids in mānuka honey may occur faster than other floral honeys due to the presence of DHA and MGO.

Weber[292] noticed that dehydration of DHA to MGO (pH 5.5, 50 °C, anaerobic) was catalysed by primary and secondary amino acids. Sterically unhindered amines were more efficient than ammonia, secondary amines and sterically hindered primary amines. Consequently, due to the influence of the R group, the rate of DHA disappearance in the system with the primary aliphatic amine compound will not directly reflect the rate at which amino acids may bind to DHA or catalyse its conversion to DHA as it would not have reduced binding/catalytic ability from either steric complications or intra-molecular interactions. The primary amine (section 5.3.3) had a larger rate constant (over double) for DHA loss and MGO gain than for any amino acid studied.

The efficiency of DHA converting to MGO was only 22 ± 7% in the initial reaction. The secondary reaction had an average efficiency of 54 ± 11%. This was calculated using [DHA] at the start of the second reaction. If $[DHA]_0$ was used, the average conversion was only 48 ± 1%. However, in a real honey the first initial reaction would not be observed. The efficiency of the DHA-alanine system is significantly higher than the control system (18%), which also suggests alanine prevents DHA from entering a dead end route or recovers it from one. The stoichiometric factor is 0.73, which is close to the efficiency if it is calculated at the start of the second reaction.

The DHA-alanine system turned a light fawn colour over time (similar to the proline sample). A study of colour formation of DHA with amino acids on filter paper reported that when DHA reacted with alanine, colour formation occurred within 1-5 hours with alanine, while colour development with aspartic acids took 1.5-2 days. Furthermore, the alanine sample changed from light brown on day 1 to dark brown on day 4.[355]

It is likely that valine, isoleucine and leucine (all with hydrophobic side chains like alanine) would behave in a similar manner to alanine because the only difference in the R group is an increasing number of carbons. Phenylalanine, tyrosine and tryptophan also have hydrophobic side chains; they all have an

aromatic ring which is not likely to affect the ability of the amino group to donate a proton, but may cause steric hindrance. These seven amino acids make up approximately 50% of the primary amino acids in the six mānuka honeys measured in section 4.3.7.

5.3.5.6 MGO-alanine system

An MGO-alanine system which contained 5 mmol/kg alanine and 29 mmol/kg MGO was studied to investigate if MGO and alanine were binding irreversibly. Over the 100 days that the sample was monitored there was no loss of MGO indicating that alanine does not react irreversibly with MGO or catalyse a reaction to a further product on this time scale in the reaction conditions. The sample was retested at 519 days and no MGO had been consumed. This sample behaved in the same manner as the MGO-control system (only MGO added to the artificial honey) and turned yellow over time.

Later stages of the Maillard reaction favour neutral or alkaline conditions, hence will not readily occur in a honey matrix. In addition, at the low temperature of incubation (37 °C) the reaction of side products would proceed slowly. Unlike DHA, MGO does not show any interaction with alanine. This is in contrast to Weber who states that primary amines react with MGO to form imidazole at pH 5.5 at 50 °C.[292]

5.3.5.7 DHA-lysine system

The DHA-lysine system (22 mmol/kg DHA, 5 mmol/kg lysine) had an initial fast reaction for DHA loss (0.0064 day^{-1} over 42 days), which is similar to the initial reaction for the DHA-alanine system (0.0067 day^{-1} over 24 days). The amount of DHA lost in this first reaction (5 mmol/kg) is the sample as the amount of lysine added. As noted for the systems perturbed by proline and alanine, the 1:1 reaction may be a coincidence due to the amount of monomeric DHA available in the system. The stoichiometric factor of the initial reaction is only 0.09, confirming DHA is involved in a side reaction or bound to lysine and is not converted to MGO.

The secondary rate constant, k_2, for loss of DHA (0.0017 day^{-1}) was lower than for the control and DHA-alanine systems but similar to the DHA-proline system, suggesting that lysine is not able to alter the equilibrium constant for DHA dimer. This rate constant is much slower than the reported rate constant for the conversion of glycealdehyde to MGO reported by Bonsignore *et al.*[287] who reported that at pH 7.7, with lysine in the solution the rate constant was 5.7240 day^{-1}. This suggests that lysine acts as a catalyst only in basic conditions.

It was expected that lysine would enhance the rate more than alanine due to the two amine groups (on the R group and the amino), but the rate constant for MGO formation was the same for both initial and secondary reactions (0.0006 day^{-1}) and was the same as for the control system. In comparison, Adams *et al.*[14] doped clover honey (a more complex matrix) with lysine and reported an increase in MGO. They used a similar concentration of DHA (2,490 mg/kg) as used in this research, but used a much larger concentration of lysine compared to this research (6.2 g/kg and 25 g/kg) which may have been large enough to allow some surplus lysine to act as a catalyst. They also reported a decline in MGO after time, presumably from MGO reacting with lysine in side reactions. The experiment in the current research was not carried out long enough to see if there was a decline in MGO, because it was set up later than other systems. The DHA:MGO ratio was 4:1 at the end of data accumulation and was not near the point where MGO side reactions would become prominent. Furthermore, work by Bonsignore *et al.*[294] (on aqueous systems at pH 7.7) reported that two amine groups spaced by at least 5 carbon atoms was necessary for complete catalysis of glyceraldehyde to MGO; the rate enhancement increased with increasing number of carbon atoms between the two groups, possibly due to the ease in which the molecule could fold to bring the two groups closer. Lysine has five carbon atoms between the two amine groups to allow folding. However, there was no rate enhancement in MGO formation in the DHA-lysine system, as anticipated.

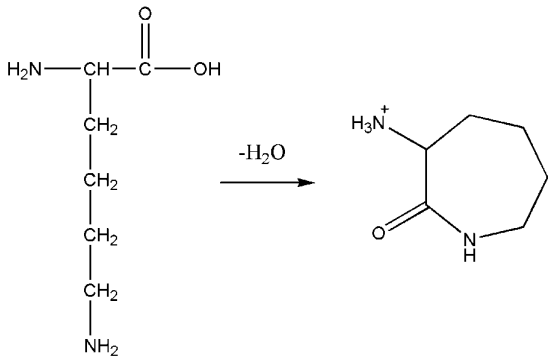
The stoichiometric factor for the secondary reaction was 0.41. This is almost double the percentage efficiency of the control sample (27 ± 8%), due to the smaller rate constant for DHA loss. There was some fluctuation in the calculated efficiency of the reaction, likely to be due to measurement error in DHA and

MGO, which would partially account for the difference between the stoichiometric factor and calculated efficiency.

The failure of lysine to show any measureable catalytic activity may be due to:

- irreversible reaction of lysine and DHA
- removal of MGO by lysine at the same rate at which it is formed
- an inherent property of lysine
- reaction of lysine with itself or another compound

The large R group ($CH_2CH_2CH_2CH_2NH_2$) of lysine may form an intra-molecular lactam with the COOH, or intra-molecular H bonding may occur. Lactams are known to form from lysine; D,L-lysine 1,6-lactam (also known as α-amino-ε-caprolactam, 3-amino-hexahydro-2-azepinone, or 3-aminoazepan-2-one) is produced by the amine on the end of the R group reacting with the OH in the carboxyl group to form a seven-membered ring (Figure 5.39). This may prevent lysine reacting in the matrix. This compound is known in the literature and has been synthesised; a patent by Frost[356] reports that upon heating lysine and NaOH in hexanol with removal of water, α-amino-ε-caprolactam was formed, after workup and treatment with HCl (75% yield). Preparations for α-amino-ε-caprolactam in the literature are carried out at high temperature[357-359] or by an enzyme,[360-361] hence this is unlikely to be the product in honey due to the harsh treatment required and the need for base. Therefore, the more likely possibility is H bonding between the OH and NH_2 on the R group which would prevent the H on the NH_2 from being labile. The amine NH_2 would still be available in both cases; however, the large ring would sterically hinder DHA from getting close.



Lysine L-lysine 1,6-lactam

Figure 5.39 Lysine may convert to L-lysine 1,6-lactam.

Another possible reason for the lack of catalytic ability of lysine is the high reactivity of lysine; Kawashima *et al.*[308] reported that lysine was the most reactive amino acid tested when reacted with DHA at pH 4 and 5 in aqueous solutions. The browning reaction increased with pH. Furthermore, lysine is more readily lost in food matrices compared to other amino acids due to its free ε-amino group. This substantial loss occurs before the formation of brown pigments.[267] Loss of lysine in food matrices due to non-enzymatic browning has been reported to occur at high temperature. This is greater when reducing carbohydrates are present above 2-5%. Significant loss of lysine (up to 40%) has been reported when the temperature is high (190-210 °C) and water content low (13%). However, at 18% moisture, the loss of lysine in much less.[305] Therefore lysine may be reacting in the honey matrix, but at a slower rate due to the temperature of storage and the water content of the matrix. A lysine-DHA complex may also be present.

Analysis of primary amino acids in the honeys used for the storage trials reported that lysine was present; the mānuka honeys tested had an average of 14.5 ± 7.4 mg/kg lysine in the samples. This is similar to the average if the clover honeys were also included in the calculation (14.7 ± 6.7 mg/kg). The similarity between fresh mānuka honey and clover honey is expected, but as storage time increases the amount of lysine will decrease.[354] Lysine may be lost more rapidly in mānuka honey if it reacts with DHA or MGO. The samples were not reanalysed part way through storage, so no conclusions can be drawn. Sanz *et al.*[354] examined the loss of amino acids by Maillard reactions in honey (formation of 2-furoylmethyl amino acids) over 12 months. Honey stored at 25 °C showed a loss of lysine (due to formation of Maillard products) compared to the control sample; 2-furoylmethyl amino acids formation was larger at 35 °C and there was no remaining lysine after 12 months in these samples. Furthermore they reported that 2-furoylmethyl concentrations decreased at later times due to conversion to advanced stage Maillard products. These results show that lysine is reactive in a honey matrix at low temperature, and is the most likely cause for lysine not having any measureable catalytic ability in the conversion of DHA to MGO.

Weber[293] reported a poly-lysine catalyst was poisoned during the course of a reaction (pH 5.5, 40 °C) due to reaction with the aldehyde reactants and products

in the solution. This may reflect what is occurring in the artificial honey system, due to the similar pH and the presence of MGO which has an aldehyde group. In contrast, in basic conditions (pH 7.7) Bonsignore *et al.*(1972)[287] reported that lysine was able to catalyse the conversion of glyceraldehyde to MGO (37 °C). Furthermore lysine was also reported to catalyse the conversion of MGO to a yellow product, which accounted for lack of stoichiometry. McLaughlin *et al.*[362] studied the effects of pH, time, solvent and chemical modification of arginine and lysine side chains of proteins with MGO. They reported that the primary step was the formation of a Schiff base (-HC=N-) linkage between the ε-amino group of a lysine side chain and the aldehyde group of MGO. The aldehyde acts an electron acceptor in charge transfer interactions with proteins. The reaction was reported to decrease below pH 7, hence may not be prominent in a honey matrix.

MGO is known to react with lysine to form methylglyoxal lysine dimer (MOLD, Figure 5.40);[150, 324, 363] but is reported to occur under physiological conditions.[113] Furthermore, in base (at 150 °C) lysine can also polymerise with itself due to the two amine groups having different reactivity which results in hyperbranched polymers.[364] However, to the authors knowledge, these reactions have not been reported under acidic conditions.

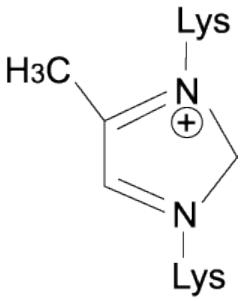


Figure 5.40 Methylglyoxal lysine dimer (MOLD).

5.3.5.8 DHA-serine system

The DHA-serine system (22 mmol/kg DHA, 5 mmol/kg serine) showed an initial fast reaction for DHA loss over 42 days where 5 mmol/kg of DHA was lost, which is equivalent to the amount of serine added. The initial rate constant for DHA loss was 0.0052 day^{-1} which was smaller than for the other three amino acids studied. However, the initial rate constant for MGO formation (0.0007 day^{-1})

was the same as the control system and those perturbed by proline and lysine. The stoichiometric factor was only 0.13.

The secondary rate constant for DHA loss was the same as for proline (smaller than the control sample) and the rate constant for the formation of MGO (0.0007 day^{-1}) was the same as the control system and those perturbed by proline and lysine (within experimental error), indicating serine does not enhance the conversion of DHA to MGO. The R group on serine (CH_2OH) is likely to interact with the amino group by H bonding; intra-molecular bonding will occur in low water activity. This prevents the H on the amine group from being labile, hence it will not be a good catalyst. Furthermore, the internal bonding makes the compound ring-like, hence there will be a steric effect. The rate constant for MGO appearance was approximately 50% greater for the system perturbed with alanine compared to the system perturbed with serine. This was also noted by Bonsignore *et al.*[287] who reported alanine was 50% more reactive than serine.

The DHA-serine system was set up later than most of the other systems so did not have a chance to reach completion. At the end of data analysis the DHA:MGO ratio was 4:1. Therefore if any MGO side reactions were being catalysed by serine, these had not yet become prominent.

The stoichiometric factor for the secondary reaction was 0.50. The calculated percentage efficiency for the secondary reaction (38 ± 12%) was lower. There was a lot of variation in the calculated efficiency of the reaction, likely to be due to measurement error in DHA and MGO, which would account for the fluctuation and large standard deviation.

Analysis of primary amino acids in six mānuka and four clover honeys used in the storage trials had an average of 10.8 ± 3.1 mg/kg serine; the average was the same if the average was calculated for only the mānuka honeys.

5.3.5.9 DHA-iron system

A sample with iron (870 mg/kg, 3 mmol/kg) and DHA (2,000 mg/kg, 22 mmol/kg) only had one rate of DHA loss suggesting that there is no interaction

between DHA and iron binding to each other, or catalysis to a side product similar to that seen for the amino acids. In addition, the sample did not change colour during storage. The rate constant for DHA disappearance (k) was 0.0047 day^{-1}, which is larger than the control (0.0025 day^{-1}), and is the largest rate constant for the secondary reaction in systems with individual compounds added. The rate constant for MGO appearance (k') was also larger than the control (0.0015 day^{-1} compared to 0.0006 day^{-1}).

The DHA:MGO ratio was less than 1:1 at the end of data accumulation, but there was no deviation in MGO when plotted against time, indicating iron was not reacting with MGO or catalysing a side reaction.

On average the efficiency of the conversion was 30 ± 15%, which is similar to the stoichiometric factor (0.32). This is higher than the efficiency of the control sample by a factor of 2. Iron may be able to shift the equilibrium of the dimer towards the monomer easier than amino acids because it will not form a side product with DHA, hence a higher proportion will be readily avaliable. Therefore monomeric DHA is avaliable to convert to both MGO and side products, hence the efficiency is low.

These results indicate that there is some truth to the folklore that doping honey with iron will speed up the conversion of DHA to MGO. However, because this concentration of iron is not naturally occurring in mānuka honey the effect of iron is probably negligible in typical commerical honeys.

5.3.5.10 DHA-proline-alanine system

An artificial honey with DHA (2,000 mg/kg, 23 mmol/kg) proline (800 mg/kg, 7 mmol/kg) and alanine (400 mg/kg, 5 mmol/kg) was studied. This is 66% proline, 33% alanine (primary amino acid). The ten honeys analysed for amino acids had an average of 74 ± 6% proline and 26 ± 6% primary amino acids, which is similar to the model system here and should approximate the effect of the primary and secondary amino acids in honey; although, as discussed above, not all primary amino acids have an effect on DHA loss or MGO formation.

The DHA:MGO ratio for the proline-alanine system was 0.7:1 at the end of data accumulation, hence side reactions with MGO are prominent (Figure 5.41); the MGO concentration reached a maximum, then started to decline. This decline is larger in the DHA-alanine-proline system compared to the DHA-proline or DHA-alanine systems, due to the DHA conversion to MGO proceeding faster, hence further reactions of MGO at later time are more noticeable. The decline observed is analogous to that which occurs in real honey matrices. This has been discussed in detail in chapter 6.

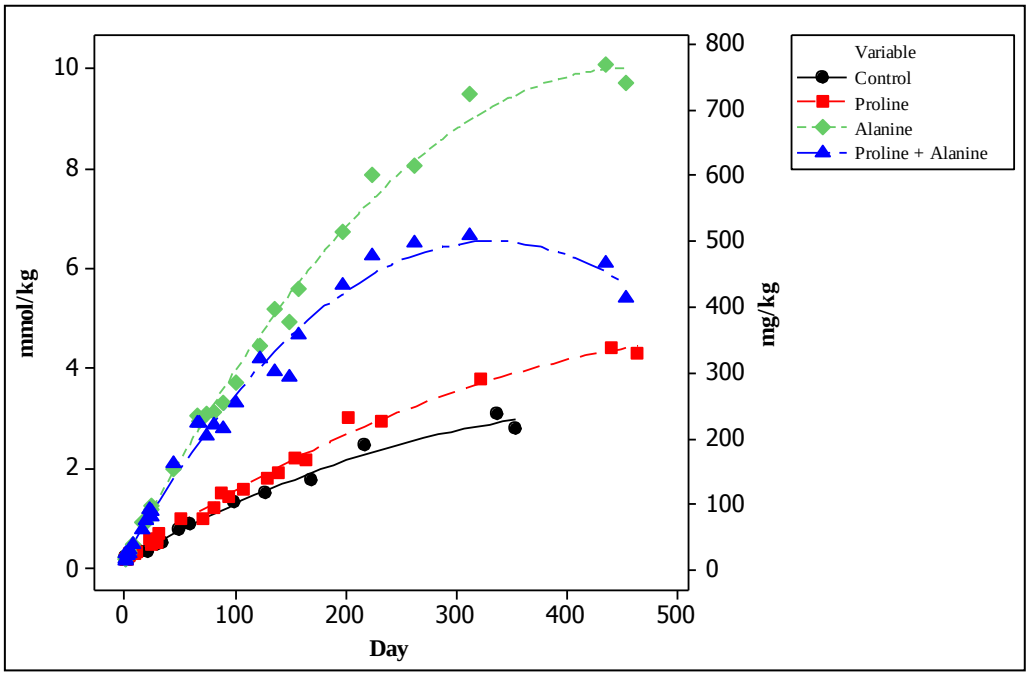


Figure 5.41 Appearance of MGO vs. Day at 37 °C. Alanine (5 mmol/kg, diamonds) accelerates the appearance of MGO compared to proline and alanine (8 and 5 mmol/kg respectively, triangles), proline (8 mmol/kg, squares) and the control (circles). The DHA-proline-alanine system shows the MGO reaches a maximum then begins to decline.

The DHA-proline-alanine system showed an initial rapid loss of DHA over approximately 44 days. However, unlike for the systems with individual perturbants this loss did not correspond to the summation of the two perturbants. In total 12 mmol/kg of the two perturbants were added but only 5 mmol/kg DHA was lost. This supports the idea of limited monomeric DHA in the system which proline and alanine are competing for.

The initial fast reaction was also seen in the first-order rate plot. The initial rate constant of DHA loss (k_1) was 0.0068 day^{-1}; however, there was some scatter in

the data which may affect the reported result. It was assumed that the loss would be the summation of the rate constants for the systems with the individual perturbants, but this was not observed. The rate constant for loss of DHA in the system with two perturbants was smaller than the DHA-proline system (0.0075 day^{-1}), but the same as the DHA-alanine system (0.0067 day^{-1}), possibly due to competition for limited monomeric DHA.

The secondary rate constant for DHA disappearance was 0.0031 day^{-1} which is the same as the addition of the individual rate constants for loss of DHA in DHA-alanine and DHA-proline systems (within experimental error); hence an accumulation effect and not synergy is observed.

The rate constant for the appearance of MGO in the initial reaction (k_1') was 0.0020 day^{-1} which is faster than for the DHA-proline system (0.0006 day^{-1}) and the same as the alanine system (0.0022 day^{-1}). The first-order plot for MGO appearance deviated from linearity at later times. The last data points were removed so that a secondary rate constant of MGO appearance could be calculated from the linear region, this was 0.0014 day^{-1}, which is similar to the DHA-alanine system (0.0016 day^{-1}) and larger than the proline system (0.0006 day^{-1}). No accumulative effect was seen, since proline does not appear to enhance MGO formation.

The stoichiometric factor was 0.45 and the average efficiency of conversion was 39 ± 7%. The decline in MGO did not affect the average efficiency at this point in time; however, over time more MGO would be consumed and the average efficiency would decrease. Weber[292] also noted that the MGO concentration increased to a maximum then decreased at a later time due to its consumption in another pathway. The author stated that this indicates that observed MGO concentration is a steady-state concentration resulting from the difference between the rate of product formation and rate of product removal by another reaction.[*]

[*] A steady state is an unvarying condition in a physical process. In the honey matrix MGO superficially takes on the appearance of a steady state in a certain portion of the reaction when the amount of MGO formed is equal to the amount of MGO lost. However, it does not obey a steady state over the entire reaction so cannot be classed as a steady state in the true sense of the definition.

Proline is able to catalyse the three-component Mannich reaction, between an aldehyde, ketone and amine.[335] This may occur in the DHA-proline-alanine sample and be one cause of the MGO disappearing at later times.

5.3.5.11 DHA-alanine- potassium phosphate system

A system with DHA (23 mmol/kg), alanine (5 mmol/kg) and potassium phosphate (2 mmol/kg) was created to examine the possibility that phosphate enhances the conversion of DHA to MGO in an acidic medium. This sample was compared to the DHA-alanine system (Figure 5.42); however, a DHA-potassium phosphate system was not set up. In addition, a system with another cation was not used (e.g. calcium phosphate) to identify if the effect was from potassium or phosphate.

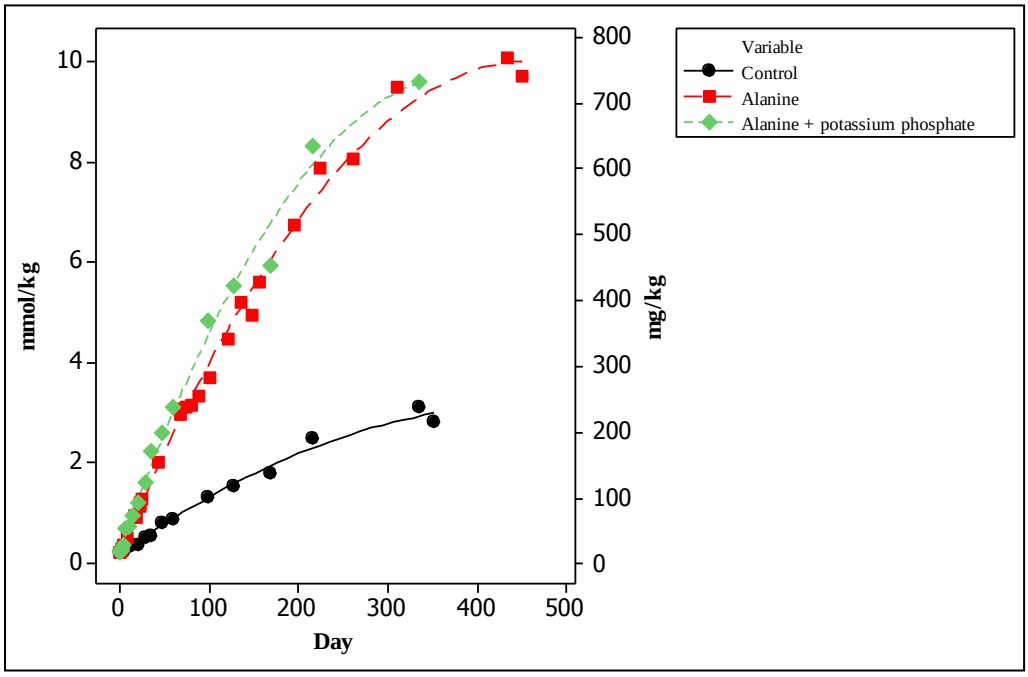


Figure 5.42 Appearance of MGO vs. day at 37 °C for artificial honeys perturbed by alanine (5 mmol/kg) or alanine and potassium phosphate (5 mmol/kg and 2 mmol/kg respectively); samples started with DHA (22 mmol/kg).

The DHA-alanine- potassium phosphate system did not have a break in the DHA disappearance, indicating that potassium phosphate may prevent alanine binding quickly to DHA or removing it in a side reaction. Potassium phosphate will not bind to DHA, hence it could convert dimeric DHA to monomeric DHA, similar to that seen for iron. Therefore even though alanine binds to DHA, dissociation of the dimer can still occur. However, the disappearance of DHA in the sample with

alanine and phosphate was the same within experimental error as for the sample with only alanine (k_2 = 0.0026 cf. 0.0021 day^{-1}), indicating that potassium phosphate does not affect the secondary rate constant for loss of DHA.

The sample reached a ratio of 0.7:1 DHA:MGO at the end of data accumulation and showed the same slight deviation in MGO at later times as the DHA-alanine system. The rate constant for formation of MGO was also the same as for the system perturbed with alanine within experimental error (0.0020 day^{-1} compared to 0.0016 day^{-1}), indicating that potassium phosphate does not enhance the conversion of DHA to MGO. This is in contrast to Bonsignore *et al.* [287] who reported that there was a co-operative effect of lysine and phosphate in a physiological system with glyceraldehyde. This may be due to the pH at which the reaction was carried out (pH 7.7) or the amino acid used and the influence of the R group. The phosphate may prevent lysine from reacting with itself. Furthermore, Riddle and Lorenz[105] reported that at pH 7.4 phosphate catalysed the conversion of DHA to MGO. They reported that in aqueous solutions with a pH above 6, the conversion of DHA to MGO in the presence of phosphate was first-order with respect to the phosphate concentration. At the lowest phosphate concentration, the reaction was linear for the first six hours, compared to the other samples which began to level off. It appears that this catalytic effect does not occur in the acidic honey medium.

The efficiency of the reaction (70 ± 20%) was within experimental error with the alanine sample (50 ± 10%). The stoichiometry factor was high (0.77) and similar to the alanine sample (0.73). In comparison, Riddle and Lorenz reported a maximum net conversion of DHA to MGO was only 25% efficient in an aqueous solution (pH 7.4) with phosphate.[105] The sample with alanine and potassium phosphate turned a light yellow, indicating a reaction – possibly due to alanine reacting with DHA or sugars reacting in unrelated pathways.

5.3.5.12 DHA-proline-iron system

The DHA-proline-iron system (23 mmol/kg DHA, 8 mmol/kg proline and 3 mmol/kg iron) did not have a break in the first-order plot for the disappearance of DHA, similar to the systems perturbed by iron and alanine + potassium phosphate.

The system reached a DHA:MGO ratio of 0.5:1 at the end of data accumulation; it would be expected that further reactions of MGO would be prominent at this time; however, only a slight deviation in the [MGO] vs. t plot (Figure 5.43) and first-order plot were observed. Therefore iron appears to protect DHA and MGO from side reactions that are catalysed by proline. This is in comparison to two reactions (initial and secondary) observed when proline is the only compound in the system. The loss of DHA in the DHA-proline-iron system has a rate constant of 0.0040 day^{-1}, which is within experimental error compared to the sample perturbed by iron only (0.0047 day^{-1}), but it is faster than the secondary proline system (0.0014 day^{-1}).

The rate constant for MGO gain is 0.0017 day^{-1} which is the same as the rate constant for iron (within experimental error), showing that there is no accumulative effect of proline and iron occurring in the formation of MGO.

The efficiency of the reaction was 40 ± 8%, which is the same as the DHA-proline system but slightly higher than for the system perturbed with iron (30 ± 15%). The stoichiometric factor is the same as for the system perturbed by iron (0.43).

Proline may coordinate to the iron instead of acting as a catalytic species. Šišková *et al*.[365] suggested that amino acids become part of the solvation sphere of iron. They hypothesised that at pH 3, glutamic acid could replace water in the solvation sphere (Figure 5.44). It is probable that proline can also coordinate to the solvation sphere by the COO^- group. Iron is known in biology to chelate readily to functional groups. This includes amino acids side chains (e.g. amine, carboxylate and imidazole). Fe^{III} is a hard Lewis acid and prefers ligation to hard Lewis base donors whereas Fe^{II} is a borderline soft Lewis acid and prefers ligation to soft Lewis base donors (e.g. S and pyrrole N).[366] The degree of complexation of iron is influenced by the ligand pK_a and pH of the matrix. Ferrous glycinate is 4-coordinate iron with 2 glycine coordinated by carboxyl and amino groups. It is stable between pH 3 and pH 10; either side of this the chelate bonds are weakened.[367] Citric acid has been reported to protect ferrous iron from oxidation and has been used during preparation of amino acids chelated to iron.[367-368]

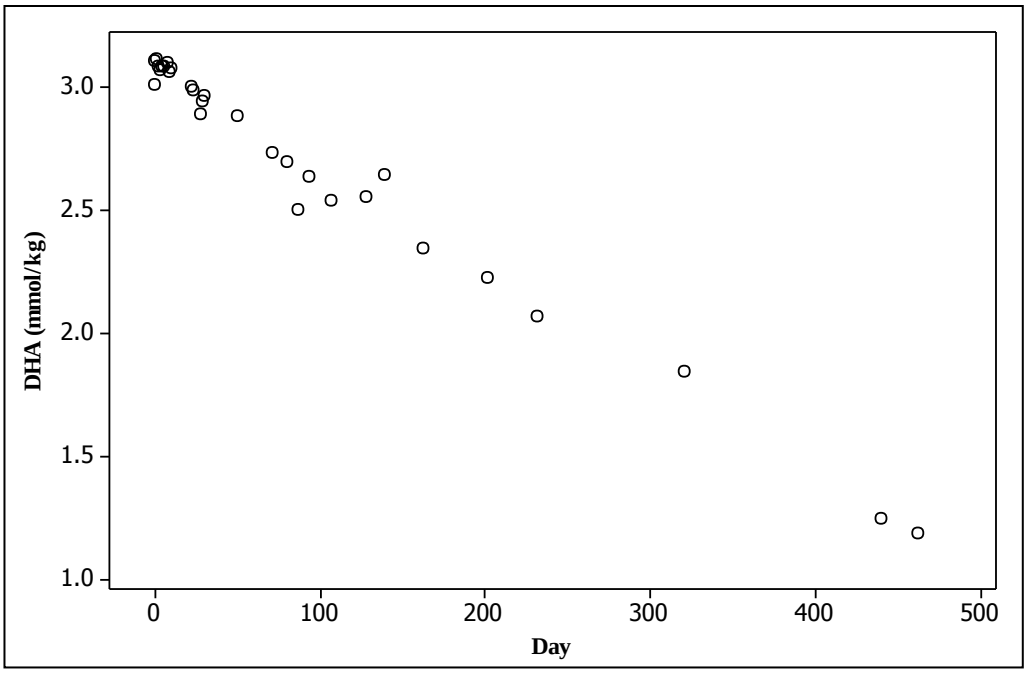


Figure 5.43 Decrease of DHA (23 mmol/kg initial concentration) in artificial honey perturbed by proline (8 mmol/kg, 880 mg/kg) and iron (3 mmol/kg, 900 mg/kg). There is not two rates of reaction caused by an initial loss of DHA from rapid reaction with proline suggesting that iron protects DHA from binding to or reacting with proline.

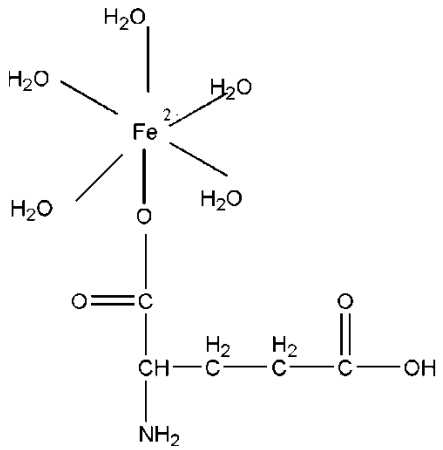


Figure 5.44 Example of glutamic acid in iron coordination sphere.

Adapted from Šišková *et al.*[365]

5.3.5.13 DHA-alanine-iron system

The DHA-alanine-iron system (23 mmol/kg DHA, 5 mmol/kg alanine and 3 mmol/kg iron) showed two rates of DHA loss. The first reaction occurs up to 10 days, with a total of 5 mmol/kg DHA lost. This is equivalent to the amount of

alanine added. The rate constant for disappearance of DHA in the initial reaction is 0.0119 day^{-1} which is larger than the initial rate constant seen for just alanine (0.0067 day^{-1}) over 24 days. In comparison, the DHA-proline-iron system only had one rate of DHA disappearance, suggesting that iron has more of an effect on proline than alanine. However, the initial reaction in the DHA-alanine-iron system is small and not very prominent so a rate constant for DHA loss was calculated over the entire range to examine if there was any influence. The rate constant was 0.0042 day^{-1} which is no different, within experimental error, to the secondary rate constant (0.0037 day^{-1}). This rate constant is between that of the systems perturbed by the individual compounds. This may arise from only a limited amount of DHA available in the monomeric form.

The rate constant for the initial formation of MGO was 0.0037 day^{-1} and the secondary reaction was 0.0026 day^{-1}. This secondary rate constant is the same, within experimental error, as for the addition of the rate constants of the systems with the individual perturbants (0.0030 day^{-1}). This suggests that there is no cooperative effect from alanine and iron on the formation of MGO.

The stoichiometric factor for the initial reaction was only 0.36, but increased to 0.70 in the second reaction. The calculated efficiency, using [DHA] at the start of the secondary reaction, was 86 ± 12% which is slightly higher than calculated by the stoichiometric factor. This is similar to the addition of the efficiencies of the systems perturbed by the individual compounds. If $[DHA]_0$ was used then the calculated efficiency was only 59 ± 3%.

5.3.5.14 DHA-proline-alanine-iron system

The DHA-proline-alanine-iron system (23 mmol/kg DHA, 7 mmol/kg proline, 5 mmol/kg alanine and 3 mmol/kg iron) is expected to have the largest rate constant for DHA disappearance and MGO gain of all systems examined because it has the most perturbing species. Only one rate of DHA disappearance was seen possibly due to the matrix containing multiple compounds that can alter the equilbrium constant for the dissociation of the dimer. The rate constant for DHA disappearance was the largest for all systems (0.0056 day^{-1}), but was not as large as the accumulative rate of all three individual systems (0.0082 day^{-1}). The rate

constant for MGO gain (0.0024 day^{-1}) was higher than the DHA-proline-iron system (0.0017 day^{-1}), similar, within experimental error, to the DHA-proline-alanine system (0.0029 day^{-1}) and lower than DHA-alanine-iron system (0.0031 day^{-1}).

The DHA:MGO ratio was 0.5:1 at the end of data accumulation, hence side reactions have become prominent and the first-order plot for MGO appearance deviated from linearity at later time. On average, 35 ± 10% of DHA converted to MGO; this is slightly lower than the calculated stoichiometric factor (0.43).

5.3.6 Conversion of DHA to MGO in artificial honey matrices at 20 and 27 °C with added perturbants

Artificial honey systems were perturbed with various amino acids (proline, alanine, lysine and serine) and stored at 20 and 27 °C for comparison to the systems at 37 °C. The systems stored at these temperatures behaved similarly, so are discussed together. Possible reactions that may occur were discussed in section 5.3.5 and these reactions are expected to occur at the lower temperatures, albeit slower; hence discussion of these reactions will not be repeated here. As expected, the samples stored at the lower temperatures (20 and 27 °C), had smaller rate constants for DHA loss and MGO than samples stored at 37 °C. It is unknown whether or not the dimer ⇌ monomer equilibrium of DHA would alter depending on the temperature. Investigation of this is currently being carried out in a separate project.

There was scatter in the reported DHA concentration early in the reaction, which most likely arises from a longer equilibration time at lower temperatures. It may take longer for the molecules to initially distribute in the matrix; this phenomenon was also seen in the real honey matrices doped with DHA. This made it difficult to obtain a first-order rate constant for DHA loss. There was no scatter in the reported concentration of MGO at any time in the reaction. The error in the rate constants were the same as for the higher temperature (0.0004 day^{-1} for DHA and 0.0002 day^{-1} for MGO); this was discussed in section 5.3.1.4.

A complication arose with the reported concentration of DHA for some of these systems; the UV lamp in the detector blew shortly after starting some of the artificial honey trials at 20 and 27 °C. After replacement of the bulb, the reported concentration of DHA was higher than expected. An example of the problem in DHA concentration is shown in Figure 5.45 for artificial honey perturbed by lysine. A natural increase in DHA is not possible because the conversion to MGO is an irreversible reaction. Reported concentrations of HMF (279 nm) and MGO (244.3 nm, Figure 5.46) were not affected. Furthermore the peak area of HA, which was measured at the same wavelength as DHA (260 nm) was not affected, hence it is unlikely to be due to the replaced UV bulb. The DHA may have been incorrectly calibrated – although further calibrations also showed the same phenomenon. Therefore the origin of this problem is unknown. This problem affected the control samples and those perturbed by alanine, proline, lysine and serine for samples stored at 20 and 27 °C (20 samples in total); however, the systems perturbed by alanine and proline did not have an increase in DHA concentration as big as systems perturbed by lysine and serine.

A correction calculation was not used to adjust the DHA data because not enough data points were collected on each side of the discrepancy and it is unknown whether a break in the reaction naturally occurred (similar to that seen for samples at 37 °C). Secondary rate constants were still able to be calculated for all samples because data after the DHA calibration complication was used; although the absolute concentration was incorrect, the linear trend is expected to be correct. Systems perturbed by lysine and serine have enough data points on either side of the break to calculate both the initial and secondary rate constants. However, samples perturbed by alanine and proline do not have enough data points to calculate before the break. Hence it is unknown whether an initial fast reaction relating to the added perturbant occurred, similar to that seen in systems stored at 37 °C. Other systems were not affected as they were either no longer measured or had not yet been set up.

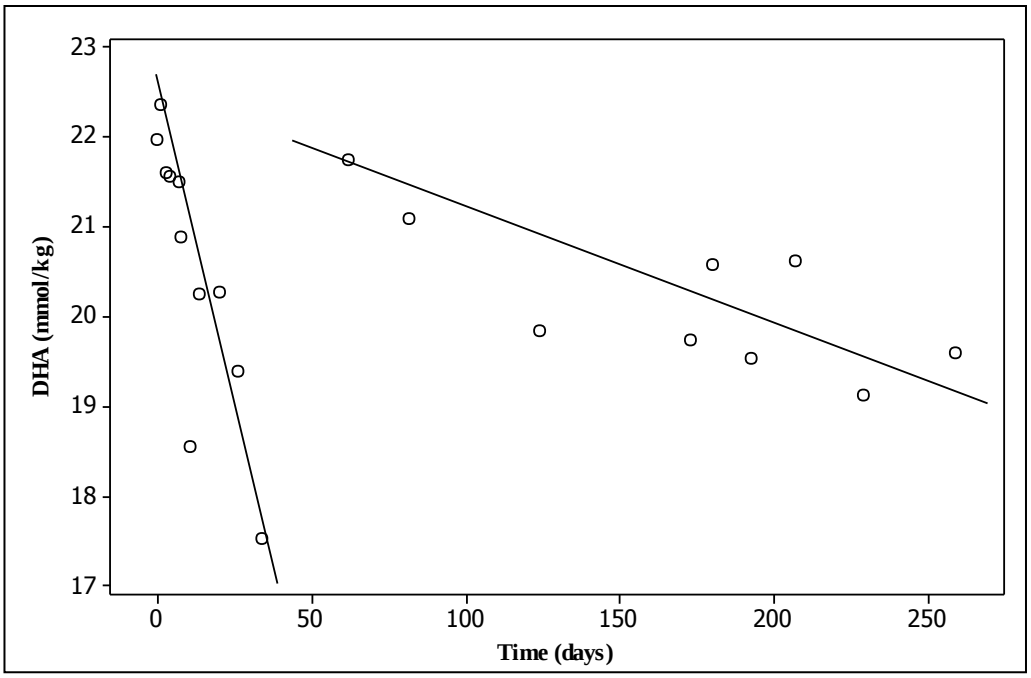


Figure 5.45 DHA vs. time for artificial honey system perturbed by lysine. There is a break in the DHA concentration at 50 days which corresponds to a new UV lamp.

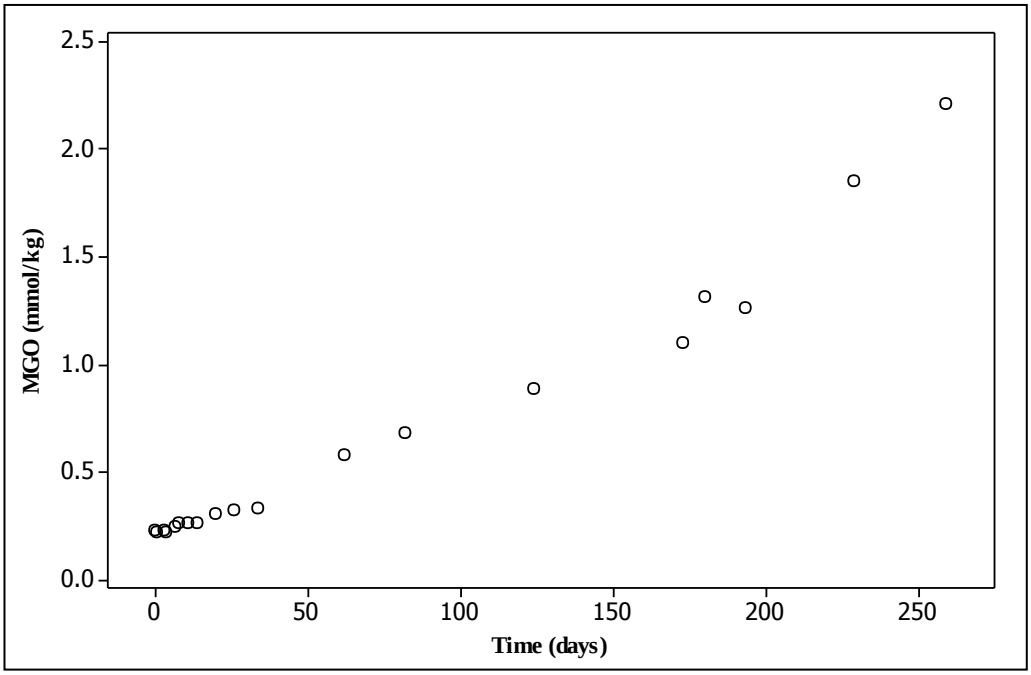


Figure 5.46 MGO vs. time for artificial honey perturbed by lysine and stored at 27 °C.

Another complication for this set of data was that the 27 °C oven was found turned off 190 days into the lysine and serine storage trials and 179 days into the control, alanine and proline storage trials. Samples had been tested on day 180 and 169 days respectively prior to this, hence the oven would have only been off for a maximum of 10 days. When discovered, the temperature was 23 °C. This should not affect the reaction substantially because other samples show that between 20-27 °C there is very little difference in the rate of conversion.

A summary of the rate constants for samples stored at 27 and 20 are given in Table 5.5 and Table 5.6 respectively. The same trends observed at 37 °C were also observed at the lower temperatures. Appendix D summarises the starting concentrations for samples stored at 27 and 20 °C. As expected, the rate constants were smaller than for samples at 37 °C, but there was not enough variation in the data between 20 and 27 °C to calculate the activation energies for each system.

Table 5.5 Summary of rate constants for first-order disappearance of DHA and appearance of MGO in artificial honey with 2,000 mg/kg DHA added and stored at 27 °C.

Perturbant	Initial reaction			Secondary reaction		
	Rate constant for DHA disappearance (k'_1, day^{-1})	Rate constant for MGO appearance (k'_1, day^{-1})	Stoichiometric factor ($x_1 = k'_1/k_1$)	Rate constant for DHA disappearance (k'_2, day^{-1})	Rate constant for MGO appearance (k'_2, day^{-1})	Stoichiometric factor ($x_2 = k'_2/k_2$)
Control				0.0010	0.0004	0.40
Proline				0.0008	0.0003	0.38
Alanine				0.0008	0.0008	1.00
Lysine	0.0048	0.0002	0.04	0.0004	0.0004	0.80
Serine	0.0035	0.0003	0.09	0.0007	0.0004	0.57
Proline + Alanine	0.0030	0.0007	0.23	0.0011	0.0008	0.72

†The stoichiometric factor ($x_2 = k'_2/k_2$) calculates the efficiency of the conversion of DHA to MGO. This is calculated as the gross percentage of MGO gained relative to the amount of DHA loss and takes into account all side reactions.

Table 5.6 Summary of rate constants for disappearance of DHA and appearance of MGO in artificial honey with 2,000 mg/kg DHA added and stored at 20 °C.

Perturbant	Initial reaction			Secondary reaction		
	Rate constant for DHA disappearance (k'_1, day^{-1})	Rate constant for MGO appearance (k'_1, day^{-1})	Stoichiometric factor ($x_1 = k'_1/k_1$)	Rate constant for DHA disappearance (k'_2, day^{-1})	Rate constant for MGO appearance (k'_2, day^{-1})	Stoichiometric factor ($x_2 = k'_2/k_2$)
Control				0.0008	0.0001	0.13
Alanine				0.0010	0.0005	0.50
Lysine	0.0040	0.0002	0.05	0.0004	0.0003	0.75
Serine	0.0038	0.0002	0.05	0.0003	0.0003	1.00

† The stoichiometric factor ($x_2 = k'_2/k_2$) calculates the efficiency of the conversion of DHA to MGO. This is calculated as the gross percentage of MGO gained relative to the amount of DHA loss and takes into account all side reactions.

5.3.6.1 DHA control system

The control system contained approximately 2,000 mg/kg DHA (22 mmol/kg) DHA. It is expected that the loss of DHA will be linear throughout the entire reaction, similar to that seen for the control system stored at 37 °C, because there are no perturbants in the matrix for DHA to react with. Therefore the data collected after the measurement difficulty was used to calculate the rate constant for loss of DHA. At 27 °C, the first-order rate constant for loss of DHA is 0.0010 day^{-1}, which is approximately half that seen for the control system stored at 37 °C (0.0025 day^{-1}). As a rule of thumb, for a first-order reaction, the rate of a reaction doubles with every 10 °C increase in temperature.[369] This holds true for the loss of DHA in the honey matrix. The first-order rate constant for the gain of MGO is 0.0004 day^{-1}, which is almost half of the rate constant for samples stored at 37 °C (0.0006 day^{-1}), but due to such small values, it is also within experimental error. The stoichiometric factor, x, is 0.40. This is higher than for the control stored at 37 °C (x = 0.24), suggesting that at the lower temperature side reactions are not as prominent, allowing more DHA to convert to MGO. The DHA/MGO ratio was still 13:1 at the end of data accumulation, which indicates that the reaction is not near completion.

At 20 °C, the rate constant for the loss of DHA was 0.0008 day^{-1}, which is within the experimental error (0.0004 day^{-1}) for the 27 °C data. The rate constant for MGO appearance was 0.0001 day^{-1}, which is smaller than for 27 °C data, but is still within experimental error. The stoichiometric factor was 0.13, which is lower than for samples at the higher temperatures. The DHA/MGO ratio was still 18:1 at the end of data accumulation, which indicates that the reaction is not near completion.

5.3.6.2 MGO control system

Artificial honey with only MGO (approximately 2,000 mg/kg, 28 mmol/kg) was stored at 20 and 27 °C. There was no change in MGO concentration over 125 days, indicating that MGO does not react irreversibly with itself or the matrix at these temperatures. This was also observed in the 37 °C data. Furthermore, similar to the MGO-control sample stored at 37 °C, the MGO-control sample stored at 27 °C was more viscous than samples that started with DHA.

5.3.6.3 System perturbed by proline

DHA-proline systems stored at 20 and 27 °C contained approximately 2,000 mg/kg (22 mmol/kg) DHA and 830 mg/kg (7 mmol/kg) proline. The systems perturbed by proline were affected by the aforementioned measurement complication. Therefore it is unknown whether or not a fast initial reaction occurred and if it did, if it had the same rate constant as the sample at the higher temperature. Furthermore, due to time restraints not enough data was obtained to get an accurate rate constant at 20 °C, hence they cannot be reported here.

At 27 °C, the secondary rate constant for the loss of DHA was 0.0008 day^{-1}, which is smaller than for the control sample. This was also observed when examined at 37 °C, but it is still within experimental error (0.004 day^{-1}). The rate constant is approximately half the rate constant for the same system stored at 37 °C.

The rate constant for the gain of MGO was 0.0003 day^{-1}, which is the same as for the control sample, but is a factor of 2 times smaller than the reported rate constant at 37 °C. The stoichiometric factor was 0.38, which is similar to the control sample but slightly lower than that calculated for the DHA-proline system stored at 37 °C (0.43). The DHA:MGO ratio was 9:1 at the end of data accumulation, indicating that the reaction has not neared completion.

A plot of MGO vs. time for the three temperatures examined (Figure 5.47) shows that there is not a large difference in gain of MGO between 20 and 27 °C compared to 27 and 37 °C. The samples at the two lower temperatures were not analysed for as long as the system at 37 °C, hence it is unknown whether or not the samples at the lower temperatures will plateau or if MGO will continue to increase linearly and exceed the concentration of MGO in the 37 °C system. If the lines for 20 and 27 °C data are extrapolated linearly, they will exceed the concentration of MGO in the 37 °C system around 400 days.

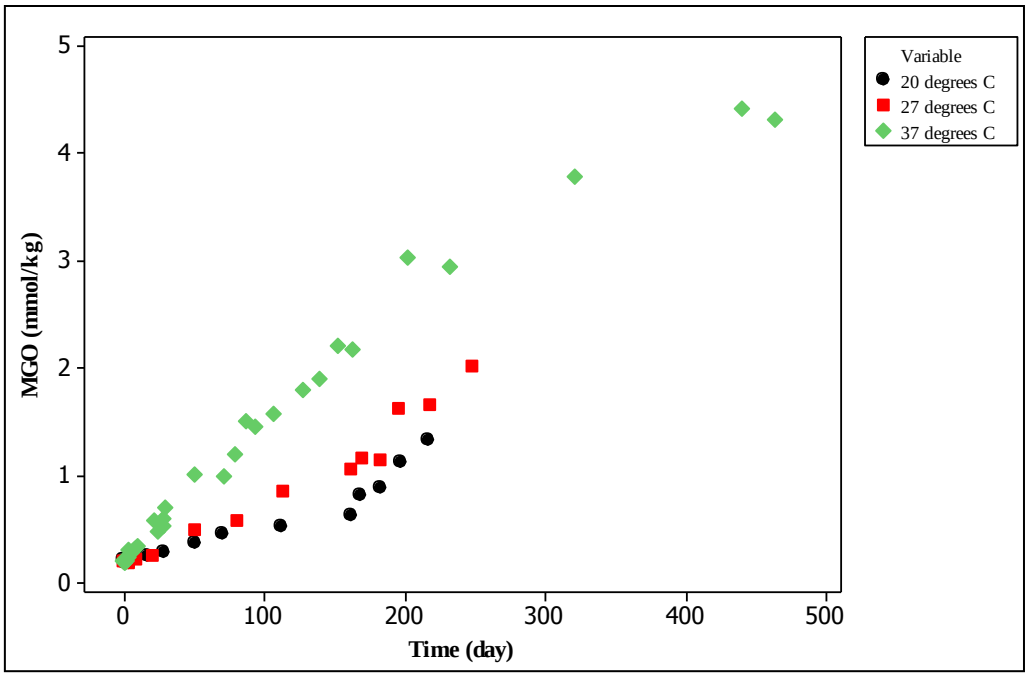


Figure 5.47 MGO vs. time for DHA-proline system. The difference between 20 and 27 °C is not as large as the difference between 27 and 37 °C.

5.3.6.4 System perturbed by alanine

The DHA-alanine systems stored at 27 and 20 °C both contained approximately 2,000 mg/kg (22 mmol/kg) DHA and 430 mg/kg (4 mmol/kg) alanine. The DHA measurements in this system (Figure 5.48) were not influenced as much as the lysine system. There does not appear to be a fast initial reaction; at a lower temperature side reactions may not occur as readily hence will not be so prominent. However, there are not enough data points in the first part of the graph to calculate a rate constant or draw any conclusions. The rate constant for DHA loss was calculated using only data after the known problem with DHA, in case there was some influence.

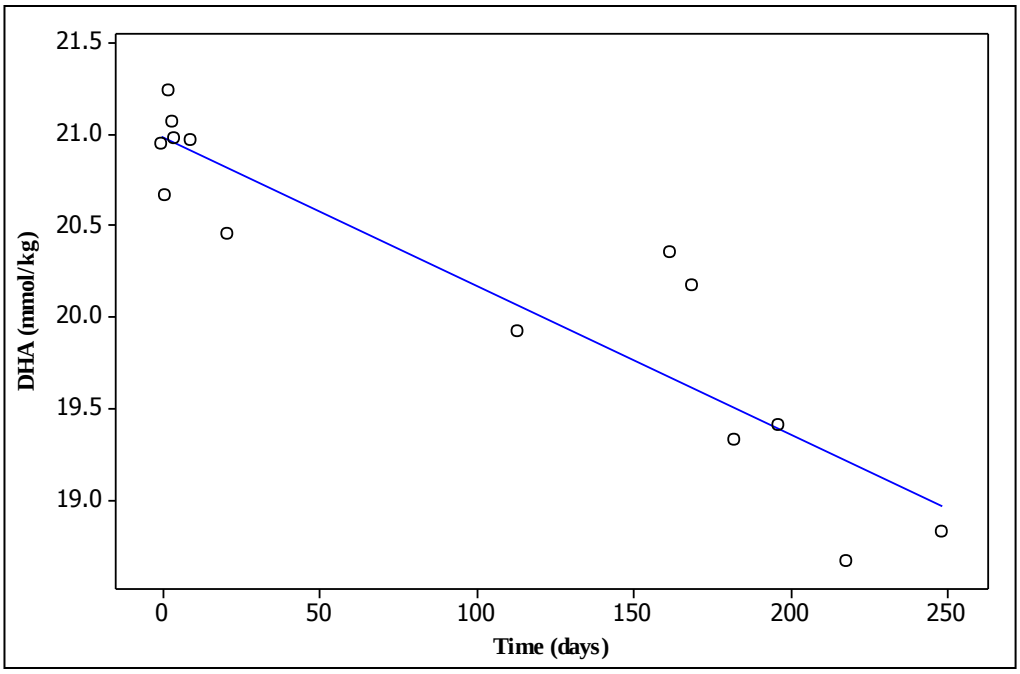


Figure 5.48 DHA (mmol/kg) vs. time for systems perturbed by alanine and stored at 27 °C. There does not appear to be a break in the loss of DHA which corresponds to the amount of alanine added.

At 27 °C, the rate constant for the loss of DHA is 0.0008 day^{-1}, which is similar to the control sample stored at 27 °C. The control system and that perturbed with alanine stored at 37 °C also had similar rate constants to each other for DHA loss. The rate constant for the sample at 27 °C is approximately 2.6 times smaller than for the system perturbed by alanine stored at 37 °C. The rate constant for the gain of MGO at 27 °C was 0.0008 day^{-1} which is double the rate constant for the control sample. Rate constants for DHA loss and MGO gain are half of the reported value for the rate constants in the DHA-alanine system stored at 37 °C.

Due to time restraints not enough data was obtained to get an accurate rate constant at 20 °C for the loss of DHA. ln[DHA] vs. time was plotted and although there was scatter (R^2 = 67%), the rate constant for DHA loss was obtained (0.0010 day^{-1}); however, this value needs to be treated cautiously. This value is similar to the DHA-alanine system stored at 27 °C and the control samples stored at both 20 and 27 °C. The rate constant for MGO gain must also be treated with caution because the rate constant for DHA loss was used in the calculation. The rate constant for MGO gain was 0.0005 day^{-1}; this is five times larger than the rate

constant for the control sample. As expected, the rate constant is slightly smaller than for the DHA-alanine system stored at 27 °C. A plot of MGO vs. time for each temperature (Figure 5.49) shows that as temperature increases, so does the formation of MGO, which is reflected by the rate constants. In addition, the difference between 20 and 27 °C is larger than that seen for the DHA-proline system.

The stoichiometric factor at 27 °C was 1, but needs to be treated with caution due to the scatter in the data. The stoichiometric factor at 20 °C was only 0.5, which is similar to that seen at 37 °C. At the end of data analysis the DHA:MGO ratio was 4.5:1 for the sample at 27 °C and 9:1 at 20 °C, which indicates both reactions still have potential for further conversion.

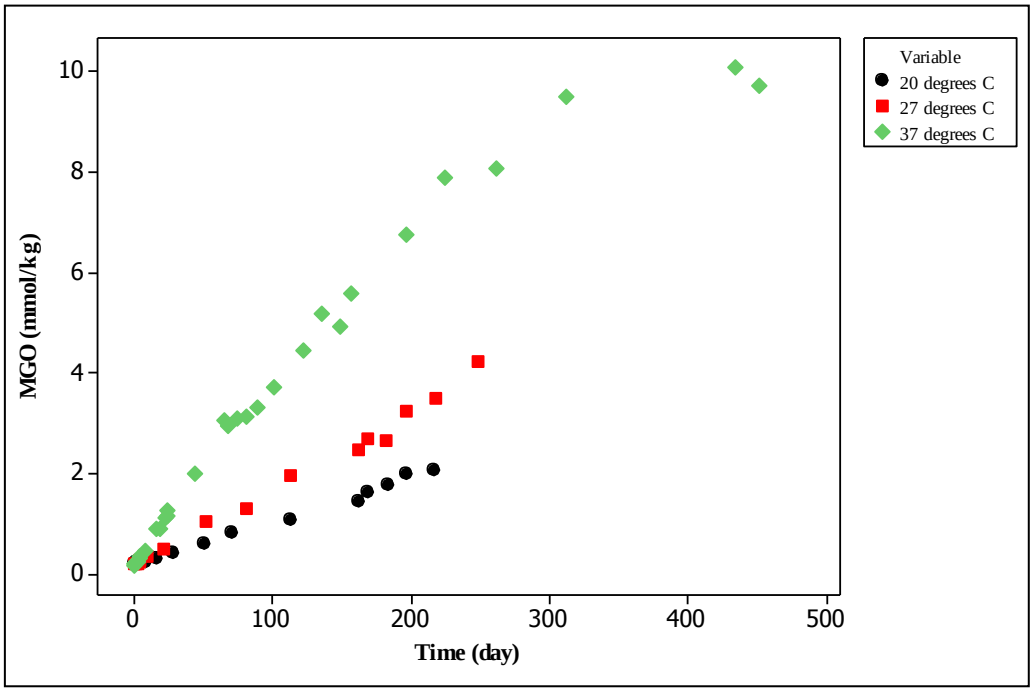


Figure 5.49 MGO vs. time for DHA-alanine systems stored at 20, 27 and 37 °C. There is an increase in MGO concentration with increasing temperature.

5.3.6.5 System perturbed by lysine

DHA-lysine systems contained approximately 2,000 mg/kg (22 mmol/kg) DHA and 700 mg/kg (5 mmol/kg) lysine. Data was collected for 34 days before the system was affected by the calibration problem. Therefore there was enough data

to calculate a rate constant for the initial reaction. However, the data to calculate k_2 was scattered and only one of the duplicate samples was used.

At 27 °C, the initial rate constant for loss of DHA was 0.0048 day^{-1}, this occurred over 34 days and removed 4.5 mmol/kg DHA, which is equivalent to the amount of lysine added. A similar result was seen for the DHA-lysine system stored at 37 °C which showed an initial fast reaction (0.0064 day^{-1}) over 42 days which lost a similar amount of DHA as the amount of lysine added and also at 20 °C (0.0040 day^{-1}), suggesting either a reaction between the two compounds or limited monomeric DHA available for conversion to a side product. However, it is unknown if this initial rate would have continued had there been no problem with the analysis of DHA.

The initial rate constant for MGO appearance (during the fast loss of DHA) was only 0.0002 day^{-1} for systems at 20 and 27 °C (0.0006 $days^{-1}$ at 37 °C) which suggest that DHA is being lost in a side reaction. The stoichiometric factor was only 0.04 and 0.05 for 27 and 20 °C data respectively.

At 27 °C, the secondary reaction for DHA loss had a much smaller rate constant (0.0004 day^{-1}) than the initial reaction. It was also smaller than the control sample; this phenomenon was also seen for some samples stored at 37 °C. The calculated initial and secondary reactions are different which indicates that there are two rates of reaction, similar to that observed at the higher temperature. If there was no change, the two rates would have been the same because the calculated slope for DHA loss would be the same despite the reported concentration of DHA being higher in the second part.

The rate constant for appearance of MGO was the same as the control sample (0.0004 day^{-1}), which was also seen for the samples held at 37 °C. The stoichiometric factor was 0.80. Samples stored at 20 °C had similar secondary rate constants for DHA loss and MGO gain as were seen for the sample stored at 27 °C. However, when MGO vs. time is plotted for the three temperatures (Figure 5.50) there is a difference between the 20 and 27 °C data which is not reflected in the rate constant. This may be due to the complication in reporting the concentration of DHA for these samples.

The DHA:MGO ratio was 9:1 for the sample at 27 °C and 14:1 for the sample at 20 °C, indicating that both reactions are not near completion.

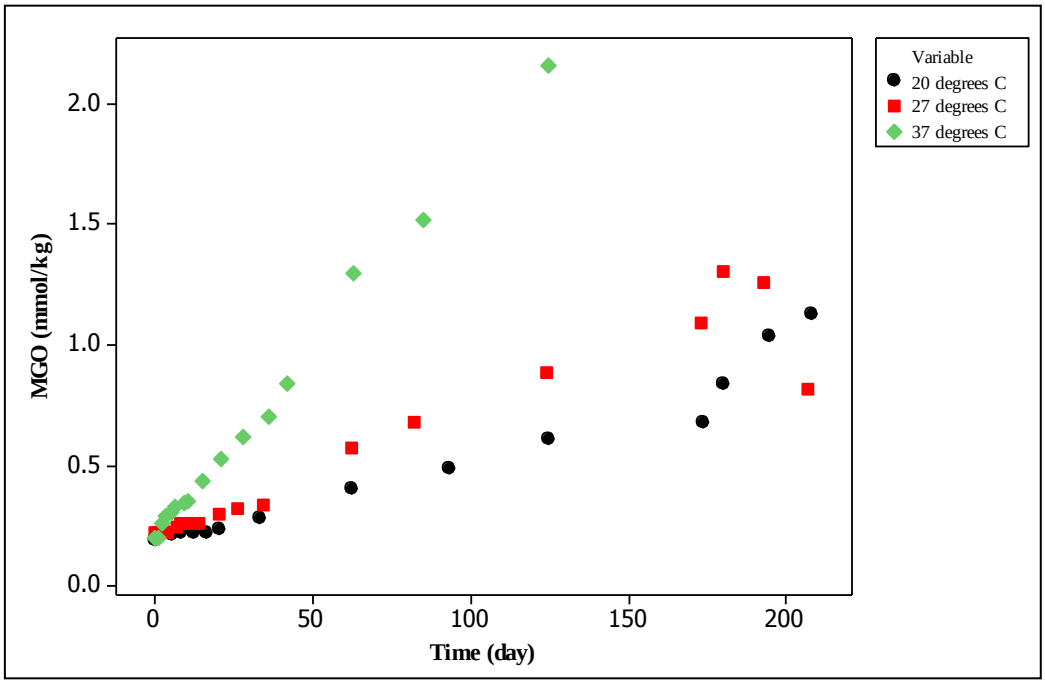


Figure 5.50 MGO vs. time for DHA-lysine systems stored at 20, 27 and 37 °C. The amount of MGO formed increases with increasing temperature for the time period observed.

5.3.6.6 System perturbed by serine

DHA-serine systems contained approximately 2,000 mg/kg (22 mmol/kg) DHA and 500 mg/kg (5 mmol/kg) serine. Data was collected for 34 days before the system was affected by the calibration problem. Therefore there was enough data to calculate a rate constant for the initial reaction. However, this region did not consume as much DHA as the amount of serine added; only 2.36 mmol/kg had been consumed by this point and it is unknown for how long the initial reaction would have continued had there been no problem with analysis of DHA.

The initial rate constant for the loss of DHA before the calibration problem was similar for samples at 27 and 20 °C (0.0035 and 0.0038 day^{-1} respectively). The rate constant for the gain of MGO was 0.0002 and 0.0003 day^{-1} at 27 and 20 °C respectively. This system behaves similarly to the lysine sample; there is no fast formation of MGO hence the loss of DHA is due to side reactions.

The secondary rate constants after the measurement problem were calculated and the rate constant for disappearance of DHA was smaller than the control sample for samples stored at both 27 and 20 °C (0.0004 and 0.0003 day^{-1} respectively). This is much smaller than the rate constant for the appearance of MGO at 37 °C (k'_2 = 0.0014 day^{-1}), possibly due to slower dissociation of the dimer.

The calculated initial and secondary reactions are different, which indicates that there are two rates of reaction seen. This was also observed at the higher temperature. If there was no change, the two rates would have been the same because the calculated slope for DHA loss would be the same despite the reported concentration of DHA being higher in the second part.

The DHA:MGO ratio was 9:1 for the sample at 27 °C and 14:1 for the sample at 20 °C, indicating that both reactions are not near completion.

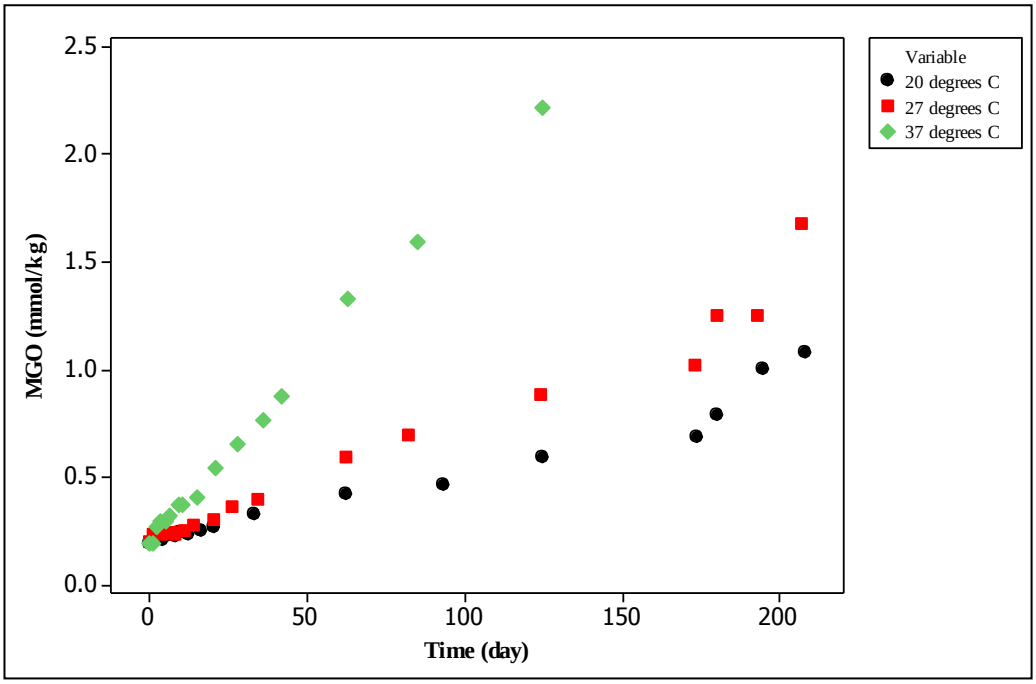


Figure 5.51 MGO vs. time for DHA-serine systems stored at 20, 27 and 37 °C. The amount of MGO formed increases with increasing temperature over the time period observed.

5.3.6.7 System perturbed by alanine and proline

DHA-proline-alanine systems contained approximately 2,000 mg/kg (22 mmol/kg) DHA, 850 mg/kg (8 mmol/kg) proline and 450 mg/kg (5 mmol/kg) alanine. Only data from one duplicate could be used for samples stored at 27 °C due to too much scatter in the other sample. There appeared to be an initial fast reaction, but it is difficult to tell because it may just be the break in the reaction due to the measurement problem. Rate constants were calculated for the initial and secondary reactions. The rate constant for the initial loss of DHA and gain of MGO were 0.0030 and 0.0007 day^1 respectively, which are approximately a factor of 2 and 4 smaller than calculated for the same sample stored at 37 °C. The initial reaction has a stoichiometric factor of 0.23, which is similar to the sample at 37 °C (0.29).

The rate constant for the secondary loss of DHA and gain of MGO were 0.0011 and 0.0008 day^1 respectively. The initial and secondary rate constants for loss of DHA are different, indicating that there was an initial fast reaction. The secondary loss of DHA at 37 °C was 0.0031 day^{-1} which is approximately three times faster than at the lower temperature. The gain of MGO was approximately double (k'_2 = 0.0014 day^{-1}) at 37 °C.

The secondary reaction for the 27 °C system has a stoichiometric factor of 0.72, which is larger than the sample stored at 37 °C (0.45). As proposed earlier, this may be due to side reactions being less prominent.

There was too much scatter in the samples stored at 20 °C to calculate rate constants.

5.4 Conclusion

The conversion of DHA to MGO in honey was confirmed to be a first-order reaction. In the artificial honey systems an initial and secondary reaction were observed in some systems (depending on the perturbant). The initial reaction possibly corresponds to a side reaction of DHA, catalysed by the perturbant, and is halted by the limited about of monomeric DHA available in the matrix. Amino acids may form imines with DHA hence are unavailable to speed up the

dissociation of dimeric DHA by donating a proton. This is suggested to be the rate limiting step for all reactions. The conversion of DHA to MGO was not a 1:1 reaction in any of the systems studied, suggesting that DHA can form side products with itself.

The artificial honey systems allowed individual reactions to be isolated so that perturbants affecting the conversion of DHA to MGO and side reactions could be identified. Amino acids had differing effects on the reaction. Proline, a secondary amino acid, enhanced side reactions of DHA which lowered the efficiency of the DHA to MGO reaction. Furthermore, not all primary amino acids behaved in the same manner. Alanine appeared to prevent DHA from entering a side reaction, or recovered it from a side reaction. Therefore the efficiency of the conversion of DHA to MGO was high in samples that contained alanine. In comparison, lysine and serine did not appear to have an effect on the DHA to MGO conversion.

Chapter Six

6 Storage trials in real honey matrices

This chapter describes the investigation of the conversion of DHA to MGO in mānuka and doped clover honey matrices. Storage time and temperature are the only two factors that can be altered to maximise the MGO concentration without adulterating the honey. The concentration of HMF must stay below the recommended limit of 40 mg/kg during this process. There is very little research in the literature reporting the effect of time and temperature on the conversion of DHA to MGO in real honey matrices or similar model systems. There is extensive work on HMF formation at different temperatures in sugar systems[370] and honey matrices.[186, 260, 371-372] HMF is addressed in chapter 7.

Fresh mānuka honey, as well as mānuka and clover honey to which DHA had been added, were tested. Honeys were stored at 37, 32, 27, 20 and 4 °C (although not all honeys were stored at all temperatures). Rate constants for disappearance of DHA and appearance of MGO, stoichiometric factors and activation energies were calculated. This chapter extends work carried out in chapter 5 by examining the influence of compounds that were identified as having an effect on conversion of DHA to MGO or side reactions in the artificial honeys.

The ability of high pressure processing (HPP) was also investigated as a way to enhance the conversion of DHA to MGO. Artificial honey and clover honey doped with DHA and fresh and stored mānuka honey were analysed to see if high pressure could accelerate the conversion of DHA to MGO.

6.1 Overview of storage trials in real honey matrices

This section gives an overview of some important concepts that are discussed throughout this chapter. They have been compiled here for ease of referral. These ideas extend on those in chapter 5, which first introduced the first-order rate calculations and concept of the stoichiometric factor. Homogeneity of the honey matrix, the ratio of DHA and MGO, and first-order plots of DHA and MGO and their deviations are discussed in this section.

Samples were treated as having first-order rate constants for DHA loss and MGO gain. A discussion of first-order reactions and calculations for rate constants for disappearance of DHA and appearance of MGO can be found in section 5.3.1. Furthermore, as first mentioned in section 5.3.1, the amount of DHA that converted to MGO was calculated in two different ways – stoichiometric factor, $x = \frac{k'}{k}$, and efficiency, $\frac{MGO}{DHA} \times 100$. In the real honey samples, the efficiency varied too much between days, presumably due to measurement error in both DHA and MGO and also due to side reactions. Therefore a value cannot be confidently stated. The stoichiometry factor only applies to the initial formation of MGO; at later times in the reaction the value decreases due to increased side reactions.

Mānuka and clover honeys were used in storage trials to examine the rate of DHA conversion to MGO and side reactions in real honey matrices. The samples were also compared to the artificial honey systems (chapter 5). Clover honey allowed the initial part of the reaction to be followed to see if DHA might initially bind to compounds present in honey that had not previously contained DHA. Fresh and old mānuka honeys were used to see if there was a difference in rate of reaction. The old honey had proline replenished to observe the effect it had on DHA and MGO. Eleven mānuka honeys were used as matrices; samples 946, 953, 1394 were from Steens Honey Ltd.; samples 14, 25, 32, 41, 66, 78 and 84 were from Gibbs Honeys and sample 1404 was from New Zealand Honey Traders. Four commercial clover honeys bought from various supermarkets were used; these were Happy Bee (HB), Airborne (AB), Hollands (Hol) and Katikati (Kat).[*] Clover

[*] Happy Bee (HB), Airborne (AB), Hollands (Hol) and Katikati (Kat) have been abbreviated in tables and figures.

and some mānuka honeys were doped with DHA (2,000 or 10,000 mg/kg). Aliquots were removed periodically for analysis by HPLC. Details of the initial DHA concentration and water content for the honeys used throughout this chapter can be found in Appendix G.

6.1.1 Homogeneity of honey matrices with added DHA

It is important to ensure that DHA added to a honey matrix is evenly distributed and dissolved so that it can react the same as it would if it were naturally occurring in the honey matrix. In addition, it is expected that the sub-samples taken for analysis are representative of the entire sample.

DHA is a crystalline solid that would not likely give a homogenous distribution if it were added as a solid to the honey matrix. In order to obtain a homogenous mixture, DHA was pre-dissolved in water and the pH was adjusted to ~3.8, using gluconic acid,[*] before adding it to the honey matrix. It was critical to not upset the water content because the system may behave differently if excess water was added. For example, the proportion of DHA found as the monomer may be larger in a higher proportion of water and may allow reactions to occur more rapidly. There was a compromise between dissolving DHA in sufficient water and not adding too much water to the matrix. Therefore 1.0000 g[†] of DHA stock solution was chosen to add to the honey (40.00 ± 0.5 g). This is 2.4% of the total weight. The National Honey Board of the United States Department of Agriculture (USDA)[155] reported that the moisture content of honeys range from 13.4-22.9%. The average of 23 mānuka honeys tested during this research ranged from 20-22.4%.

The homogeneity of DHA in a mānuka honey (sample 66, 40.00 ± 0.02 g) doped with DHA stock solution (1.00 ± 0.02 g) was examined. The sample was thoroughly mixed and replicate samples (n = 8) were analysed on the same day that the honey was doped. The standard deviation for replicate analyses of DHA was compared to the standard deviations of MGO and HMF as these compounds were naturally occurring in the honey, and hence should be evenly distributed.

[*] Only a small amount of gluconic acid was necessary in order to adjust the pH.
[†] Stock solutions were weighed instead of delivered by volume. This was to ensure consistent delivery of moles between different compounds.

The theoretical concentration of DHA, based on the standard solution added, in the sample is 3,548 mg/kg; there is only 3% difference between this and the measured value. The percentage RSD is less than 5% and is similar to the variation in MGO and HMF, which were naturally occurring (Table 6.1). Therefore the sample is well mixed and DHA is distributed evenly. This technique was used for doping all real honey matrices.

Table 6.1 Summary of compounds in mānuka honey matrix on day one after DHA was added (n = 8).

	DHA	MGO	HMF
Average (mg/kg)	3291	276	8.9
Standard deviation	140	12	0.5
RSD (%)	4.24	4.28	5.47

Even though the above results show that DHA was evenly distributed in the mānuka honey, the storage trials with 10,000 mg/kg DHA had variable reported concentrations when multiple measurements were taken over the first day (the first measurement in the storage trial was taken as soon as the sample was made up, followed by a measurement approximately three hours later). This effect was not as big as that seen for the aliphatic amines in artificial honey systems (chapter 5), so is not likely to be due to rapid binding of DHA to compounds in the honey. This effect only occurred in the first few hours, not over a series of days. This variation in concentration may arise from inadequate mixing, slow diffusion, or fast reaction/binding of DHA with other compounds in the matrix. In a mānuka honey it is likely that compounds that might react with or bind to DHA would have already been exhausted in other reactions, or are in equilibrium in other reactions. Hence a high concentration of these compounds would not be available for quick binding to DHA as was observed in the artificial honeys, or alternatively such compounds may be present in such low concentrations that they would not have a large effect on the DHA concentration. Therefore it is more likely that the small change over the first day is due to a mixing effect. At the molecular level mixing occurs via diffusion; this can be slow (order of days), especially in a viscous liquid, such as honey. In some cases the reported concentration was higher than the theoretical value indicating incomplete mixing. After the initial discrepancy, the concentration of DHA followed a linear decrease due to its

conversion to MGO and side products. Therefore, in cases where the initial data point did not reflect the theoretical concentration, this point was removed to accommodate a mixing time so that the results were not skewed. Honey samples with 2,000 mg/kg DHA added did not show this initial discrepancy. This suggests that it is a diffusion issue and that there was insufficient time for equilibration of the DHA to occur when 10,000 mg/kg DHA was used and a sub-sample was analysed immediately.

6.1.2 Ratio of DHA:MGO

Theoretically, there should be a 1:1 conversion of DHA to MGO; however, as discussed throughout this research, some of the DHA is consumed in side reactions and MGO reacts further in various pathways. In addition, because the conversion of DHA to MGO is an irreversible reaction, it should act as a 'sink' with all of the DHA converting to MGO. However, this does not occur and complete conversion was only observed in one sample analysed during this research – sample 946 (mānuka honey with 2,000 mg/kg DHA) had no DHA remaining at 441 days.[*] In comparison, sample 1349 (harvested in 2003 and stored at ambient temperature until 2012 when it was analysed) still had 189 mg/kg DHA remaining. A possible cause for this residual DHA is that it is bound to other compounds in equilibrium and is not available to convert to MGO. When the honey is prepared for analysis, the conditions may favour a shift in the equilibrium and cause a release of DHA, hence it will be detected as free DHA in the analytical method. Strain and Spoehr[286] noted incomplete conversion of DHA to MGO in aqueous solutions. They reported that this occurred in control samples and samples with added amines.

Superficially it looks like there is an equilibrium between DHA and MGO, or that the system is in a steady state. Weber[292] defined the reaction as a steady state resulting from the difference between the rate of product formation and rate of product removal by another reaction. The true definition of a steady state is an unvarying condition in a physical process. Furthermore, a steady state approximation is used when the major part of the reaction occurs when the

[*] Correspondence with Steens Ltd. reported that sample 946 had a high moisture content (21.5%) and fermented in the storage drum,

reactive intermediates are at virtually constant concentrations.[373] However, the slow conversion of DHA to MGO balanced by the equal amount of MGO removed in a side reaction is not the true definition of a steady state, nor can it be approximated as a steady state because the steady concentration of MGO only occurs in a small portion of the reaction.

Fresh mānuka honey initially has a high DHA concentration and low MGO concentration. Atrott *et al.*[106] reported seven- to sixteen-fold excess of DHA compared to MGO in fresh mānuka honeys. This ratio would depend on a number of factors, such as contribution from other floral types, level of DHA in the nectar and the time and storage conditions since harvest. Over time as DHA converts to MGO and both compounds are lost in side reactions, this ratio gets smaller. Atrott *et al.*[106] reported that the mean ratio of DHA:MGO was 2:1 in 18 commercial honeys. In addition, correspondence with various beekeepers[*] suggests that MGO attains a maximum at the point where there are 2 DHA remaining in the system for every 1 MGO present. Personal communication with other beekeepers has revealed that they do not sell their honey at a 2:1 ratio because they believe that there is still potential to gain more MGO. One company aim for 1.2:1 before they sell their honey. The figures calculated by the beekeepers are in mg/kg, not mmol/kg, but the conversion is similar when working in mmol/kg due to MGO and DHA having similar molecular weights.[†]

The current research also observed a plateau in MGO formation when the ratio was around 2DHA:1MGO. However, the reaction continued past this point. In some cases the ratio was as low as 0.6:1 at the end of data accumulation, but significant extra MGO is not gained. Furthermore, if the honey is left too long, MGO is consumed in further reactions, which are more noticeable because there is less DHA to convert to MGO and balance the consumption of MGO. The origin of the ratio is discussed in more detail in section 6.1.3.2.

Atrott *et al.*[106] suggested that the incomplete conversion and stability at 2:1 may arise from the water content being a limiting factor. However, it is unlikely that this is the reason because most of the water is hydrogen bonded to sugars in the

[*] To protect their identity the beekeepers have not been referenced.
[†] For example, a honey with 500 mg/kg DHA and 250 mg/kg has a 2:1 ratio. This is 5.56 and 3.47 mmol/kg respectively, which is a 1.6:1 ratio.

matrix. In addition, the reaction appears to slow down at this ratio regardless of the water content of the honey or the initial DHA concentration. It most likely arises from the amount of MGO gained and lost being equivalent – similar to a steady state.

6.1.3 Initial and secondary rates of reaction

6.1.3.1 DHA

Three honeys (946, 953 and Happy bee) were doped with both 10,000 mg/kg and 2,000 mg/kg DHA. There was no significant difference between the rate constants within each of the two starting concentrations for each sample. Therefore samples with different starting concentrations of DHA were compared. The similarity of rate constants for samples with different initial concentrations of DHA has previously been mentioned in section 5.3.1.2. All samples were carried out in duplicate and the average rate constants are reported.

All honeys (doped with either 2,000 or 10,000 mg/kg) had only one rate of DHA loss (Figure 6.1), except for sample 946. Therefore the secondary rate constants for DHA loss and MGO gain in artificial honey systems were used to compare to the real honey matrices in order to give a closer comparison. Sample 946 had an initial fast rate of DHA loss (6.8 mmol/kg over 10 days), followed by a slower loss. This is comparable to the initial and secondary rates of reaction seen in the DHA-proline and DHA-alanine artificial honey systems studied. In the artificial honeys, the initial loss of DHA corresponded to the amount of perturbant added, suggesting a 1:1 reaction, but may be coincidental and may arise from the amount of monomeric DHA available for reaction. The reason for this initial loss in sample 946 is unknown but could be due to the high water content.

In sample 946, the initial rate constant was 0.0201 day^{-1} compared to 0.0077 day^{-1} for the secondary rate constant. If a single trend line is fitted through the data, the rate constant is 0.0084 day^{-1} which has a small influence from the initial rate. The initial rate would not likely be observed commercially in honeys with naturally occurring DHA because the DHA is introduced from the nectar and hence is present from the beginning. Therefore an equilibration time is not required. The secondary rate constant for DHA disappearance, k_2, was used for comparison with

other samples. Furthermore, this is the rate constant used to calculate the appearance of MGO.

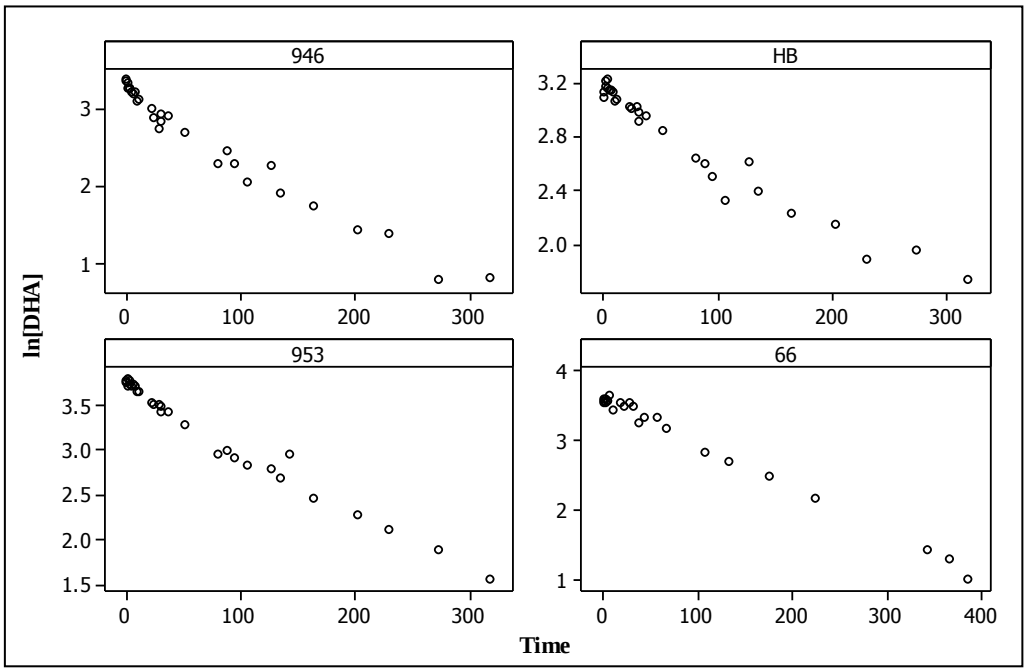


Figure 6.1 First-order plot for loss of DHA (2,000 mg/kg, 22 mmol/kg) in four doped honeys (946, HB, 953 and 66) at 37 °C. The first-order plot for sample 946 showed a short initial fast disappearance of DHA. An initial fast rate of DHA loss was not observed for the other three samples.

6.1.3.2 MGO

A first-order plot for the appearance of MGO, k', ($k * \frac{MGO}{DHA_0}$ $vs. 1 - e^{-kt}$) was not linear for the entire range of most of the real honeys analysed (honeys doped with either 2,000 or 10,000 mg/kg), indicating that after a time the reaction no longer obeys first-order kinetics, due to multiple side reactions of MGO becoming more prominent at longer times. Examples are shown in Figure 6.2 for four honeys. The decline is larger in mānuka honey suggesting that it contains one or more compounds that are absent in clover honey which contribute to the removal of MGO. Therefore clover honey is not a good matrix for modelling the fate of MGO at later times in the reaction. Fresh mānuka honeys or those with low NPA would be better to observe MGO at later times.

Three areas were identified on the first-order plot (Figure 6.3) and were named (as indicated in parentheses) for ease of discussion in this text; the plot initially has a

positive linear slope (initial rate of MGO formation, k'_1), then begins to deviate from linearity (transition period, k'_{trans}) before moving into the third region where there is a negative slope (rate of MGO decay).

The initial formation of MGO, k'_1, is the fastest period of MGO production because the concentration of DHA is high. Furthermore, side reactions of MGO are not prominent as the MGO concentration is low. Hence, overall in this region, more MGO is formed than removed. This region obeys first-order kinetics and was used to calculate the first-order rate constant for MGO formation.

The start of the transition period occurs when the first-order plot of MGO formation begins to deviate from linearity, indicating that it no longer obeys first-order kinetics. This typically occurs when the DHA:MGO ratio is near 2:1; however, this may be a coincidence. This region has a slower rate of MGO formation compared to the initial region due to side reactions of MGO being more prominent at later times. In some samples this region has a slope close to 0 and is similar to a steady-state. The transition period lasted longer in samples with 10,000 mg/kg initial DHA than for the samples with 2,000 mg/kg DHA.

The third region of the graph was labelled as 'rate of MGO decay'; side reaction of MGO will be prominent in this region because the MGO concentration is high, hence the rate of loss will be faster. Moreover the DHA concentration is low, so the rate of conversion to MGO will be low.

The DHA-proline-alanine artificial honey system also showed a deviation from linearity in the first-order plot for the appearance of MGO. Other artificial honey systems did not show this deviation, either because the reactions were occurring a lot slower so they did not reach this point, or because the perturbants were not reacting with MGO or catalysing side reactions.

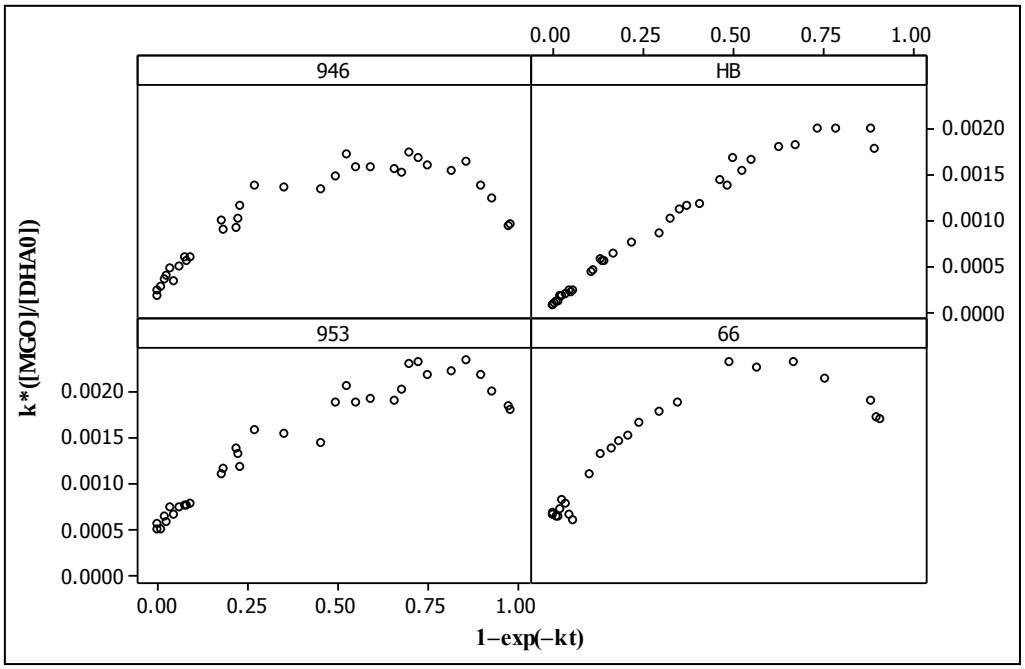


Figure 6.2 First-order plot for MGO appearance at 37 °C for samples 946, HB, 653 (up to 457 days) and 66 (up to 386 days), where k = first-order rate disappearance of DHA. The starting concentration of DHA was approximately 2,000 mg/kg (22 mmol/kg) for all samples. The mānuka samples have an initial increase in MGO formation, followed by a transition period then a decline in MGO.

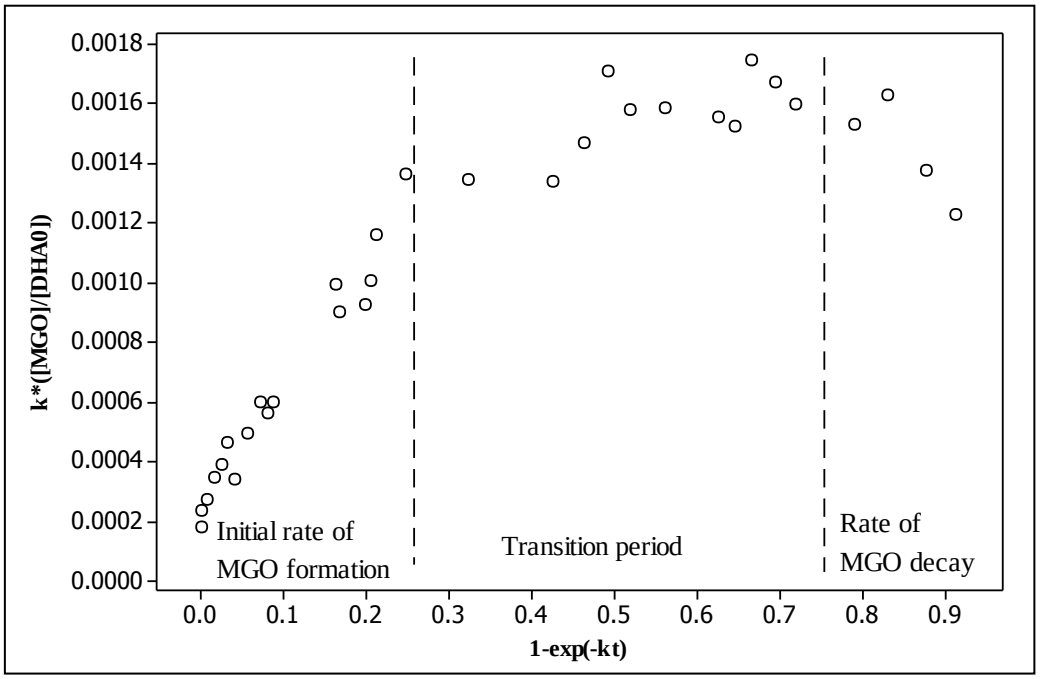


Figure 6.3 First-order plot for sample 946 (2,200 mg/kg/ 24 mmol/kg DHA), incubated at 37 °C , where k = first-order rate disappearance of DHA. The regions of initial rate of MGO formation, transition period and MGO decay are indicated on the graph.

Riddle and Lorenz[105] examined the conversion of DHA to MGO in physiological model systems (aqueous solutions in basic conditions) with various perturbants; and noted a slower rate of MGO formation later in the reaction. They reported that the rate of MGO formation was linear for up to 3 hours, then changed to a lower rate by 9 hours, presumably from an increase in side reactions of both DHA and MGO. Many of these reactions will be different side reactions compared to those occurring in acidic conditions; for example, MGO converts to lactic acid in basic conditions. The authors also incubated MGO and phosphate at various pH values (6.0 to 11.0) and reported linear first-order decay curves up to pH 8.0, after which they became exponential. They suggested that further reaction of MGO was only due to the pH of the system and was independent of the ions that were tested. However, at low pH, the conditions are suitable for the initial stages of the Maillard reaction to occur, and hence reactions would be influenced by amines in the matrix.

MGO formation appeared to slow down when approximately 200 mg/kg DHA remained in most samples. This is similar to the concentration of DHA in the sample stored at ambient temperature for nine years and may be due to most of the remaining DHA being bound to other compounds, but still being able to be measured by the analytical method. Differences in the decay of MGO between samples in the third region are likely to arise from varying concentrations of one or more compounds involved in the removal of MGO. The compound may bind to MGO in a reversible or irreversible manner. Furthermore, the compound may be a catalyst for MGO removal without itself being consumed, or the compound may be poisoned by other compounds in the matrix or products of side reactions. MGO is known for its reactivity as a precursor of advanced glycation end products (AGEs) in food and *in vivo*; it readily react with *N*-termini of proteins and also lysine and arginine side chains.[115] Majtan e*t al.*[57] reported MGO binds to the enzyme glucose oxidase in honey, indicating it most likely also binds to other enzymes, R groups on proteins and amino acids. Additionally, MGO may also polymerise.

Stephens and Schlothauer[151] reported that warming above ambient may cause sugar-bound MGO to be released in the initial stages of heating. However, the [MGO] vs. time plot and the first-order plot for mānuka honeys that contained

MGO before heating do not show a discrepancy at the beginning of the reaction. The difference may arise from the size of samples tested. They did not state the size of their samples, but if they were large commercial drums there may be a stratified effect, where the middle portion of honey takes longer to warm up. Honey nearest the side of the drum will heat faster and hence conversion of DHA to MGO and side reactions will initially occur more rapidly here until all of the honey is at the same temperature.

In sample 946 all DHA was consumed by day 441 before the end of the experiment, hence the loss of MGO was larger than for other samples because no more MGO was formed. It is unknown why all of the DHA was consumed, but it could be due to a greater amount of an unknown compound which removed DHA compared to other honeys studied. Alternatively the difference observed may be the influence of the high water content or even proliferating microbes since the honey began to ferment.

6.2 Kinetics of conversion of DHA (2,000 and 10,000 mg/kg) to MGO in real honeys at 37 °C

Six mānuka and one doped clover honey (2,000 mg/kg DHA) and two mānuka and four doped clover honeys (10,000 mg/kg) were stored at 37 °C to examine the conversion of DHA to MGO in real honey matrices. As expected, mānuka and clover honeys stored at 37 °C did not all have the same rate constants for the loss of DHA and gain of MGO, indicating that not all real honeys behave in the same manner. The rate constants for DHA loss and MGO gain and the stoichiometric factor for the mānuka and clover honeys with 2,000 mg/kg DHA are summarised in Table 6.2; samples doped with 10,000 mg/kg DHA are summarised in Table 6.3. The error in the rate constants is ± 0.0004 day^{-1} for DHA loss and ± 0.0002 day^{-1} for MGO gain, as deduced in section 5.3.1.4.

Mānuka honeys had higher rate constants for both DHA loss and MGO gain. Four clover honeys doped with 10,000 mg/kg DHA had an average rate constant of 0.0050 ± 0.0005 day^{-1} for DHA disappearance compared to two mānuka honeys that had an average of 0.0081 ± 0.0011 day^{-1}. Furthermore, one clover honey doped with 2,000 mg/kg had a rate constant of 0.0048 day^{-1} for DHA

disappearance compared to five mānuka matrices that had an average of 0.0070 ± 0.0006 day^{-1}. As expected, and discussed in section 5.3.1.2, honeys used as matrices for both 2,000 and 10,000 mg/kg initial DHA had similar rates of DHA loss; this was also seen for artificial honeys with the same perturbant and different starting concentrations.

Table 6.2 Summary of the rate constants for DHA loss and MGO gain and stoichiometric factor (x) for mānuka and clover honeys stored at 37 °C with 2,000 mg/kg DHA.

Sample*†	k (day^{-1})	k'† (day^{-1})	Stoichiometric factor (x)
946‡ (M)	0.0077	0.0044	0.57
953 (M)	0.0070	0.0034	0.49
66 (M)	0.0064	0.0040	0.64
78 (M)	0.0075	0.0053	0.71
84 (M)	0.0064	0.0050	0.59
HB (2,000, C)	0.0048	0.0032	0.67
1404 (M)	0.0056	0.0035	0.63
Total average ± σ	0.0065 ± 0.0010	0.0041 ± 0.0008	0.61 ± 0.07
Mānuka average ± σ	0.0068 ± 0.0008	0.0043 ± 0.0008	0.61 ± 0.07
Clover average ± σ	0.0048	0.0032	0.67

*M = mānuka, C = clover

† Calculated as the initial rate of MGO appearance

‡ This sample had a high moisture content (21.5%) and fermented.

Table 6.3 Summary of the rate constants for DHA gain and MGO loss and stoichiometric factor (x) for mānuka and clover honeys stored at 37 °C with 10,000 mg/kg DHA.

Sample*	k_1 (day^{-1})	k'_1 (day^{-1})	x_1‡	k'_{trans}* (day^{-1})	x_{trans}‡
946 (M)	0.0089	0.0051	0.57	†	-
953 (M)	0.0073	0.0051	0.70	†	-
HB (10,000, C)	0.0056	0.0035	0.63	†	-
Katikati (C)	0.0045	0.0035	0.78	0.0014	0.40
Hollands (C)	0.0052	0.0049	0.94	0.0014	0.27
Airborne (C)	0.0045	0.0032	0.71	†	-
Total average ± σ	0.0060 ± 0.0018	0.0042 ± 0.0009	0.72 ± 0.12	0.0014	0.34 ± 0.09
Mānuka average ± σ	0.0081 ± 0.0011	0.0051	0.64 ±0.09		
Clover average ± σ	0.0050 ± 0.0005	0.0038 ± 0.0008	0.76 ± 0.13	0.0014	0.34 ± 0.09

* M = mānuka, C = clover

† There was too much scatter in the secondary formation/disappearance of MGO to calculate k'_{trans}

‡ x = stoichiometric factor

The highest rate constant for DHA disappearance in the artificial honey systems was for the DHA-proline-alanine-iron system (0.0056 day^{-1}), which is similar to the clover honeys, but smaller than the mānuka honeys. It should be noted that iron does not occur naturally in honey at the concentration used in this study (~800 mg/kg). The DHA-proline-alanine system had similar concentrations of total primary and secondary amino acids as real honey. The rate constant of 0.0031 day^{-1} for the loss of DHA in this sample, was smaller than seen in the real honey matrices indicating that while proline and primary amino acids (represented by alanine) account for part of the loss of DHA one or more compounds are still unaccounted for.

Large rate constants for DHA disappearance did not necessarily correspond to large rate constants for formation of MGO. This concurs with results from the model systems (chapter 5), and is due to a loss of DHA in side reactions. For example, sample 953 (2,000 mg/kg DHA) had a large rate constant for DHA loss (possibly due to a high proline concentration, see section 6.3), but the rate constant for MGO appearance was one of the smallest recorded.

The initial rate constant for MGO appearance was also different between the mānuka and clover matrices. Mānuka honeys with 10,000 mg/kg initial DHA had an average rate constant for MGO appearance of 0.0051 ± 0.0000 day^{-1} compared to clover honeys which had a smaller rate constant (0.0038 ± 0.0008 day^{-1}). Mānuka honeys and one clover honey doped with 2,000 mg/kg initial DHA had similar rate constants for MGO appearance (0.0043 ± 0.0008 day^{-1} and 0.0048 day^{-1} respectively). The highest rate constant for MGO appearance in artificial honey was for the DHA-proline-alanine system (0.0029 day^{-1}), which is smaller than for any real honey matrices. The artificial honey systems indicated that proline mainly caused a loss of DHA and MGO into side reactions and had only a very small effect of formation of MGO. Therefore the results indicate that alanine, which was used to represent all amino acids at the level which it was doped, makes up a substantial amount of the compounds responsible for the conversion, but one or more other compounds are still unaccounted for. Possible compounds have been discussed in section 6.3.

Despite the potential catalysts in the honey matrix, the rate constants for loss of DHA are smaller than those reported for DHA loss in aqueous solutions (see section 5.1.2), proposed to be due to lack of available H^+ ions for conversion of dimeric DHA to monomeric DHA in the honey matrix.

The rate constant for MGO appearance in the transition region, k'_{trans}, for two honey samples with 10,000 mg/kg was calculated; this was possible because the transition period lasted longer than in the 2,000 mg/kg samples. The rate constant was 0.0014 day^{-1} for both samples, which is much slower than the initial reaction. This shows that the loss of MGO due to side reactions is dominant at this time in the reaction. All other samples had too much scatter in the data for the rate constant to be calculated.

The average initial stoichiometric factor for the conversion of DHA to MGO was 0.61 ± 0.07 for samples with 2,000 mg/kg DHA. This suggests that approximately 60% of DHA is converted to MGO when incubated at 37 °C. Therefore 40% is lost in side reactions. This value is only relevant for the initial rate of MGO before side reactions become prominent at later times and MGO is net consumed. Samples with 10,000 mg/kg DHA had a higher stoichiometric factor during the initial formation of MGO (average of 0.70 ± 0.10). This may be due to minor side reactions being swamped by the large amount of DHA. Unfortunately the percentage efficiency could not be calculated due to too much scatter in the data. As expected the stoichiometric factor for the secondary appearance of MGO (transition period), k'_{trans}, was reduced (0.34 ± 0.09), suggesting that MGO is involved in side reactions at a later time. As previously mentioned MGO behaves differently in mānuka and clover matrices once the DHA:MGO ratio passes 2:1. Mānuka honeys have a larger decline in MGO at later times, whereas in a clover matrix this effect is not as pronounced. Therefore while clover is a reasonable model early on in the reaction it does not hold up later on and a mānuka matrix should be used to investigate the fate of MGO at longer time periods.

Adams *et al.*[14] split a clover honey and doped it with various concentrations of DHA (680-10,400 mg/kg). The rate constant for DHA disappearance (0.0041 ± 0.0002 day^{-1}) is similar to the clover honeys in this research, but the rate constant of MGO appearance is much smaller than in this research (0.0017 ± 0.0001

compared to 0.0038 ± 0.0008 day^{-1}). They also monitored fresh mānuka honeys; the initial concentrations of DHA ranged from 3,148 to 4,248 mg/kg DHA. The first-order rate constant for disappearance of DHA was between 0.0057 and 0.0081 day^{-1} which is similar to the values in this research. The rate constants for the appearance of MGO were 0.0023 day^{-1} for all three samples, which is much lower than the values in this research (0.0043 ± 0.0008 day^{-1}). This may be due to the samples having different levels of compounds that influence the conversion of DHA to MGO and side reactions, possibly due to the samples coming from different regions, and also different seasons. Due to the small gain in MGO, the stoichiometric factor was low (0.28 to 0.42) compared to samples in this research (0.61 ± 0.07).

To examine whether the age of honey affected the rate of conversion of DHA to MGO or any of the side reactions due to loss of catalytic compounds, an experiment was carried out with the 2003 honey (sample 1394). DHA (10,000 mg/kg) was added to the sample. The sample was split into two portions and one had proline (780 mmol/kg) added to it. A summary of the rate constants and stoichiometric factor for the samples are in Table 6.4. The rate constant for DHA disappearance in the sample doped with only DHA was 0.0061 day^{-1}; initial MGO appearance was 0.0042 day^{-1} and the transition rate constant for MGO appearance was 0.0022 day^{-1}. The rates are similar to those seen for the fresh mānuka and clover honeys doped with DHA, suggesting that a compound required for catalysis is still available. The sample with added proline had a larger rate constant for DHA disappearance (0.0070 day^{-1}), but it did not affect the formation of MGO (0.0040 day^{-1}). This observation is similar to that seen in the artificial honey systems for the control (DHA only) the system perturbed by proline. There was too much scatter in the transition period of MGO formation for sample 1394 with proline to report a value. The stoichiometry factor for sample 1394 doped with DHA alone was 0.69, which was higher than the sample doped with both DHA and proline (0.57).

Table 6.4 Summary of rate constants of DHA loss and MGO gain and the stoichiometric factor for an old mānuka honey with and without added proline.

Sample	k (day^{-1})	k' (day^{-1})	x_1*	k'_{trans} (day^{-1})	x_{trans}*
1394 (M)	0.0061	0.0042	0.69	0.0022	0.36
1394 + Proline† (M)	0.0070	0.0040	0.57	‡	

* x = stoichiometric factor

6.3 Influence of compounds on loss of DHA and MGO, and formation of MGO

The mānuka and clover honeys used in the storage trials were analysed for a number of chemical and physical properties, before they were doped with DHA (chapter 4). Significant differences between the samples may help to explain differences in the rate constants for disappearance of DHA and appearance of MGO. Sample 78 was an outlier in almost all plots; the rate constant for DHA loss was higher than expected. A compound that was not examined in this research may be the cause of the large loss of DHA in this sample and may not be present in large quantities in the other samples tested. This sample was excluded from the following discussion. A large number of samples from a wide variety of geographical areas and varieties of mānuka need to be analysed to see if the trends observed here also hold true for other honeys.

Table 6.5 summarises some chemical and physical properties of the honeys. All samples were within 1 pH unit of each other (3.43 to 4.27) and no correlation between pH and k, k' or x was observed. The free acidity (15.40 to 34.75 meq/kg) and total acidity (17.09 to 37.34 meq/kg) had more variation; a positive correlation between the rate constant for DHA loss and each of these parameters were observed which were similar to each other. Figure 6.4 shows the correlation between k and total acidity (R^2 = 87%). The clover honey had the smallest rate constant and also the lowest total acidity. This concurs with observations from chapter 5 that DHA reacts faster when H^+ donors are present. There was no correlation between k' and total acidity.

Table 6.5 Chemical and physical properties of honeys used as matrices for storage trials.*

Sample†	DHA (mg/kg)			HMF (mg/kg)			MGO (mg/kg)			Proline (mg/kg)	% Moisture	pH			Free Acidity (meq/kg)			Lactone (meq/kg)			Total Acidity (meq/kg)		
946 (M)	322	±	5	9.56	±	0.02	67	±	1	809	21.5	3.90	±	0.04	34.75	±	0.64	2.35	±	0.21	37.1	±	0.85
953(M)	2675	±	97	10.07	±	0.47	246	±	10	746	19.4	3.57	±	0.00	35.34	±	2.06	2.01	±	0.13	37.34	±	2.19
1349 (M)	189	±	4	195.45	±	5.80	761	±	9	302	19.7	3.57	±	0.10	22.41	±	8.83	1.96	±	0.75	24.37	±	9.57
Airborne (C)	N/D			13.39	±	0.45	N/D			361	20.7	3.50	±	0.01	20.61	±	0.40	1.15	±	0.69	21.76	±	0.29
Holland (C)	N/D			13.09	±	0.15	N/D			480	19.4	3.82	±	0.06	17.59	±	1.03	2.32	±	1.36	19.91	±	0.33
Happy Bee (C)	N/D			12.95	±	0.85	N/D			422	19.8	3.84	±	0.05	15.41	±	0.86	1.43	±	0.62	17.09	±	0.78
Katikati (C)	N/D			58.31	±	1.21	N/D			637	19.6	3.43	±	0.00	26.46	±	0.58	1.78	±	4.08	28.23	±	3.49
66 (M)	1552	±	7	8.63	±	0.22	255	±	4	572	20.1	3.69	±	0.04	20.11	±	2.78	8.61	±	1.82	28.72	±	0.96
78 (M)	1037	±	50	6.74	±	0.52	165	±	5	391	18.4	4.27	±	0.01	15.40	±	1.29	9.71	±	1.89	25.11	±	0.6
84 (M)	1968	±	18	8.45	±	0.26	302	±	4	550	22.0	3.74	±	0.02	24.23	±	1.03	6.42	±	0.12	30.65	±	1.14
1404 (M)	1539	±	87	1.87	±	0.36	44	±	4	372	17.8	‡			‡			‡			‡		
25 (M)	2376	±	30	7.24	±	0.40	289	±	3	380	22.0	3.76	±	0.01	22.38	±	1.28	12.19	±	0.42	34.57	±	0.86
41 (M)	3242	±	50	3.45	±	0.24	304	±	65	368	20.5	3.70	±	0.02	17.37	±	0.01	11.42	±	0.71	28.78	±	0.72
Average	1656	±	1031	28.02	±	54.75	270	±	208	491	20.1	3.73	±	0.22	22.67	±	6.70	5.11	±	4.27	27.80	±	6.51
Minimum	N/D			1.87			N/D			302	17.8	3.43			15.40			1.15			17.09		
Maximum	3242			195.45			761			809	22.0	4.27			35.34			12.19			37.34		

* All analyses were carried out before the honeys were doped and stored at 37 °C.

† M = mānuka, C = clover

‡ Due to the limited sample size of sample 1404, pH and acidity were not tested.

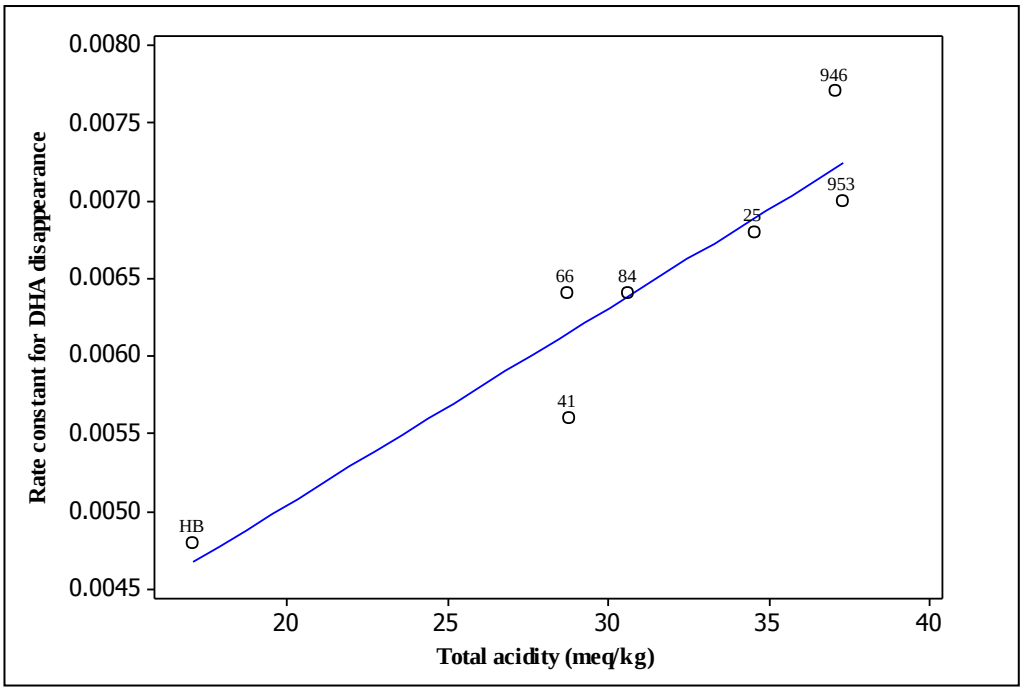


Figure 6.4 Rate constant for DHA loss (k, day^{-1}) vs. total acidity. There is a strong positive linear correlation (R^2 = 87%).

Proline

The proline concentration of the honeys used in the storage trials (8 mānuka and 4 clover) varied from 302 to 808 mg/kg (ninhydrin method). The artificial honey storage trials (chapter 5) showed that proline enhanced side reactions of DHA, hence the efficiency of the conversion to MGO was lowered. In a real honey matrix, multiple reactions occur simultaneously which may lessen the effect of proline. Relationships between proline and k, k' and x were examined to see if proline has an effect on the loss of DHA in a real honey matrix. A positive linear correlation between k and proline (68%) was observed for the eight mānuka honeys; sample 25 sits above the trend line. If this sample is removed and 3 of the 4 clover honeys are added (Katikati was an outlier) the R^2 is 82% (Figure 6.5).

The amount of proline in the honey matrix has an effect on how much DHA will be consumed but this does not equate to a higher rate of MGO formation, as observed in the model system due to side reactions of DHA rather than rate enhancement to form MGO; no trend was observed between k' and proline. This supports observations made in the DHA-proline artificial honey system (chapter 5), where the rate constant for MGO formation was the same as for the control

sample. Furthermore no correlation was seen between the stoichiometry factor and proline.

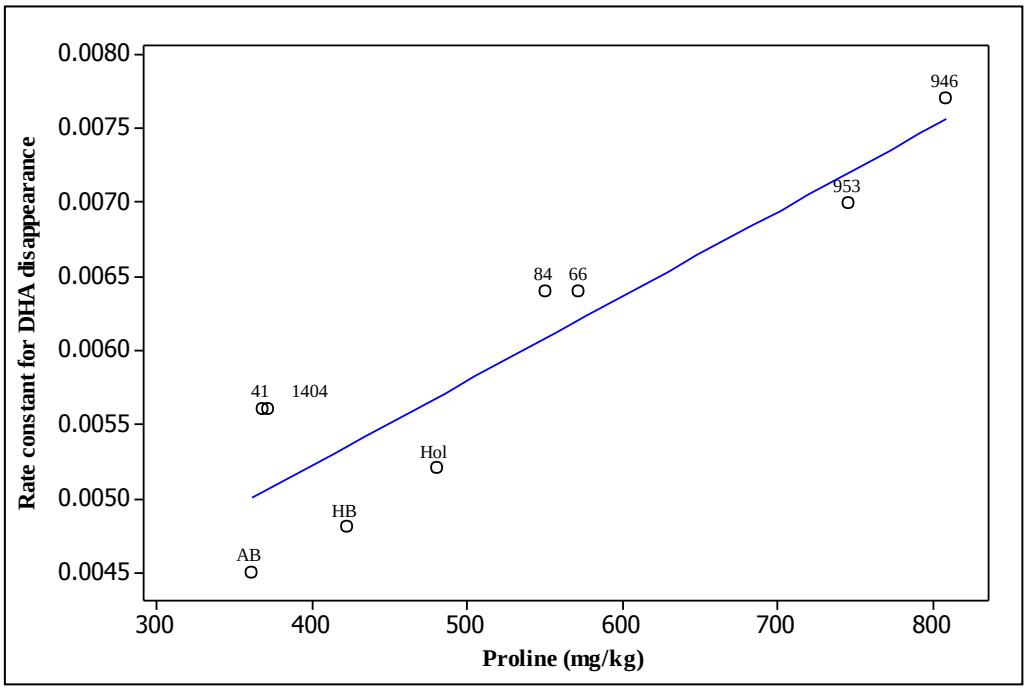


Figure 6.5 Rate constant for DHA loss vs. proline (mg/kg) for mānuka and clover honeys stored at 37 °C. (R^2 = 82%).

There was also a positive linear trend between k and proline for the storage trials with 10,000 mg/kg DHA if the data from the ninhydrin sampling was used. The R^2 value was 68%; the Katikati sample lowered the fit. If the Katikati value was removed the R^2 increased to 92%. If the proline data analysed by the HPLC method was used no correlation was seen with k.

Primary amino acids

The honeys used in the storage trials were analysed for primary amino acids. Concentrations of individual primary amino acids for each sample are summarised in Table 6.6. Primary amino acids account for 26 ± 6% of all amino acids in the storage trial matrices. Relationships between primary amino acids (individual and total) and k, k' and x were examined.

The rate constant for DHA disappearance vs. total primary amino acids was plotted for all mānuka and clover honeys (both 2,000 and 10,000 mg/kg starting DHA); a different trend was observed for three clover honeys doped with 10,000

mg/kg compared to all other samples (Figure 6.6). Two of the clover honeys (Hol and Kat) had very high levels of phenylalanine, which may partially contribute to the difference observed. Samples 946, 953 and HB were doped with both 2,000 and 10,000 mg/kg DHA, but did not have large differences in reported rate constant for DHA loss; hence the amount of DHA does not appear to be the cause of the different trends. Further work could be carried out on the influence of phenylalanine on conversion of DHA to MGO.

There was a strong correlation between total primary amino acids and rate constant for DHA loss when only the mānuka honey samples with 2,000 mg/kg DHA were plotted (R^2 = 86%, Figure 6.7). In addition, if total amino acids (including proline) are plotted against k, the R^2 value is 92%.

Table 6.6 Amino acids (mg/kg) in storage trial matrices.

Amino acid	HB	AB	Hol	Kat	946	953	1404	66	78	84
	mg/kg									
Aspartic Acid	11.0	11.4	9.0	11.4	20.2	16.9	31.1	4.6	5.9	4.6
Threonine	1.8	3.6	3.8	3.0	6.9	6.0	3.4	2.8	1.9	1.9
Serine	8.4	11.8	13.9	9.2	16.0	13.8	6.6	10.4	7.1	10.9
Glutamic Acid	14.5	15.2	13.6	10.0	15.1	14.9	22.9	9.6	10.3	8.8
Glycine	2.2	3.6	3.4	3.0	4.6	4.5	1.7	2.2	1.5	2.4
Alanine	7.0	10.8	9.6	9.7	20.7	18.3	5.0	11.6	7.3	12.3
Valine	5.1	9.2	9.0	8.1	10.9	12.2	4.9	7.2	4.8	8.0
Methionine	0.1	0.2	0.4	0.2	0.5	0.5	0.6	0.2	0.2	0.4
Isoleucine	2.6	5.6	4.5	6.1	6.6	7.5	2.3	4.6	2.8	4.8
Leucine	2.7	5.8	6.3	9.6	7.2	5.0	1.7	3.0	2.0	2.8
Tyrosine	7.5	12.6	65.4	33.9	20.1	17.5	2.8	13.6	7.1	10.0
Phenylalanine	27.1	47.8	480.8	135.7	87.8	54.3	5.9	42.1	19.2	19.6
Histidine	5.7	9.8	6.2	5.7	19.9	18.9	4.7	5.5	2.4	3.1
Lysine	9.3	24.4	12.3	14	23.9	22.2	7.5	16.1	6.8	10.2
Arginine	5.2	8.1	5.3	9.4	13.7	8.9	4.3	5.2	3.5	1.2
Tryptophan	0.6	0.5	9.5	0.1	1.1	0.2	0.2	0.2	0.2	4.1

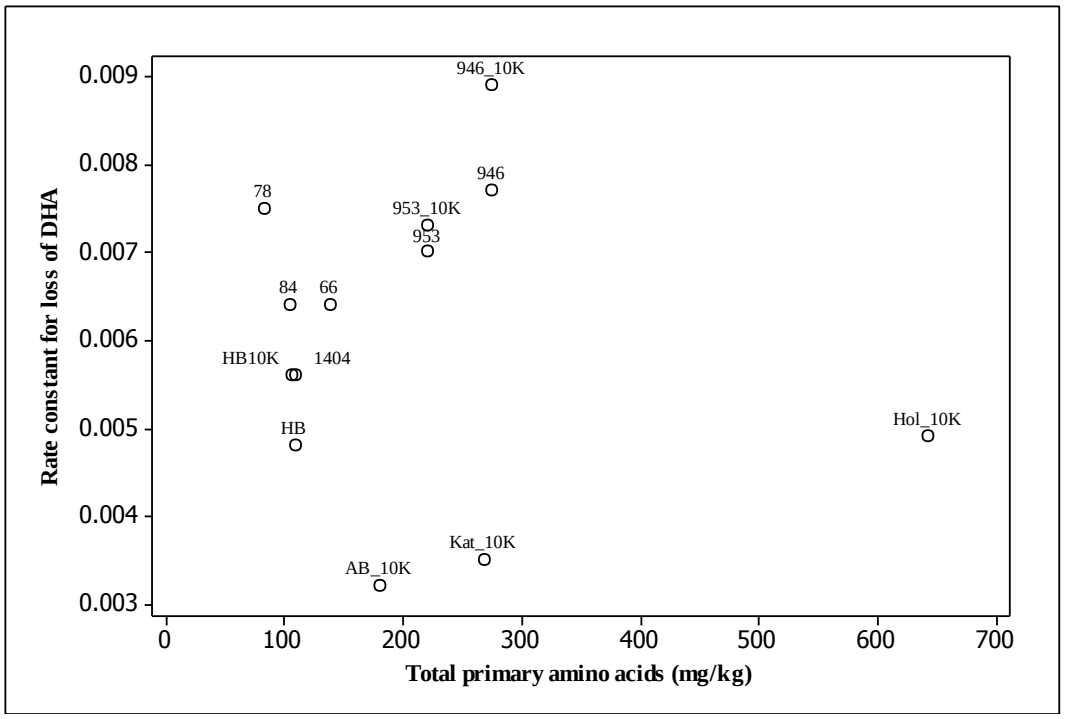


Figure 6.6 Rate constant for DHA loss (k, day^{-1}) vs. total primary amino acids for mānuka and clover honeys doped with either 2,000 or 10,000 mg/kg DHA. Samples with 10,000 mg/kg are shown with '10K' after the sample ID. AB, Kat and Hol have a different trend to the other samples, possibly due in part to the influence of phenylalanine.

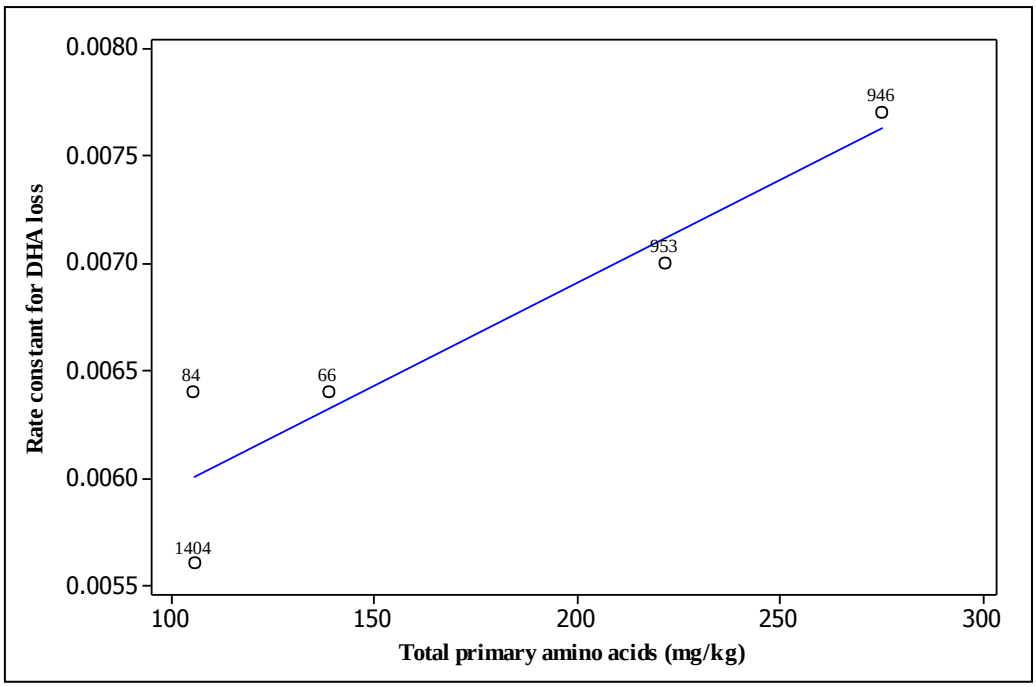


Figure 6.7 Rate constant for DHA loss (k, day^{-1}) vs. total primary amino acids for mānuka honey samples with 2,000 mg/kg DHA (R^2 = 86%).

Correlations between k and individual amino acids were examined for the mānuka honey samples (Table 6.7). Serine, alanine, leucine, phenylalanine and tyrosine had the highest correlations (R^2 < 90%). However, amino acids with similar R groups did not necessarily have similar correlations. For example, alanine, valine had R^2 values of 96% compared to isoleucine and leucine which only had R^2 values of 78%, although all four amino acids have similar R groups. Furthermore methionine is similar, but contains sulfur in the R group; no correlation with k was observed; however, this may be due to is low concentration (it was the least abundant amino acid in the honeys analysed). Tyrosine and phenylalanine have similar hydrophobic R groups and had similar positive correlations with k. However, tryptophan also has a similar R group, but no correlation is observed; this may be due to the small variation in concentration over the samples analysed. There was no correlation of k with aspartic acid or glutamic acid, which have very similar structures; the R groups contain COO^- groups, which may H bond with the amino group preventing the H from being labile.

Table 6.7 R^2 values (as a percentage) for plots of rate constant of DHA disappearance (k) vs. amino acid.

Amino acid	R^2 for k vs. AA (%)*
Serine	99
Alanine	96
Leucine	96
Phenylalanine	93
Tyrosine	92
Lysine	86
Glycine	85
Valine	78
Isoleucine	78
Histidine	73
Arginine	68
Threonine	60
Aspartic Acid	-
Glutamic Acid	-
Methionine	-
Tryptophan	-

* This excludes samples 78 and HB

* This excludes samples 78 and HB.

No apparent trend between the rate constant for formation of MGO (k') and primary amino acids or total amino acids was observed. Only tryptophan had a positive correlation with k' (72%). As discussed in chapter 5, alanine increased the rate constant of MGO appearance compared to the control sample, but the real honey matrices examined here did not show a correlation between MGO appearance and alanine. The honey matrix is very complex with many reactions occurring which will all influence the rate constant of MGO appearance. Aspartic

acid and glutamic acid both have weak negative correlations with k' (R^2 = 26 and 38% respectively when all 7 samples are plotted, Figure 6.8, or when samples 78 and HB are removed R^2 increases to 35 and 43%). These results suggest that the concentration of amino acids at time zero are not a good predictor by themselves for the rate constant of MGO appearance and that there are one or more other unidentified compounds that enhance the conversion of DHA to MGO. Alternatively, there may be a synergistic effect between some compounds.

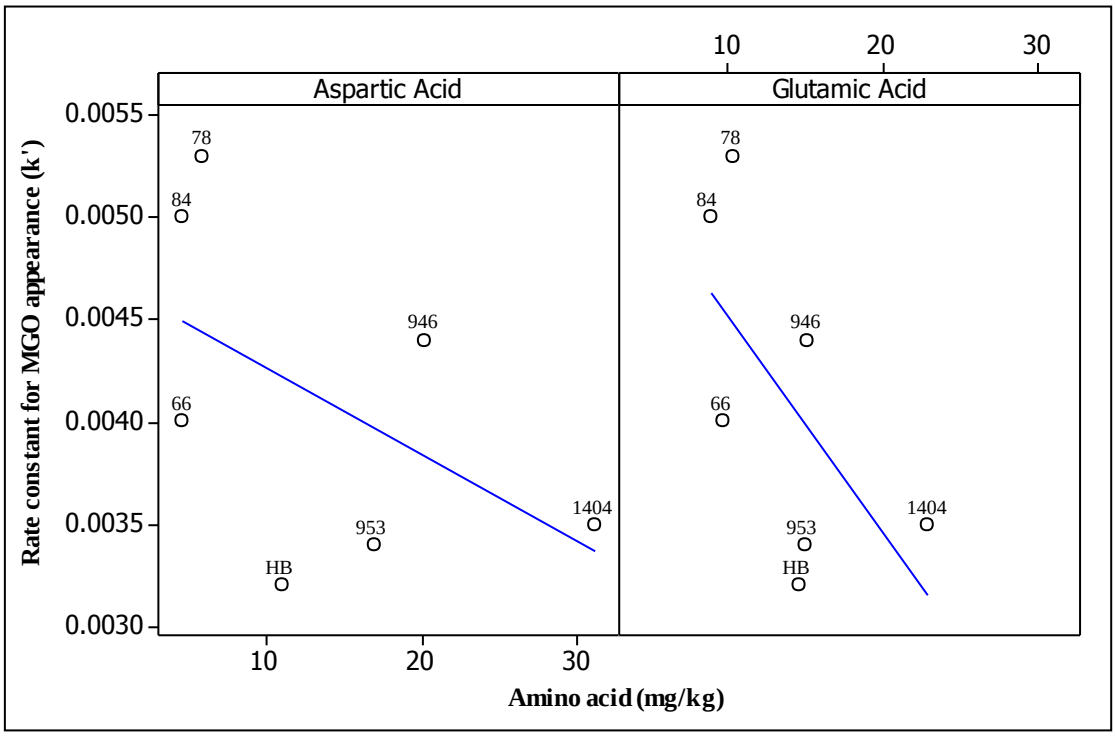


Figure 6.8 Rate constant for MGO appearance (k') vs. amino acid. Aspartic acid and glutamic acid have weak negative linear correlations with k' (R^2 = 26 and 38% respectively, with all 7 honeys plotted).

Elemental analysis

Eighteen elements were analysed in the honeys by ICP-MS (section 4.3.9.2). A summary of the elements for the honeys used in this storage trial are shown in Table 6.8 A and Table 6.8 B. Sodium, magnesium, aluminium, phosphorus, potassium and calcium were found in the highest level, while other elements were in trace amounts.

Table 6.8 A Trace element data for honeys used as matrices in storage trials.

	B			Na			Mg			Al			P			K			Ca			V			Cr		
946	2.65	±	0.01	20.30	±	2.62	36.72	±	0.76	16.07	±	14.86	100.21	±	2.61	1097.35	±	1.15	70.95	±	1.56	0.25	±	0.00	0.45	±	0.03
953	2.11	±	0.04	12.68	±	0.03	21.95	±	0.55	20.50	±	8.41	42.85	±	1.00	1093.81	±	12.29	58.94	±	3.56	0.25	±	0.00	0.48	±	0.02
1349	2.47	±	0.02	51.68			14.83	±	1.02	33.07	±	5.74	16.77	±	1.45	824.95	±	31.94	44.58	±	11.38	0.27	±	0.00	0.59	±	0.00
Airborne	4.38	±	0.21	17.60	±	1.71	13.02	±	2.01	8.02	±	0.33	29.40	±	0.09	322.09	±	0.10	41.53	±	9.26	0.28	±	0.01	0.56	±	0.07
Happy Bee	1.39	±	0.07	38.29	±	1.69	14.95	±	3.08	13.88	±	1.99	24.17	±	3.94	455.40	±	9.20	31.37	±	0.24	0.27	±	0.00	0.54	±	0.04
Hollands	3.80	±	0.86		ND		11.37	±	1.09	3.54	±	4.68	32.67	±	3.49	414.26	±	80.45	33.71	±	11.23	0.26	±	0.02	0.45	±	0.06
Katikati	2.58	±	0.25		ND		10.89	±	3.16	10.68	±	9.05	38.63	±	0.37	326.67	±	10.23	36.32	±	12.12	0.28	±	0.00	0.51	±	0.01
66	3.62	±	0.72	188.11	±	69.74	23.54	±	2.73	1.27	±	0.54	42.69	±	5.59	1067.08	±	70.46	100.33	±	14.62	0.30	±	0.03	0.17	±	0.19
78	4.01	±	0.20	91.39	±	6.15	25.61	±	0.11	0.63			37.33	±	1.33	1575.91	±	45.84	93.77	±	1.95	0.34	±	0.03	0.09	±	0.02
84	3.89	±	0.01	44.65	±	0.60	18.76	±	0.05	42.09			39.74	±	0.55	1124.46	±	13.47	74.19	±	15.66	0.38	±	0.00	0.19	±	0.04
14	2.99	±	0.83	27.61	±	11.21	28.43	±	0.82	7.57	±	0.03	24.15	±	0.02	1213.45	±	57.23	37.56	±	6.92	0.30	±	0.03	0.02	±	0.01

Table 6.8 B Trace element data for honeys used as matrices in storage trials.

	Ni			Cu			Zn			Sr			Ag			Pb			Fe			Mn		
946	0.05	±	0.00	0.97	±	0.90	4.16	±	1.45	0.12	±	0.01	0.01	±	0.00	0.46	±	0.42	1.99	±	0.39	4.23	±	0.10
953	0.01	±	0.01	0.10	±	0.00	5.68	±	0.03	0.09	±	0.00		ND		0.05	±	0.06	1.16	±	0.24	1.59	±	0.02
1349	0.01			0.23	±	0.15	7.18	±	0.44	0.01	±	0.00	0.01	±	0.00	0.19	±	0.11	1.23	±	0.08	0.41	±	0.01
Airborne	0.04	±	0.05	0.17	±	0.18	1.82	±	0.24	0.01			0.02	±	0.01	0.33	±	0.19	1.04	±	0.00	0.05		
Happy Bee	0.18	±	0.21	0.03	±	0.01	2.86	±	0.99		ND		0.05	±	0.04	0.07	±	0.03	1.32	±	0.04	0.85	±	0.03
Hollands	0.03	±	0.01	0.13			1.56	±	1.82		ND			ND		0.12			0.98	±	0.59	0.07	±	0.00
Katikati	0.03			0.46	±	0.16	3.35	±	0.14		±		0.04	±	0.00	0.08	±	0.09	1.24	±	1.01	0.02	±	0.01
66	0.16	±	0.13	0.73	±	0.79	1.94			0.84	±	0.10	0.05	±		0.80	±		7.70*	±	4.69	2.49*	±	0.23
78	0.09	±	0.09	0.23	±	0.16		ND		0.80	±	0.02	0.21	±	0.00	0.19	±	0.03	3.94*	±	4.17	3.07*	±	0.11
84	0.01	±	0.01	0.80			0.23			0.41	±	0.04	0.06	±	0.24		ND		1.10*			1.65*	±	0.08
14	0.02	±		0.02	±	0.01	0.29			0.03	±	0.10	0.11	±		ND	±		1.19	±	0.01	7.82	±	0.01

* Fe and Mn were not analysed by DRC for samples 66, 78 and 84

Sodium had a large range (13 to 190 mg/kg). Sodium is ubiquitous and contamination from glassware or handling cannot be ruled out. Potassium and calcium had noticeably different concentrations in the mānuka and clover honeys, with mānuka honey having ~60% more potassium and calcium. Sample 946 had over 50% more phosphorus (100 ppm) than any of the other honeys and also the highest proline level (808 mg/kg). This sample also had the highest water content (21.5%) and was later found to have fermented in the storage drum.

Real honeys doped with 2,000 mg/kg DHA had very similar potassium levels so a trend of potassium concentration with k, k' and x could not be examined. Honeys doped with 10,000 mg/kg DHA had varying potassium concentrations (322-1,097 mg/kg). A positive linear correlation between potassium and disappearance of DHA was seen – the more potassium present the higher the rate of DHA disappearance. However, trends need to be interpreted carefully, because they may be coincidental rather than causative. Potassium phosphate was examined in a model system with alanine and did not show any significant differences for DHA loss or MGO gain between the samples with or without the potassium phosphate. Potassium may have a synergistic effect with an unknown compound in the honey.

Phosphorous levels in the storage trial matrices range from 24 to 100 mg/kg. Samples 946 and 953 were harvested at the start of 2012 and were used soon after as matrices for the kinetics trials, yet, they have very different levels of P (100 and 43 mg/kg respectively). Sample 946 has at least double the concentration of any other honey used. Phosphate has been reported to catalyse the conversion of DHA to MGO in basic media.[105] No trends were observed between phosphorous and k, k' or x in the real honey matrices. A trial with only potassium phosphate was not carried out, nor was an experiment carried out to isolate each element, e.g. potassium chloride or sodium phosphate. Hence conclusions cannot be drawn.

No apparent trends are seen with Ca or Al and k, k' or x. There is a positive trend between Mg and k; however, this may be coincidental and not causative. Further research would have to be carried out. Riddle and Lorenz[105] studied the conversion of DHA to MGO at pH 7.4 and reported that calcium (nitrate),

aluminium (ammonium sulphate), manganese, magnesium, barium, stannous and cadmium ions did not influence the conversion.

A folklore suggests that addition of iron to mānuka honey helps to convert DHA to MGO faster. Samples that started with 10,000 mg/kg initial DHA did not show a trend between iron and k, k' or x. Not all samples doped with 2,000 mg/kg had iron analysed by DRC mode so no conclusions can be drawn from the data.

Phenolic compounds

Eight mānuka honeys stored at 37 °C were tested for seven phenolic compounds (section 4.3.8). Phenolic compounds that are able to act as acids, may influence the conversion of DHA to MGO by altering the equilibrium constant of the DHA dimer and monomer. The rate constants for DHA disappearance and MGO appearance were plotted against the concentration of each of these compounds to see if there was a correlation (Figure 6.9 and Figure 6.10 respectively). Sample 78 was an outlier in all plots, so it was removed for reporting the coefficient of determination (R^2) of each compound with k and k' (Table 6.9).

Table 6.9 Correlation (R^2, %) between k and k' with selected phenolic compounds with sample 78 removed.

Compound	pKa	R^2 for k vs. compound (%)	R^2 for k' vs. compound (%)
Phenyllactic acid	3.72	98†	66
4-Methyoxyphenyllactic acid	*	89	7
Syringic acid	4.34, COOH 9.49, 4-OH	86	14
Methyl syringate	8.7, 4-OH	70	20
2-Methoxybenzoic acid	4.09	66 (negative)	7
Leptosperin	N/A	0.2	2
Luteolin	N/A	0.2	0
Sum of phenyllactic acid and 4-methyoxyphenyllactic acid		91	23
Sum of acids		91	32

* Lactic acid has a pKa of 3.86 and phenyllactic acid has a pKa of 3.72; it is expected that the pKa of 4-methyoxyphenyllactic acid will be slightly lower due to the due to the –I effect from the methyoxy group.
†This sample had sample 84 removed because it was an outlier.

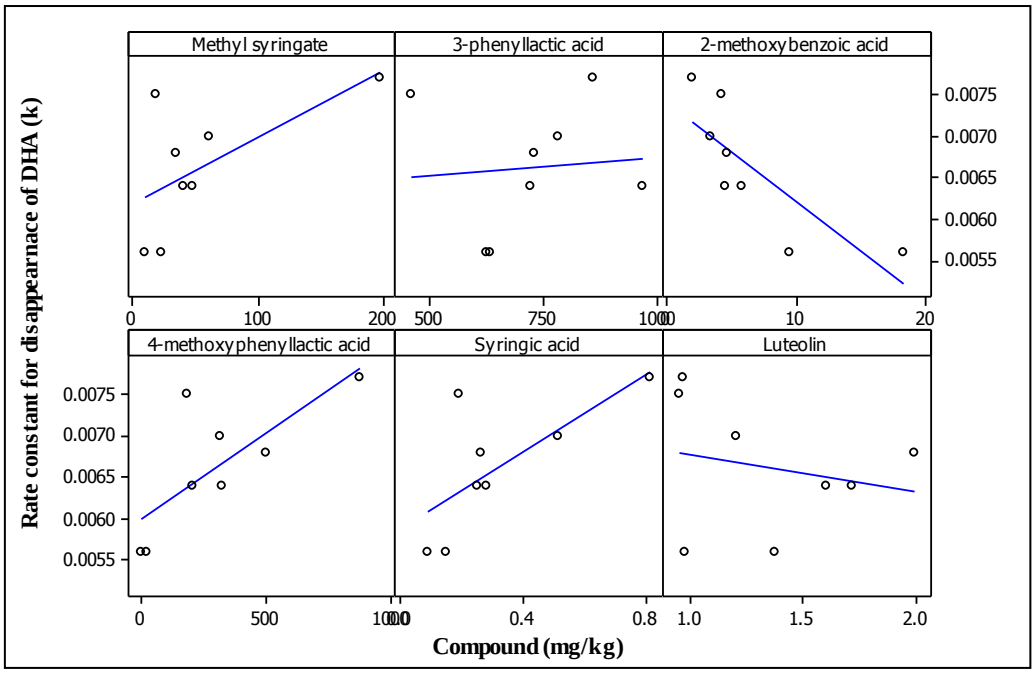


Figure 6.9 Plots of rate constant for DHA disappearance (k) vs. phenolic compounds. All samples are included on the plot.

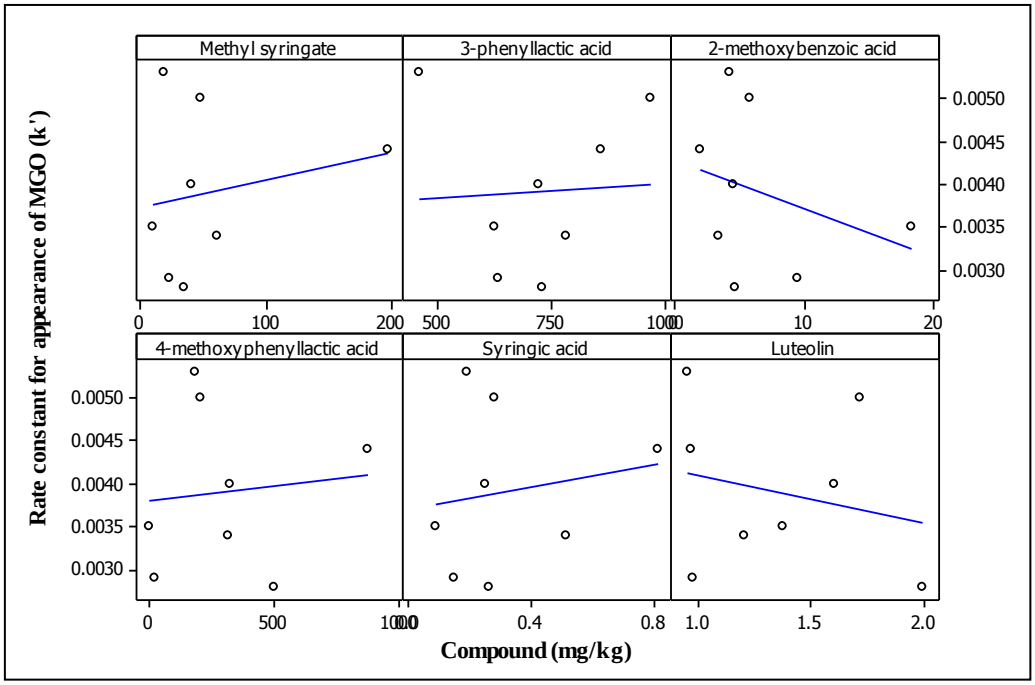


Figure 6.10 Plots of rate constant for appearance of MGO (k') vs. phenolic compounds. All samples are included on the plot.

All acidic compounds analysed here would act as moderately strong acids (low pKa values), hence they are expected to act as proton donors. This may allow

them to catalyse conversion of the DHA dimer to monomer, allowing reactions of DHA to occur faster. The rate constant for disappearance of DHA, k, has the strongest correlations with phenyllactic acid (R^2 = 98%, pKa = 3.72) and 4-methyoxyphenyllactic acid (R^2 = 89%, the pKa is unknown but expected to be close to that for phenyllactic acid); both compounds will act as moderately strong acids in a honey matrix. Donation of a proton to the DHA dimer may alter the rate at which it converts to the monomer. Furthermore, these two compounds are found in high abundance in mānuka honey, hence will have a larger influence in the honey. The sum of phenyllactic acid and 4-methyoxyphenyllactic acid was plotted against the rate constant for the loss of DHA, k; this gave a very strong correlation (R^2 = 91%, Figure 6.11). Furthermore the sum of all phenolic acids also had a strong correlation with k (R^2 = 91%). Syringic acid also had a good correlation with k (R^2 = 86%), but is only found in low levels in mānuka honey. However, 2-methyoxybenzoic acid has a negative correlation with k (R^2 = 66%). This implies that the more 2-methyoxybenzoic acid present the smaller the rate constant for DHA disappearance. It is unknown what would cause this.

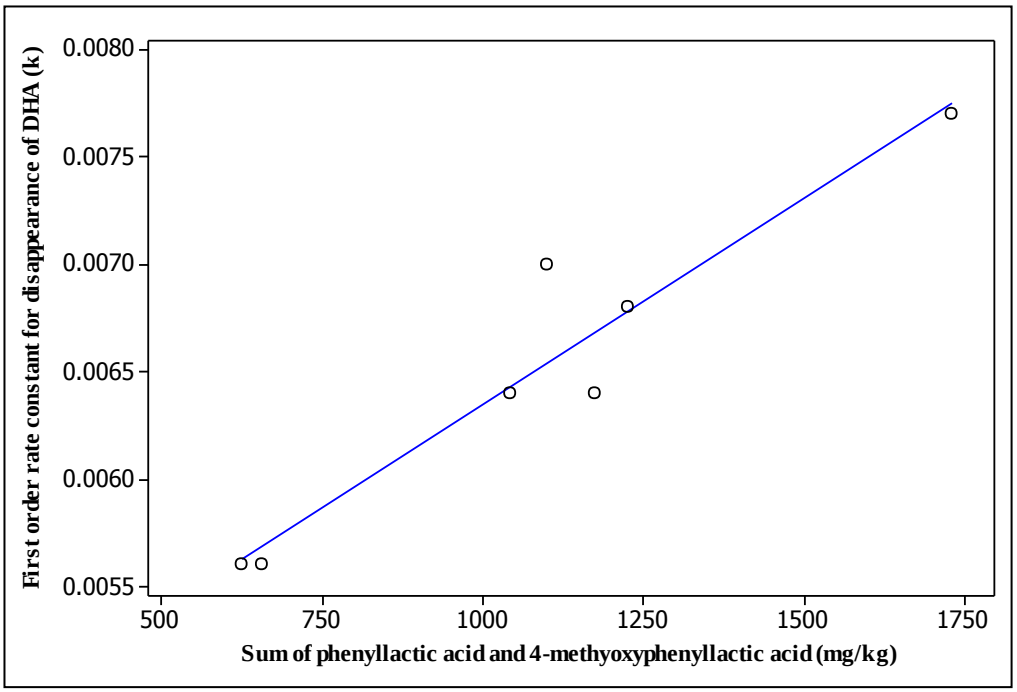


Figure 6.11 Plot of the first-order rate constant for the loss of DHA (k) vs. the sum of phenyllactic acid and 4-methyoxyphenyllactic acid (R^2 = 91.1%).

Methyl syringate does not have a COOH group and the pKa of the OH is high, hence will not readily donate a proton. However, it still had a good correlation

with k (R^2 = 70%), but the reason for this is unknown. There was no correlation between k with leptosperin and only a weak correlation with luteolin; this is expected because neither compound has the ability to donate a proton to catalyse the conversion of DHA to MGO or side products.

Phenyllactic acid had the best correlation with the rate constant for appearance of MGO, k' (R^2 = 66%). As mentioned above, the compound has a low pKa and can be stabilised by the –I effect. The correlation of k' with all other compounds was 20% or less. Furthermore, there was no correlation between the stoichiometric factor (x) and any of the phenolic compounds.

The compounds that have the largest effect on the loss of DHA, may also influence the conversion to MGO or the loss of MGO to side products at later times. However, loss of MGO would not be apparent by examining k', which is the appearance of MGO before it reaches a maximum and begins to decline. Figure 6.12 shows the difference in MGO concentration over time for four mānuka honeys. Table 6.10 shows the sum of phenyllactic acid and 4-methyoxyphenyllactic acid in the mānuka honey samples, in decreasing concentration and the pH and total acidity of each sample.

Sample 946 has the highest concentration of the two phenolic acids and does not have a large decrease in MGO at later times. Sample 78 has a low concentration of the two compounds, but a large decrease in MGO at later times. This suggests that these compounds allow more DHA to convert to MGO, possibly by altering the equilibrium constant between the DHA dimer and monomer. Therefore the loss of MGO is not as great. For most samples, the total acidity reflects the concentration of the two phenolic acids; the higher the concentration of phenolic acids, the higher the total acidity. Furthermore, a high correlation between total acidity and k was discussed at the beginning of this section; additionally, the Happy Bee clover honey had the lowest total acidity, and also the lowest concentration of phenolic acids.

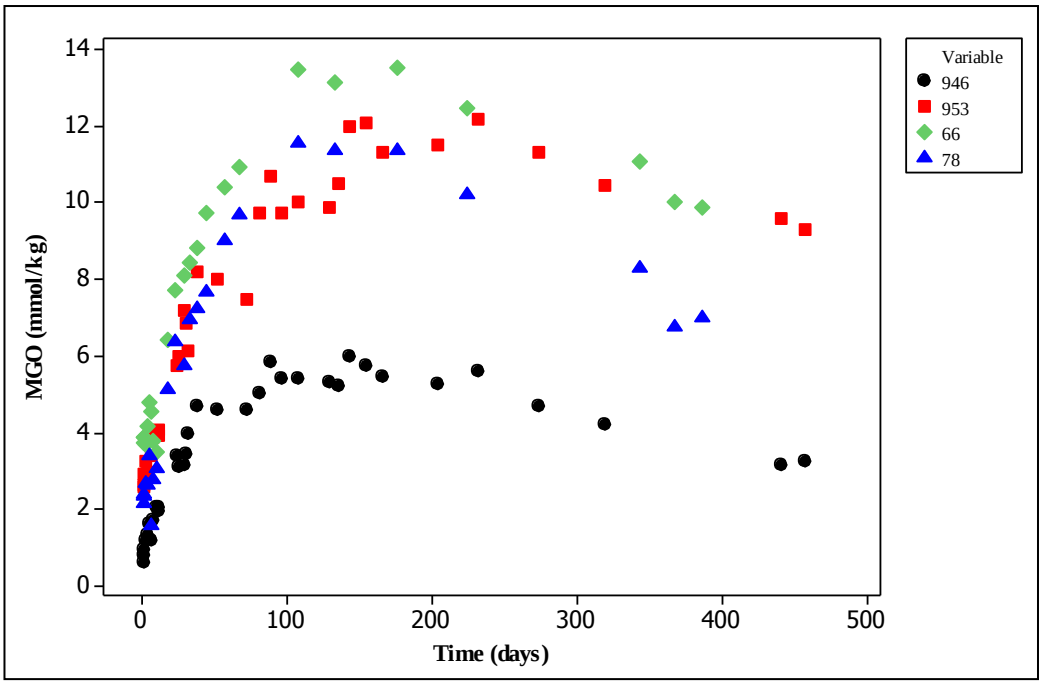


Figure 6.12 Plot of MGO (mmol/kg) vs. time for four mānuka honeys. There are different rates of decline of MGO at later times, which may be influenced by the concentration of phenyllactic acid and 4-methyoxyphenyllactic acid.

Table 6.10 Sum of phenyllactic acid and 4-methyoxyphenyllactic acid (mg/kg, decreasing concentration) and pH and total acidity in mānuka honey samples.

Sample	Sum of phenyllactic acid and 4-methyoxyphenyllactic acid (mg/kg)	pH	Total acidity (milliequiv/kg)
946	1730	3.90	37.10
25	1226	3.76	34.57
84	1175	3.74	30.65
953	1100	3.57	37.34
66	1042	3.69	28.72
41	654	3.70	28.79
78	637	4.27	25.11
1404	623	*	*

*pH and total acidity were not determined due to the sample size.

6.4 Temperature dependence of conversion of DHA to MGO

Fresh and doped honey samples were divided into portions and stored at different temperatures to examine the effect temperature has upon the conversion of DHA to MGO and side reactions of both compounds in real honey matrices. Four fresh (non-doped) mānuka honeys were stored by Gibbs Honeys in incubators in their

warehouse at 27, 32 and 37 °C (samples 14, 25, 32 and 41). They were periodically analysed four times over 8 months. During the summer months the warehouse became warm and the 27 °C incubator reached 33.5 °C, hence the conversion behaved in the same manner as the samples at 32°C for the last part of the storage trial; this data point was removed from the data analysis. Sample 1404, from New Zealand Honey Traders was a fresh mānuka honey but was doped with extra DHA. This sample was split and stored at 20, 27 and 37 °C. Three clover honeys (Happy Bee, Airborne and Hollands) doped with DHA (2,000 mg/kg) and incubated at 4, 20 and 27 °C were tested more frequently over 17 months. Happy Bee doped with 10,000 mg/kg DHA was also incubated at 37 °C. Temperatures from 20 to 37 °C were chosen because this encompasses most temperatures at which beekeepers might store their honey. A summary of the initial DHA and water content of the clover and mānuka honeys used can be found in Appendix G and Table 6.11.

6.4.1 Mānuka honeys

Fresh non-DHA doped mānuka honey stored at 27, 32 and 37 °C

The four fresh mānuka honey samples stored at 27, 32, 37 °C were not repeated in duplicate, but each data point is an average of triplicate analyses. The samples had varying natural starting concentrations of DHA (Table 6.11). The reaction is first-order so this variation will not affect the rate constant for the reaction. All samples had a DHA:MGO ratio of 1.5:1 or lower at all temperatures by the end of data collection.

Table 6.11 Initial starting concentrations of DHA for fresh mānuka honeys stored at 27, 32 and 37 °C.

	Naturally occurring DHA concentration*	
Sample	**mg/kg**	**mmol/kg**
14	1612 ± 40	17.9 ± 0.5
25	2376 ± 30	26.4 ± 0.3
32	3266 ± 60	26.3 ± 0.7
41	3242 ± 50	36.0 ± 0.6

* Triplicate analyses

The rate of DHA loss increased as the temperature of storage increased (Figure 6.13). Error bars are shown on the plot; however, some are so small that they are

difficult to see. Although there was an increase in the rate of DHA disappearance with increased temperature, there was not a corresponding stoichiometric gain in MGO concentration. Initially, MGO was produced faster at 37 °C than at the lower temperatures; however, the higher temperature appears to increase the rate of side reactions of both DHA and MGO more than the reaction of interest. Figure 6.14 shows the concentration change of MGO over time at the 3 temperatures for all 4 samples. The error in the triplicate measurements are small and have been omitted from the graph. At longer storage times more MGO was seen at lower storage temperatures.

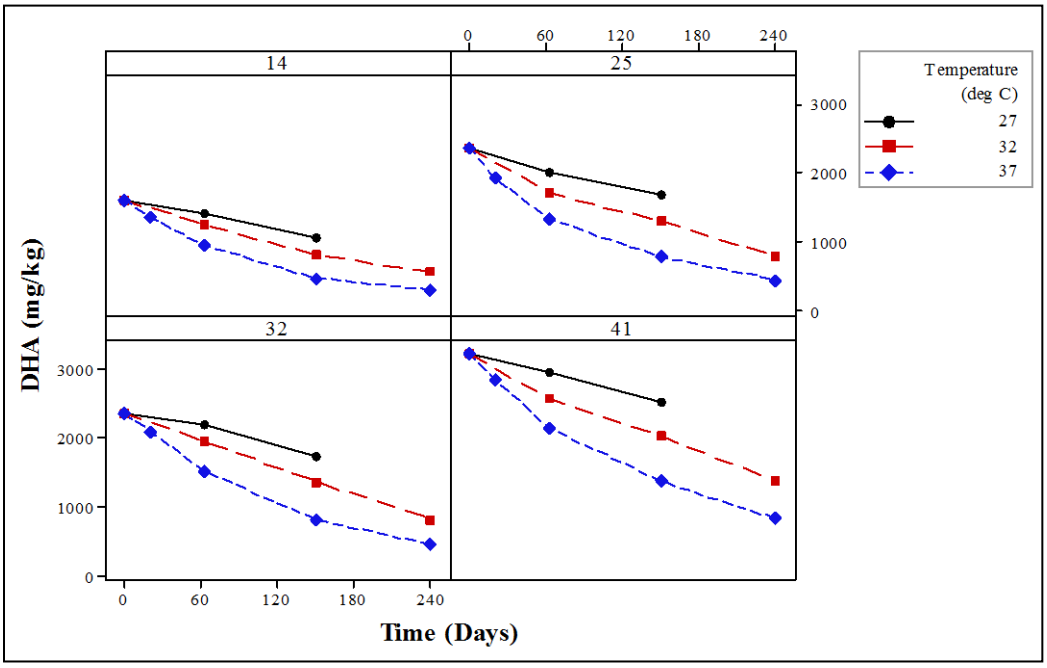


Figure 6.13 DHA consumption vs. time for all four samples. Increasing the temperature increased the loss of DHA in mānuka honey, but did not produce an equivalent amount of MGO.

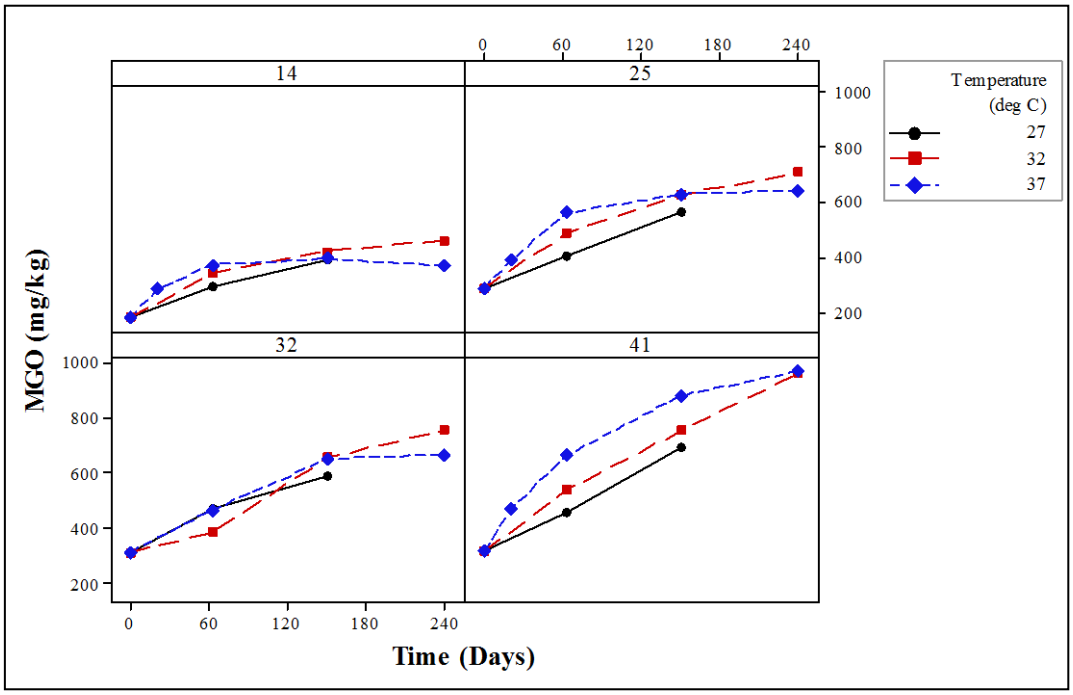


Figure 6.14 MGO appearance vs. time for all four samples. Initially MGO increased fastest at higher temperatures, but its consumption in one or more separate pathways was greater with increasing temperature. Hence at longer times, more MGO was formed at lower temperatures.

First-order plots for DHA loss were created and rate constants were obtained (Table 6.12). An example of the plots for sample 14 at 27, 32 and 37 °C are shown in Figure 6.15. The rate constants for DHA loss of the four mānuka honeys are significantly different at each temperature. As the temperature increases so does the deviation from first-order kinetics at longer time periods. This deviation is not noticeable at 27 °C and is only slight for samples at 32 °C. At 32 °C it does not make a significant difference to the calculation of the rate constant for DHA loss if the last point is included or left out of the calculation. However, at 37 °C this deviation is pronounced and data points at 241 and 314 days were removed for the calculation of the rate constant of DHA disappearance (there were four points to create the line because an extra day had been analysed at this temperature). In comparison, artificial honey systems did not show deviation in the first-order plot for loss of DHA at longer times, presumably because side reaction were limited as only one, two or three perturbants were present in the matrix.

Table 6.12 Rate constants for DHA disappearance (day^{-1}) in mānuka honeys stored at 27, 32 and 37 °C over time.

Temperature (°C)	14	25	32	41	Average
	Rate constant for DHA disappearance (k, day^{-1})				
27	0.0029	0.0023	0.0021	0.0017	0.0023 ± 0.0004
32	0.0044	0.0044	0.0044	0.0035	0.0042 ± 0.0005
37	0.0063	0.0068	0.0069	0.0056	0.0064 ± 0.0007

The variation in the rate constants between the samples is small at each temperature. This is presumably due to the four samples coming from the same honey producer and hence having similar properties due to geographical region.

The four fresh honeys stored at 37 °C were compared to the honeys stored at 37 °C in section 6.2. The average for DHA disappearance in the storage trials was 0.0070 ± 0.0006 day^{-1} which is similar to that of the fresh honeys (0.0064 ± 0.0007).

First-order plots for MGO appearance in sample 14 are shown in Figure 6.16. Samples incubated at 27 °C were linear for the entire duration, samples stored at

32 °C had a slight deviation at longer times, whereas samples at 37 °C deviated substantially from linearity at later times, indicating that at long periods MGO

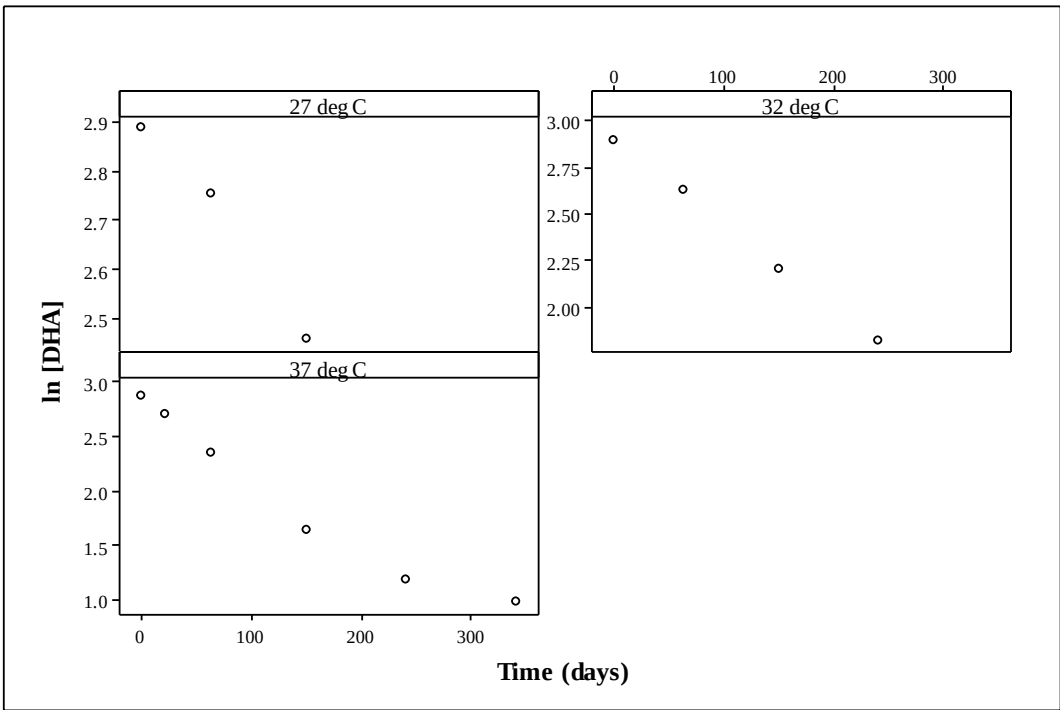


Figure 6.15 First-order plots for DHA loss (ln[DHA] vs. t) in fresh mānuka honey (sample 14) at 27, 32 and 37 °C. At 37 °C the sample begins to deviate from first-order at later times.

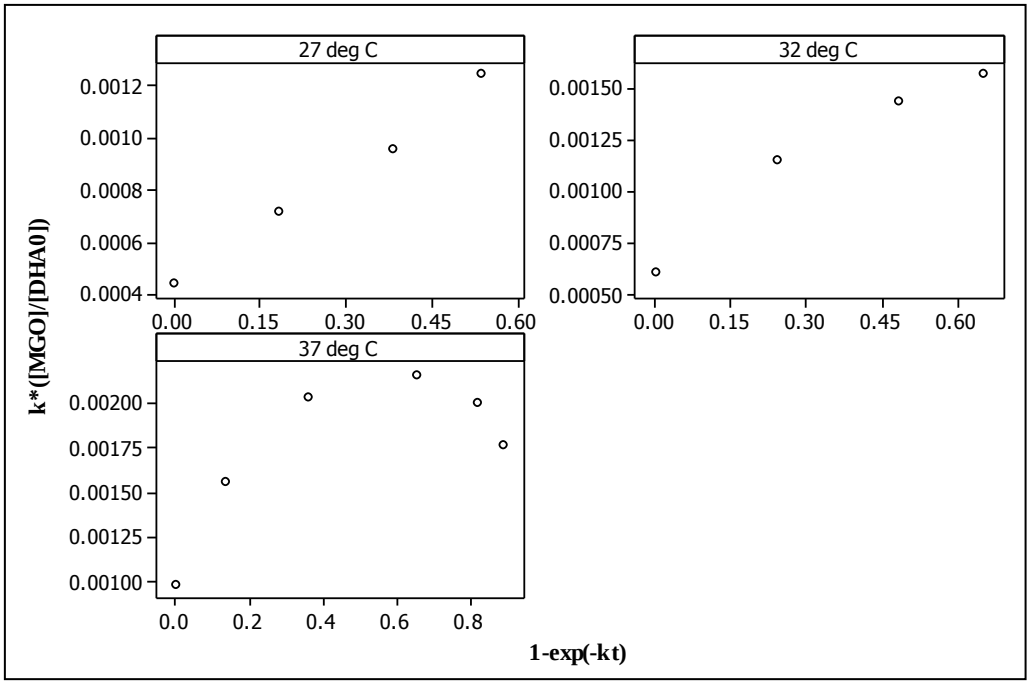


Figure 6.16 First-order plot for the appearance of MGO in mānuka honey (sample 14) stored at 27, 32 and 37 °C. As temperature increases, the deviation from first-order is more prominent at longer times.

does not obey first-order kinetics at high temperature. The pronounced deviation at the highest temperature studied is caused partially by the reaction occurring faster so that it goes through the transition period sooner than at lower temperatures. Furthermore, it is possible that at higher temperatures side reactions become more pronounced. The same effect was seen in artificial honeys, with first-order plots of MGO appearance for some systems (e.g DHA-proline-alanine) deviating from linearity at later times due to further reaction of MGO.

Only the linear portion of the graph (initial formation of MGO, k'_1) was used to calculate the rate constant for MGO appearance, which meant that only three data points were used for the calculation of k'. The calculation of a slope with only three data points is not very reliable, but because the reaction obeys first-order kinetics within this time period it is expected to be linear. Multiple samples have been analysed during this research with a high density of points in this region of the reaction and show a linear increase in MGO; the samples presented here are expected to behave in the same manner.

The initial rate constant for first-order appearance of MGO for the three temperatures for each sample are summarised in Table 6.13. There is an increase in the rate constant for MGO appearance with increasing temperature. However, it is important to remember that after an extended time MGO lost through other pathways becomes dominant, which is not accounted for in the initial rate constant; this effect is enhanced with increased temperature. Hence, initially more MGO is formed at a high temperature, but for storage over a longer period of time a lower temperature gains more MGO.

Table 6.13 Initial rate constants for formation of MGO in mānuka honeys stored at 27, 32 and 37 °C.

Temperature (°C)	14	25	32	41	Average
	Rate constant for MGO appearance (k', day^{-1})				
27	0.0010	0.0007	0.0007	0.0006	0.0008 ± 0.0002
32	0.0017	0.0016	*	0.0014	0.0016 ± 0.0002
37	0.0028	0.0028	0.0029	0.0025	0.0028 ± 0.0002

*The rate constant for this sample could not be calculated due to an outlier in the graph.

The stoichiometric factor ($x = \frac{k'}{k}$) was calculated for each sample at the three temperatures (Table 6.14). It is expected that x would decrease with increasing temperature. However, as k and k' are calculated for initial rates only, samples at 37 °C gained the most MGO in this timeframe, which counteracts the large loss of DHA. Hence the stoichiometric factor is largest for samples at 37 °C. This higher efficiency at 37 °C only applies early in the reaction before the loss of MGO in side reactions becomes prominent.

An average efficiency of conversion could not be calculated for the samples because MGO was not linear over the entire range at the higher temperatures and there were insufficient data points earlier on. Therefore the percentage efficiency of the conversion at the three temperatures at days 63 and 241 were calculated to give a snap shot of the efficiency (Table 6.15). As expected, the efficiency of the reaction decreased with increasing temperature. For samples stored at 37 °C, MGO formation is dominant at 63 days; however, at 241 days side reactions of MGO are more dominant and the efficiency decreased, on average, from 38 ± 4% at 63 days down to 24 ± 7% at 241 days. The efficiency has not dropped significantly for the other temperatures because the samples have not yet reached the transition period because the reactions are occurring more slowly.

The stoichiometric factor is close to the calculated percentage efficiencies at day 63 because it only takes into account the initial reaction. Whereas at 241 days, further reactions of MGO are noticeable at 37 °C, hence the stoichiometric factor does not match the efficiency for data at this temperature.

Table 6.14 Initial stoichiometric factor (x) for mānuka honeys stored at 27, 32 and 37 °C.

Temperature (°C)	14	25	32	41	Average
27	0.47	0.47	0.47	0.54	0.49± 0.04
32	0.39	0.36	-	0.40	0.38 ± 0.02
37	0.44	0.41	0.42	0.45	0.43 ± 0.02

Table 6.15 Efficiency of the conversion of DHA to MGO (%) for mānuka honeys stored at 27, 32 and 37 °C at 63 and 241 days.

Temp (°C)	14		25		32		41		Average	
	Days									
	63	241	63	241	63	241	63	241	63	241
27	70	N/A	41	N/A	-	N/A	65	N/A	59 ± 20	N/A
32	54	33	38	33	22	35	42	43	39 ± 10	36 ± 5
37	36	17	33	23	43	23	40	34	38 ± 4	24 ± 7
Initial DHA (mg/kg)	1612		2376		3266		3242			

Fresh DHA-doped mānuka honey stored at 20, 27 and 37 °C

Sample 1404 was freshly harvested and doped with DHA to give an initial starting concentration of 2,000 mg/kg. The sample was stored at 20, 27 and 37 °C and thus was not incorporated in the discussion in the preceding section due to the different temperatures and time frame. This sample was carried out in duplicate. Initial rate constants for first-order loss of DHA and gain of MGO and the stoichiometric factor are summarised in Table 6.16. The samples were not tested for as long as the honeys in the preceding section, therefore they did not reach the deviation in the first-order plots.

Table 6.16 Summary of k, k' and x for sample 1404 at 20, 27 and 37 °C.

Temperature (°C)	k (day^{-1})	k' (day^{-1})	x
20	0.0015	0.0013	0.87
27	0.0011	0.0012	1
37	0.0056	0.0035	0.63

The rate constant for DHA loss is similar to the other honeys stored at 37 °C, but rate constant for the formation of MGO is larger. The stoichiometric factor for incubation at 37 °C is also larger than seen for the other samples discussed; this could be due to the limited time that the reaction was monitored or due to the different area of harvest.

6.4.2 Doped clover honey

Three clover honeys (HB, AB, Hol) were doped with approximately 2,000 mg/kg (22 mmol/kg) DHA and stored at 4, 20 and 27 °C; Happy Bee (2,000 mg/kg DHA) was also stored at 37 °C. Additionally Airborne and Hollands were doped with

10,000 mg/kg DHA and stored at 37 °C. These results are included in the discussion below because previous results show that the initial concentration of DHA does not affect the rate constant. Samples were periodically tested to monitor the conversion of DHA to MGO.

Samples stored at 4 °C did not lose DHA and consequently did not gain MGO, showing that storage at this temperature will essentially halt the reaction. Samples stored at 20 and 27 °C had scatter in the DHA results, which was more prominent in the early stages. This was also seen in the artificial honey systems stored at these temperatures. The scatter seen for the DHA data may arise from a longer equilibration time of the system at lower temperatures. This is the time taken for the DHA to become homogenous in the sample. Molecules at a lower temperature move more slowly; therefore it may take longer for the molecules to initially distribute in the matrix. The viscosity of the honey due to the lower temperature may also increase equilibration time. Another cause of the scatter may be due to the slower conversion at lower temperatures, which makes it harder to measure changes in concentration due to experimental error. An example of the scatter is shown in Figure 6.17, which shows the disappearance of DHA in Happy Bee honey at 37, 27, 20 and 4 °C. In contrast, there is no scatter in the measurements of the formation of MGO at the lower temperatures (Figure 6.18), which suggests the scatter from DHA arises from equilibration time of the system. The least squares line on the first-order plots for DHA data at 20 and 27 °C over the entire set of data show poor fit due to the scatter.

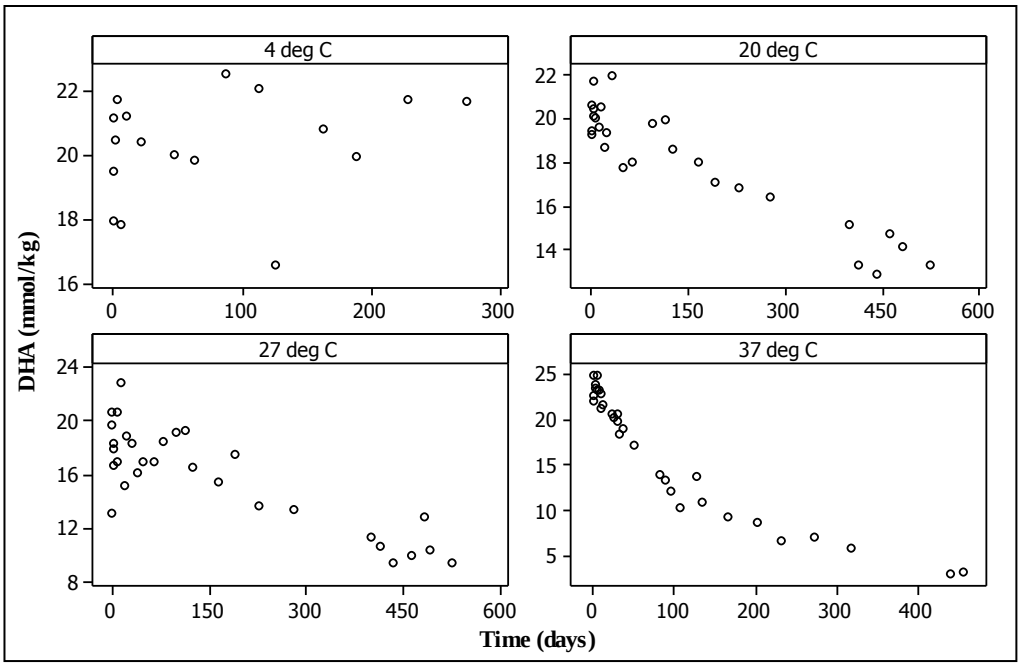


Figure 6.17 Loss of DHA vs. time for Happy Bee honey stored at different temperatures. Sampled incubated at 27, 20 and 4 °C have a large amount of scatter early in the reaction proposed to be due to slow equilibration of the more viscous honey at these lower temperatures.

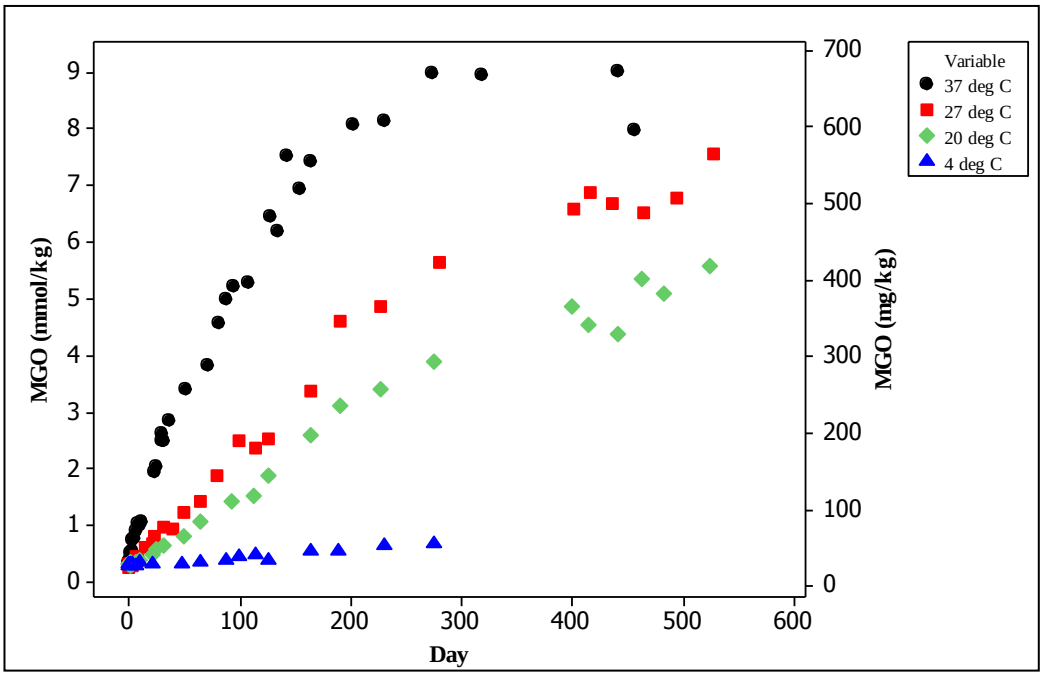


Figure 6.18 Formation of MGO vs. time for Happy Bee honey stored at different temperatures. There is no scatter in this data. As the temperature increases so does the rate of MGO formation. The concentration of MGO in the samples stored at the lower temperatures does not surpass the 37 °C, as seen in mānuka honey.

As with mānuka honey, MGO formation increased with increasing temperature for the clover honey. However, at longer times, the clover honey did not show the same trend as the mānuka honeys over the different temperatures. There was no large loss of MGO at later times when stored at higher temperatures and the concentration of MGO for honey stored at the lower temperatures does not exceed that formed at higher temperatures. For the clover matrix, the higher the temperature, the more MGO is formed in a quicker time. The clover honeys were stored for longer than the mānuka honeys and no large loss of MGO was observed, compared to the loss observed in the mānuka honeys discussed above. This is most likely due to extra compounds in the mānuka honey matrix that may influence the fate of MGO. It appears that these compounds are not present in clover honey, therefore clover may not be a good matrix to use to model the reactions that are occurring at later times. At 150 days, the MGO concentration is still climbing in the clover honey matrix in the sample stored at 37 °C; at this point the DHA:MGO ratio is approximately 1.7:1. Sample 25 analysed in section 6.4.1 is a fresh mānuka honey with a similar starting concentration of DHA to the Happy Bee sample; the MGO began to decline at 150 days when the ratio was 1:1.

Table 6.17 summarises the rate constants for DHA disappearance. As expected, DHA decreases faster with increasing temperature. However, the rate constants for DHA disappearance in clover honey were smaller than for the mānuka honey samples (DHA doped and non-doped), indicating that a compound present in mānuka honey that enhances a side reaction of DHA is not prominent in clover honey. At 27 °C, the average rate constant for DHA disappearance in clover honey was 0.0014 ± 0.0003 day^{-1} compared to 0.0029 ± 0.0004 day^{-1} in mānuka honey for non-DHA doped and 0.0011 day^{-1} for the one mānuka that was doped with DHA (but was only sampled for a short time). At 37 °C, it was 0.0048 ± 0.0004 day^{-1} in clover honey compared to 0.0064 ± 0.0007 day^{-1} in non-DHA doped mānuka honey and 0.0054 day^{-1} in the one mānuka that was doped with DHA.

Table 6.17 Rate constants for DHA disappearance (k, day^{-1}) in doped clover honeys at 20, 27 and 37 °C.

Temperature (°C)	Happy Bee	Airborne	Hollands	Average
	Rate constant for DHA disappearance (k, day^{-1})			
20	0.0008	0.0008	0.0010	0.0009 ± 0.0001
27	0.0013	0.0012	0.0017	0.0014 ± 0.0003
37	0.0048	0.0045*	0.0052*	0.0048 ± 0.0004

*AB and Hol incubated at 37 °C were doped with 10,000 mg/kg DHA.

The first-order plots of MGO appearance for 20 and 27 °C did not show deviation from linearity at longer times like mānuka honey. The clover honeys were monitored over a much longer time period and did not show deviation. The first-order plot of mānuka honey stored at 37 °C began to deviate from linearity between 60 and 150 days, compared to the clover honey which had very slight deviation around 441 days; however, due to the age of the sample it appeared to have dried out, which may be the cause of the deviation.

The rate constants for MGO appearance were calculated over the entire linear region (Table 6.18). The rate constant for MGO appearance at 37 °C in clover (0.0038 ± 0.001 day^{-1}) is larger than the non-DHA doped mānuka honeys (0.0028 ± 0.0002 day^{-1}). The DHA-doped mānuka had a first-order rate constant of 0.0035 day^{-1} which is similar to the clover honeys. The rate constant for MGO appearance at 27 °C is similar between the clover, non-doped and doped mānuka honeys (0.0012 ± 0.001, 0.0014 ± 0.0000 and 0.0012 day^{-1} respectively).

Table 6.18 Rate constants for MGO appearance, (k', day^{-1}) in doped clover honeys at 20, 27 and 37 °C.

Temperature (°C)	Happy Bee	Airborne	Hollands	Average
	Rate constant for MGO appearance (k', day^{-1})			
20	0.0007	0.0005	0.0007	0.0006 ± 0.0001
27	0.0013	0.0011	0.0012	0.0012 ± 0.0001
37	0.0032	0.0032*	0.0049*	0.0038 ± 0.0010

*AB and Hol incubated at 37 °C were doped with 10,000 mg/kg DHA.

The stoichiometric factor was calculated for each sample. Airborne and Hollands samples incubated at 37 °C had 10,000 mg/kg DHA added, which may swamp out an effect of consumption of DHA in a side pathway; these two samples had a high

factor (0.71 and 0.94), compared to the Happy Bee sample (0.67) which only had 2,000 mg/kg DHA added. It must be remembered that this is only the initial reaction and at later times the efficiency at 37 °C decreased. At 37 °C, the conversion decreased at longer times, similar to the mānuka honeys; the conversion was only 20-30%, due to other reactions taking precedence at the higher temperature. The efficiency of the conversion was not calculated for samples at lower temperatures due to the scatter in DHA data. The clover honeys have a higher stoichiometric factor than the non-DHA doped mānuka honeys, which also agrees with the idea that clover honey has less of any compounds which remove MGO.

Table 6.19 Stoichiometric factor for samples incubated at 20, 27 and 37 °C.

Temperature (°C)	Happy Bee	Airborne	Hollands	Average
20	0.88	0.63	0.70	0.7 ± 0.1
27	1.00	0.92	0.71	0.9 ± 0.2
37	0.67	0.71*	0.94*	0.8 ± 0.2

* AB and Hol incubated at 37 °C were doped with 10,000 mg/kg DHA.

6.4.3 Activation Energy

The activation energy (E_a) and frequency factor (A) of a chemical reaction are derived from the Arrhenius equation ($k_n = Ae^{\frac{-E_a}{RT}}$) in order to interpret the effect of temperature on reaction rate. Taking the natural logarithm of both sides of the Arrhenius equation gives:

$$\ln(k_n) = \frac{-E_a}{RT} + \ln(A) \quad (6\text{-}1)$$

Where:

k_n = rate constant for the disappearance of DHA, k, (day^{-1}) or rate constant for MGO appearance, k', (day^{-1})

E_a = activation energy of the reaction (J mol^{-1})

R = gas constant (8.314 J K^{-1} mol^{-1})

T = Temperature (K)

A = pre-exponential factor (day^{-1})

A plot of $\ln(k)$ or $\ln(k')$ $vs.\frac{1}{T}$ will be linear if the temperature dependence of a reaction rate is accurately described by the Arrhenius equation. The slope of this plot (when multiplied by –*R*) provides E_a of the reaction, and the intercept provides the Arrhenius pre-exponential factor, *A*.

Interpretation of the measured values of E_a and *A* for a chemical reaction may be carried out within either a molecular or a thermodynamic context. On the molecular level, the quantity *A* is interpreted as the frequency at which reactants in a bimolecular reaction (at standard state = 1 mol L^{-1}) collide with each other; E_a is interpreted as the energy difference between reactants and an activated complex close to the highest energy between reactants and products, and $e^{-(\frac{E_a}{RT})}$ is interpreted as the probability that a particular collision will lead to reaction. This probability is typically quite small, and decreases very rapidly as E_a exceeds a few kJ mol^{-1}. Measured values of E_a often fall into the range of 20-150 kJ mol^{-1}.

Alternatively, the value of *A* may be treated as:

$$A = e^2(\frac{kT}{h})e^{\frac{S_a}{R}} \quad (6\text{-}2)$$

Where:

S_a = the standard entropy change between reactants and an activated complex close to the maximum energy encountered on the route from reactants to product

k = the Boltzmann constant

h = the Planck constant

A = This generally reflects the rate at which reactants find each other in a reaction matrix (likely to be sluggish in honey due to the low water content)

Activation energies calculated for changes of [DHA] and [MGO] in mānuka and clover honeys take into consideration all reactions that DHA and MGO are involved in, not just the conversion of DHA to MGO. In comparison, side reactions of both DHA and MGO are minimised and controlled in model systems (artificial honey matrices) and are of less importance than in real honey matrices.[*] Therefore rate parameters (e.g. E_a and *A)* were derived from the early stages of the

[*] Experiments were conducted to derive E_a and *A* in artificial honey systems; however, measurement error in DHA at lower temperatures prevented this from being carried out.

reaction in real honey matrices when side reactions of MGO should be minimal; this gives a better indication of the fundamental chemistry occurring for DHA and MGO than when rate parameters at later times in the reaction are used. Side reactions of MGO may have activation energies different from those of the initial conversion of DHA to MGO. Thus the apparent E_a and A may change as the extent of the reaction increases and when side reactions become important.

Values of E_a and A for mānuka and clover honeys are derived below before a discussion on the results for both matrices. It must be noted that samples were stored at different temperatures. Mānuka honeys were stored at 27, 32 and 37 °C, while the clover honeys were stored at 20, 27 and 37 °C. Furthermore, Airborne and Hollands clover honey were doped with 10,000 mg/kg for storage at 37 °C, and doped with 2,000 mg/kg for storage at 20 and 27 °C. Further trials are necessary to obtain a more detailed comparison of the temperature dependence of these reaction rates, and the suggestions made below must be taken as tentative.

Table 6.20 summarises the activation energies and the Arrhenius pre-exponential factors (calculated from the intercept and slope, respectively, of the Arrhenius plots) for DHA and MGO for both mānuka and clover samples.

Arrhenius plots for the mānuka honey samples stored at 27, 32 and 37 °C are linear for DHA loss (Figure 6.19). This linearity suggests that the initial reaction of DHA is a one-step removal, as expected. The Arrhenius plots for MGO appearance were also linear (Figure 6.20). Arrhenius plots for both DHA and MGO in clover honeys were also linear (Figure 6.21 and Figure 6.22).

Table 6.20 Activation energy for disappearance of DHA and formation of MGO between 27 and 37 °C in mānuka honeys and 20 and 37 °C in clover honeys doped with DHA.

Sample*	Temp range (°C)	E_A for disappearance of DHA (kJ mol^{-1})	E_A for appearance of MGO (kJ mol^{-1})	*A* for disappearance of DHA (day^{-1})†	*A* for appearance of MGO (day^{-1})†
14 (M)	27 - 37	70	152	22	54
25 (M)	27 - 37	85	189	28	68
32 (M)	27 - 37	93	184	31	66
41 (M)	27 - 37	93	167	31	59
HB (C)	20 - 37	81	81	26	21
AB (C)	20 - 37	78	78	25	26
Hol (C)	20 - 37	74	74	23	29
Average (M + C)	20 - 37	82 ± 9	132 ± 52	27 ± 4	47 ± 19
Average (M)	27 - 37	85 ± 11	173 ± 17	28 ± 4	62 ± 6
Average (C)	20 - 37	78 ± 3	78 ± 3	25 ± 2	25 ± 4

*M = Mānuka, C = Clover

† *A* was calculated as the intercept of the plot.

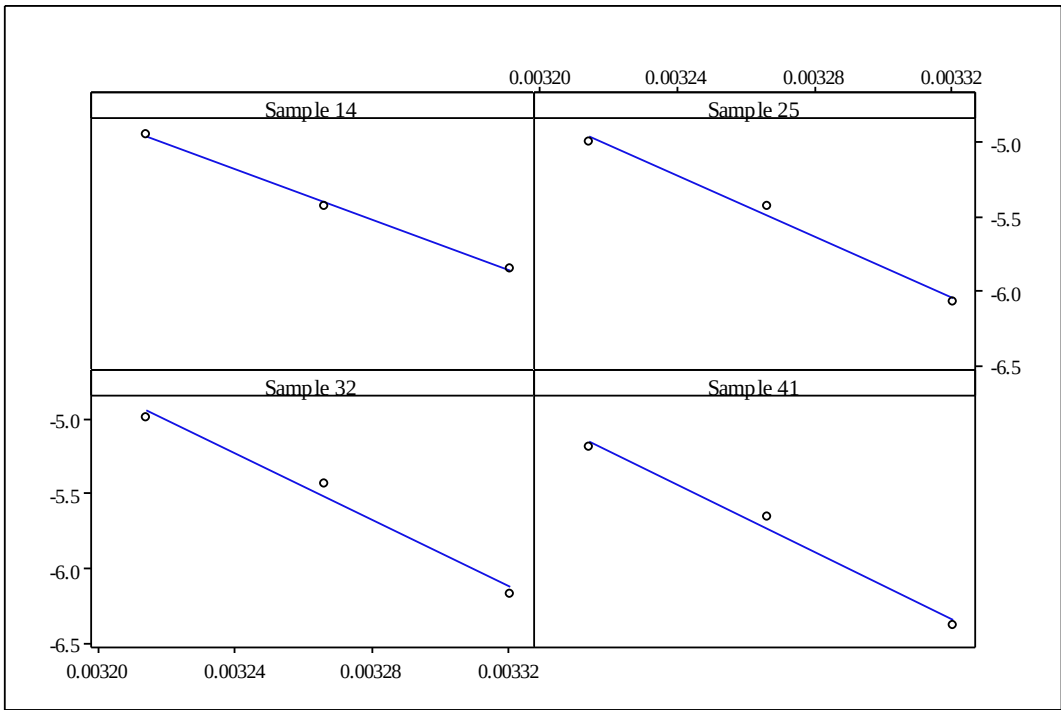


Figure 6.19 Arrhenius plots (ln(k) vs. 1/T) for DHA loss in mānuka honeys incubated at 27, 32 and 37 °C. The plots are linear for all four samples.

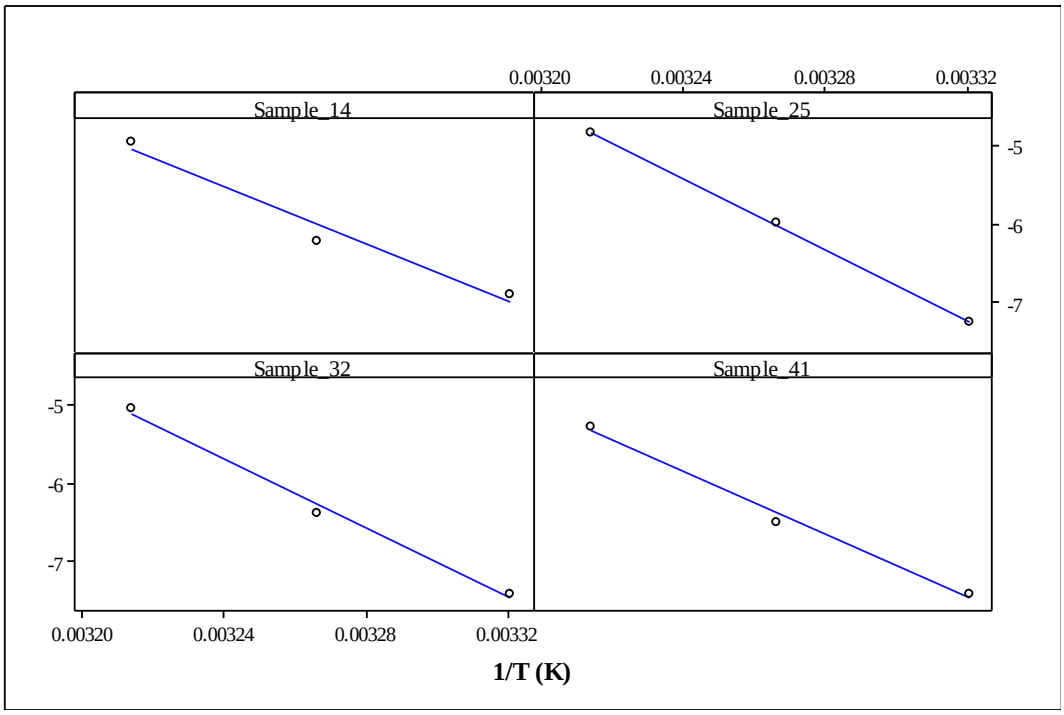


Figure 6.20 Arrhenius plots (ln(k') vs. 1/T) for formation of MGO in mānuka honeys incubated at 27, 32 and 37 °C. .

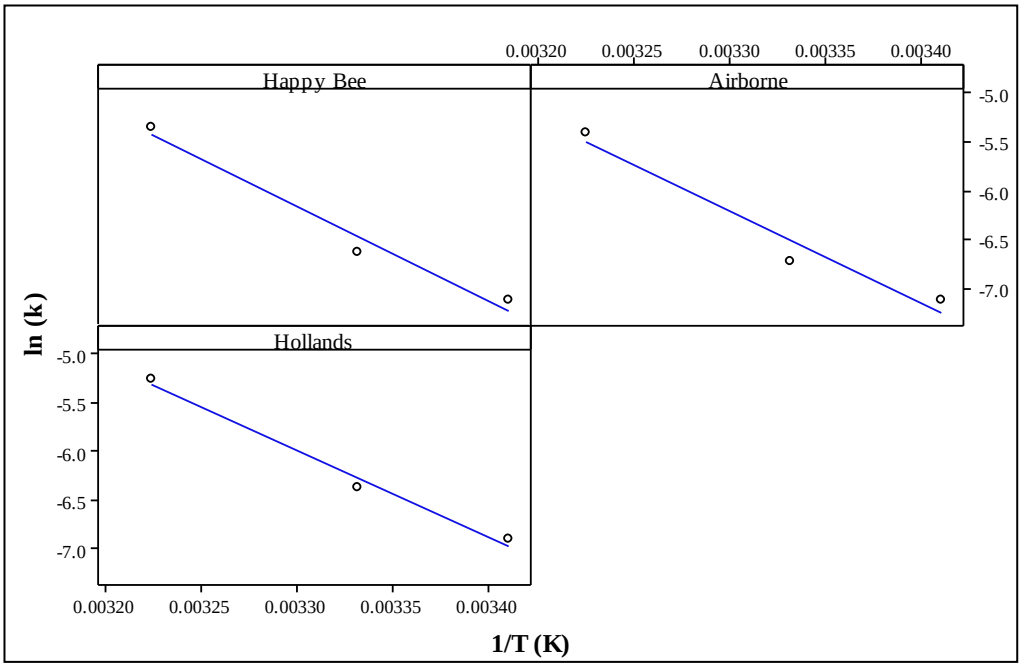


Figure 6.21 Arrhenius plots (ln(k) vs. 1/T) for doped clover honeys incubated at 20, 27 and 37 °C.

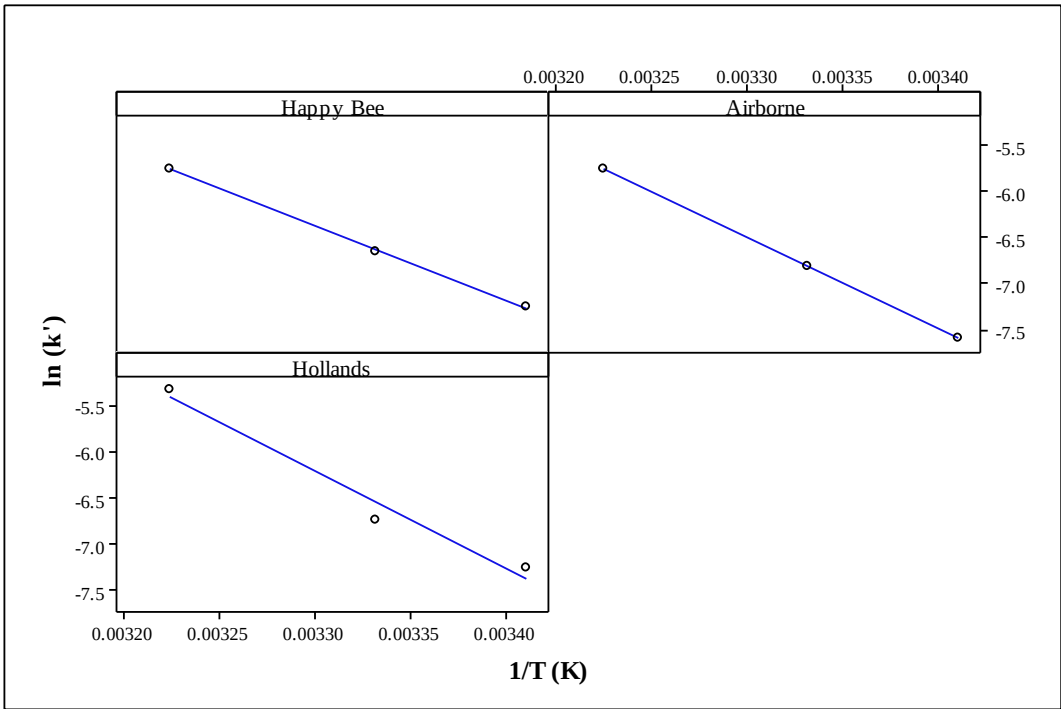


Figure 6.22 Arrhenius plots for MGO formation (ln(k') vs. 1/T) for clover doped honeys incubated at 20, 27 and 37 °C.

Due to the lack of data points for each temperatures and also the small number of temperatures examined for each honey, all mānuka honeys were plotted together on one graph to calculate E_a and *A*; this was repeated for clover honeys and both mānuka and clover honeys together. The resulting R^2 values for linear fits were large; the worst fit was for MGO in mānuka and clover honeys plotted on the same graph (R^2 = 80%). The activation energies and Arrhenius pre-exponential factor are summarised in Table 6.21. Arrhenius plots for all samples are shown in Figure 6.23.

Table 6.21 Activation energy for disappearance of DHA and formation of MGO and Arrhenius pre-exponential factor for mānuka and clover honeys between 20 and 37 °C, calculated from linear line fitted to all data points.

Matrix*	E_A for disappearance of DHA (kJ mol^{-1})	E_A for appearance of MGO (kJ mol^{-1})*	*A* for disappearance of DHA (day^{-1})	*A* for appearance of MGO (day^{-1})
Mānuka	85	173	28	62
Clover	69	79	25	25
M + C	86	99	28	32

*M = mānuka, C = clover

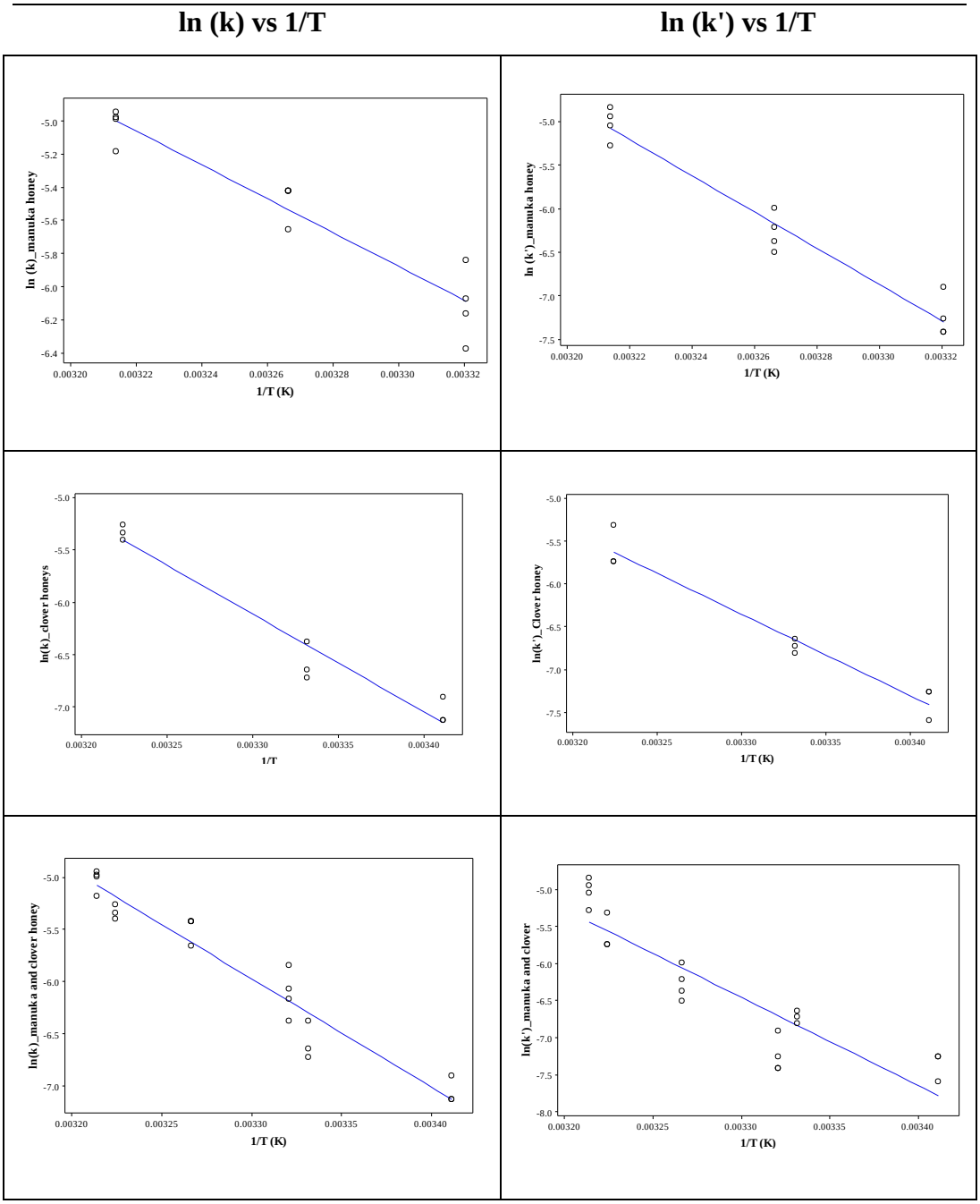


Figure 6.23 Arrhenius plots for DHA (left) and MGO (right). The top row is four mānuka honeys (27 - 37 °C), the middle row is three clover honeys (20 - 37 °C) and the bottom row is all data combined. All plots show linearity.

As previously mentioned, measured values of E_a often fall into the range 20-150 kJ mol^{-1}. The activation energy for disappearance of DHA for individual mānuka honeys are between 70 and 93 kJ mol^{-1}, which fall into the expected range. This is similar to the activation energy reported by Fedoronko *et al.*[289] for the conversion of 1,3-dimethoxy-2-propanoneto MGO in an aqueous acidic environment between 30 and 60 °C. Calculated E_a for the disappearance of DHA in three clover honeys was similar to the mānuka honeys (74 to 81 kJ mol^{-1}) and was similar when all

three were plotted together. E_a for appearance of MGO was similar in mānuka honey and clover honey (85 ±11 compared to 78 ± 3 kJ mol^{-1}). When the mānuka honeys were plotted on the same graph E_a was similar to E_a calculated from all three clover honeys (85 compared to 69 kJ mol^{-1}). This is the same as the activation energy reported by Fedoronko *et al.*[289] for the conversion of DHA to MGO in an aqueous acidic environment between 30 and 60 °C. The activation energies for loss of DHA and gain of MGO are similar, which is expected because in the initial part of the reaction side reactions of MGO are minimised. As a rule of thumb, the activation energies of first-order reactions are such that near room temperature (15-25 °C) a 10 °C increase in temperature will increase the reaction rate by a factor of two, corresponding to an activation energy of 209 kJ mol^{-1}.[374]

The pre-exponential factor (*A)* is a reflection of the rate of total reactant collisions and in Transition Sate Theory comprises the product of the typical vibrational frequency of a chemical bond (*kT*/*h*, about $10^{13}s^{-1}$ or 10^{18} day^{-1}) and a geometric orientation factor (mol L^{-1}) related to the entropy loss when the transition state is formed from two reactant species. This geometric factor may be understood as approximately the ratio of the number of quantum states available to the transition state to the number of quantum states available to the reactants.

Typical *A*-factors for gas-phase atom-transfer (metathesis) reactions are approximately 10^{10} mol L^{-1} s^{-1}or 10^{15} mol $L^{-1}day^{-1}$.[375] The experimental *A* values reported in
Table ***6.20*** and Table 6.21 are about 30 day^{-1}, which are very small values in comparison to gas-phase metathesis reactions. This decrease arises from the complexity of the sugar matrix, providing a large number of reactant quantum states in the denominator of the geometric factor. An alternate interpretation of the low experimental *A* values is that the complexity of the honey matrix greatly slows molecular migration of reactant species.

The values of the activation energies presented here are small. However, the reactions themselves are much slower than would be expected on the basis of the relatively small activation energies. The slower reactions appear to be a result of a low pre-exponential factor (A) rather than a high E_a. This suggests that the reaction rate is largely controlled by viscous matrix. For example, the rate of the

decomposition of dimeric DHA into the monomeric form may be controlled by the slow diffusion of the two DHA molecules away from each other, as well as the limited H^+ available. The temperature dependence of diffusion is given by the equation $x\ (cm) = (Dt)^{\frac{1}{2}}$, where *x*(cm) is the root-mean-square-average distance a molecule diffuses in time *t* (sec), and *D* is the diffusion coefficient ($cm^2\ s^{-1}$).

6.5 High pressure processing and temperature as a means of increasing reaction rate

In 2012, Al-Habsi and Niranjan[338] reported the use of HPP (high pressure processing) and temperature as a means of increasing the MGO content of mānuka honey. This paper evoked considerable interest because, if it worked, it could be a valuable method for increasing the MGO content because it does not require prolonged storage, which can be detrimental to the quality of the honey. Furthermore, high pressure can kill yeast spores and prevent honey from spoiling. However, while HPP is known for its effect on microbial cells and large molecules, it is not generally known for affecting small molecules such as DHA and MGO.

The work described below has been published and can be found in Appendix H.

Artificial honey and two store bought clover honeys doped with 2,000 mg/kg, one store-bought mānuka honey and two freshly harvested mānuka honeys were divided into ten sub-samples and subjected to different HPP treatments (Table 6.22). Sub-samples were analysed by HPLC (PFBHA method) for DHA, MGO and HMF and compared to the control sample to identify whether or not any changes had occurred. The concentrations of DHA, MGO and HMF for each sample at each treatment can be found in Appendix H.

Table 6.22 HPP treatment regimes of honey samples.

Treatment number	Time (min)	Pressure (MPa)
1	15	100
2	45	100
3	90	100
4	15	400
5	45	400
6	90	400
7	15	600
8	45	600
9	90	600
Control	0	0

The percentage change in DHA, MGO and HMF between each treatment and the untreated sample are summarised in Appendix H. There was less than 5% difference in the reported concentration of MGO between treatment 9 (the highest pressure and longest time) and the untreated samples for 9 of the 12 samples (Figure 6.24). The two clover and artificial honey slightly exceeded this (between 5 and 10% difference); the initial MGO concentration of these samples was an order of magnitude smaller than for the mānuka honeys (Appendix H). A similar result was obtained for DHA. However, there was more variation in the HMF results.

The results from this research are in sharp contrast to the observations by Al Habsi and Niranjan,[338] who reported a trend of time and pressure dependent increase in MGO for all treatment conditions (pressures between 100 and 800 MPa and times between 15-120 minutes). They reported a 17% change in MGO concentration for the most severe treatment. The samples subjected to treatment 9 in the current research were re-analysed using the OPD method because this was the method used by Al Habsi and Niranjan. The MGO concentration had a larger variation in MGO concentration (Table 6.23); however, it was randomly distributed and the highest variation occurred for the samples with the lowest initial MGO concentration.

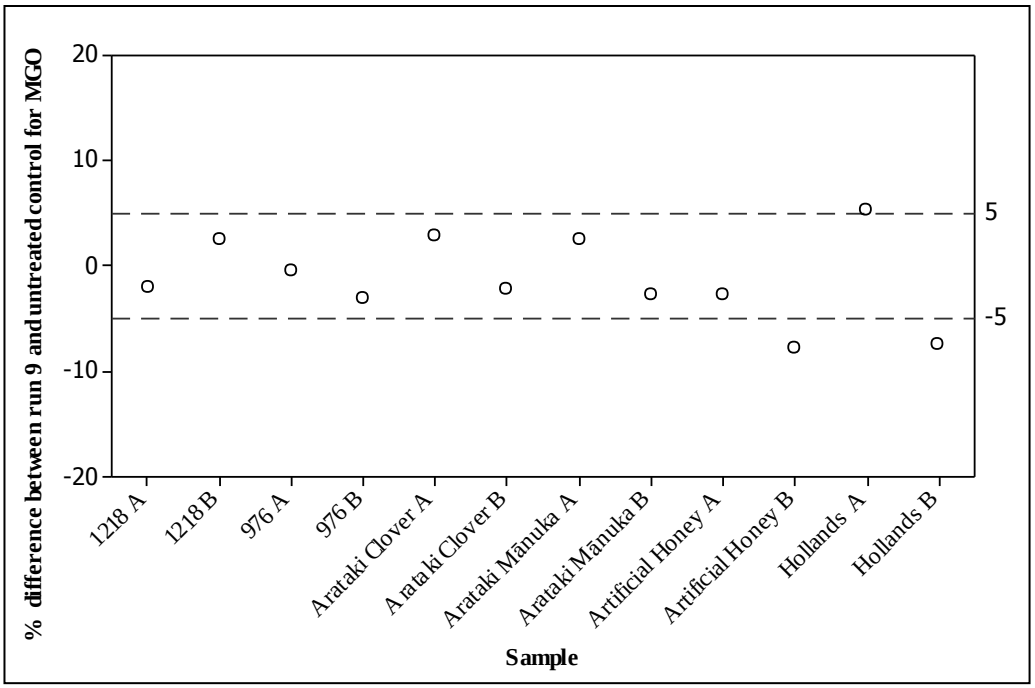


Figure 6.24 Percentage difference in MGO content of the maximum treatment pressure and duration (run 9, 600 MPa, 90 minutes) compared to the untreated sample for each matrix tested. There was less than 5% difference between the treated samples and the untreated control (with three exceptions between 5 and 10%) when analysed using the PFBHA method.

Table 6.23 Percentage change of MGO analysed by the OPD method, for the most severe HPP treatment (run 9, 600 MPa, 90 minutes) compared to the untreated control for all honey matrices.

Sample	Percentage change in MGO content after treatment 9 compared with the control
976 A	–6.37
967 B	–8.78
1218 A	–1.20
1218 B	10.78
Arataki Mānuka A	–9.79
Arataki Mānuka B	–14.60
Arataki Clover A	31.28
Arataki Clover B	5.66
Hollands A	–7.91
Hollands B	–12.48
Artificial Honey A	–20.60
Artificial Honey B	–12.98

Al Habsi and Niranjan[338] only analysed one commercial mānuka honey in their study and did not measure DHA concentration. Store bought mānuka honey is

likely to be at the end of the conversion of DHA to MGO and have a ratio around 2:1. Since the authors did not measure the DHA it is unknown if their reported change in MGO was due to conversion of DHA or from MGO being released from a reversible bond; this may be an interaction with sugar.[106] The current research examined a store-bought mānuka, as well as freshly harvested mānuka which had sufficient DHA to observe a change if it was to convert to MGO, but an increase in MGO was not observed and these samples showed the same behaviour as the store-bought mānuka honey. Therefore it does not appear that HPP can be used to quickly increase the MGO concentration of mānuka honey.

6.6 Conclusion

The work carried out in this chapter on real honey matrices has gained an insight into the behaviour of both DHA and MGO. The research has indicated that not all honeys behave the same due to various components in the honey. Mānuka and clover honey matrices behave differently at longer storage times; the MGO concentration in mānuka honeys reached a maximum then showed a decline. This decline differed between samples. In comparison, a decline in MGO concentration in the clover honeys was not observed.

Mānuka honey stored at different temperatures showed an increase in DHA disappearance as the temperature increase; however, a corresponding increase in MGO was not observed. Mānuka honey stored at lower temperature gained more MGO at longer time than honey stored at higher temperature due to the loss of MGO at extended time at the higher temperature.

Proline has been identified as contributing to the loss of DHA. There is a correlation between the rate constant for loss of DHA and the amount of proline present in a sample. This was also observed with total phenolic acids. The strength of the correlation between the rate constant for the loss of DHA and individual amino acids depends on the R group of the amino acid. Proline, primary amino acids and phenolic acids did not have a correlation with the rate constant for appearance of MGO. In the case of primary amino acids and phenolic acids, this is most likely due to the complexity of the honey matrix.

High pressure processing did not have an effect on the concentration of DHA, MGO or HMF during any of the treatment combinations studied (100, 400 or 600 MPa and 15, 45 or 90 minutes).

7 HMF formation during prolonged storage at mild temperatures

This chapter focuses on the formation of HMF in mānuka honey. Effects of time, temperature and physical and chemical properties of the honey have been examined.

Reports of studies on the formation of HMF in honey are divided between ambient temperature and long storage times to investigate shelf life, or high temperature for short times to investigate heating during processing. There is little literature reporting mild warming and extended time, as used in this study.

7.1 Literature review of the formation of HMF

7.1.1 Hydroxymethylfurfural (HMF) in honey

Hydroxymethylfurfural (HMF, Figure 4.5) also known as 5-hydroxymethyl-2-furaldehyde is a cyclic furan ring with both aldehyde and alcohol functional groups. HMF is generally not present, or is at very low levels in fresh food, but levels are increased by degradation of hexoses during storage and heating. The level of HMF in food is related to the heat treatment it has received; honey that has been heated, stored in inappropriate conditions or adulterated with invert syrup has high levels of HMF.[140] Therefore detection of its presence in honey at high concentrations can identify honey that has been inappropriately treated. HMF is heavily monitored due to being a potential toxin. Analytical methods for detection of HMF in honey have been addressed in chapter 3.

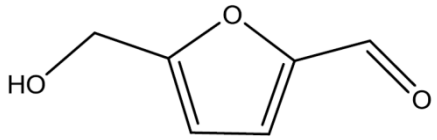


Figure 7.1 Hydroxymethylfurfural

The European quality standards and CODEX Alimentarius[174] recommends that honey should not contain more than 40 mg/kg HMF except for honeys of tropical origin which have an elevated maximum accepted level of 80 mg/kg HMF.[140] Furthermore, honey that has low enzymatic levels has a decreased limit of only 15 mg/kg.[131] In 2000, CODEX proposed to raise the limit to 60 mg/kg to allow for increase of HMF during storage in warm climate countries.[376] New Zealand and Australia do not have a level stated in the Australian and New Zealand Food Standard Code 2007.[377] Mānuka honey is the only type of honey that might be mildly warmed over a prolonged period of time to maximise the return of MGO. As most mānuka honey is exported, the honey industry have adopted CODEX regulation of 40 mg/kg HMF as a guideline so that the mānuka honey will be accepted by other countries.[136]

7.1.2 Formation of HMF in honey

Honey is an ideal matrix for the formation of HMF due to water co-existing with monosaccharides in an acidic medium. HMF can be produced by two pathways in honey:[140]

1. acidic decomposition of monosaccarides – caramelisation
2. condensation of carbohydrates with free amine groups – the Maillard reaction

HMF can be produced from all hexoses but it is more selectively produced from D-fructose.[*380-381] Both the Maillard reaction and caramelisation have been reviewed in chapter 5 and will not be reviewed again here. Furthermore, there is an abundance of literature on the formation of HMF, but this review will be restricted to its formation in honey.

Caramelisation

There are various mechanisms in the literature for the degradation of fructose to HMF involving either an open-chain pathway or a cyclic fructofuranosyl intermediate. HMF can be formed by the dehydration of fructose or glucose in an acidic environment.[134] Reports show that fructose and glucose can form HMF via a 1,2-enediol (Figure 7.2); 1,2 Enolisation occurs to form 3-deoxyosone, a key intermediate. This reacts further and is dehydrated and cyclises to yield HMF.[172] In acidic conditions the reaction can occur at low temperature, but the rate increases with increasing temperature. The second pathway for the formation of HMF from fructose involves protonation of fructose to yield a fructofuranosyl cation[†] before rearranging through intermediate species to produce HMF (Figure 7.3).[380,382] Antal *et al.*[380] reported that when sucrose was the starting sugar more HMF was formed from the resulting fructose compared to if fructose was the starting sugar (47% converted to HMF compared to 36% respectively); this is due to the ease in which the glycosidic bond of sucrose can be cleaved in acidic conditions to produce the fructofuranosyl cation. In comparison, under the same conditions, it is harder for the free fructose (which is predominately in the pyranose form) to form the fructofuranosyl cation.

[*] Fructose is unstable at pH 4.6 and is five times more reactive than glucose.[378] Fructose is able to enolise faster than glucose.[262] Pichler *et al.*[379] reported that under the same conditions, up to 40 times more HMF was formed from fructose than from glucose.

[†] This is an oxonium ion (oxygen cation with three bonds), but it is often referred to as the fructofuranosyl cation in the literature.

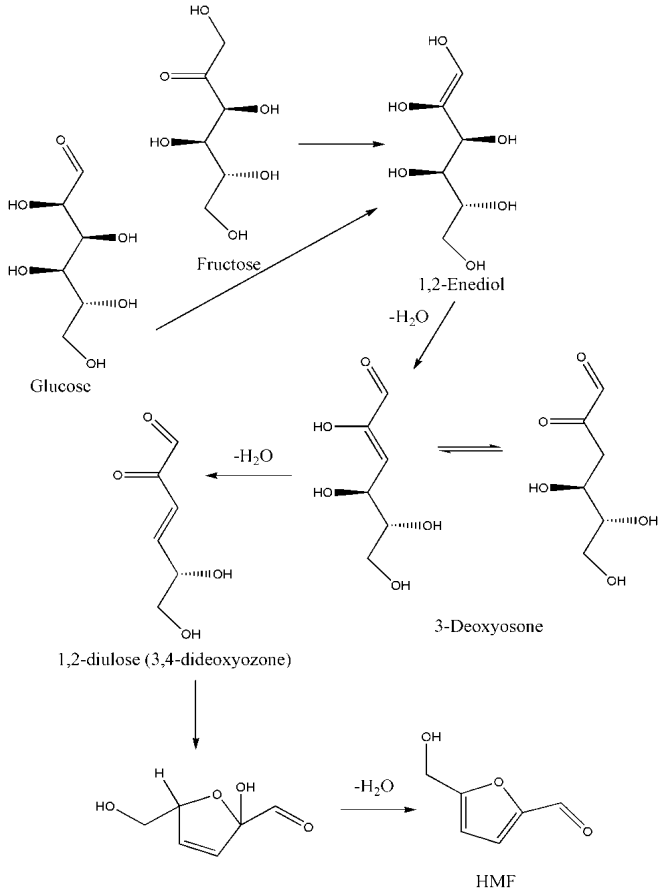


Figure 7.2 Proposed reaction scheme of fructose and glucose to HMF via 1,2-enediol. Adapted from Zirbes *et al.*[383]

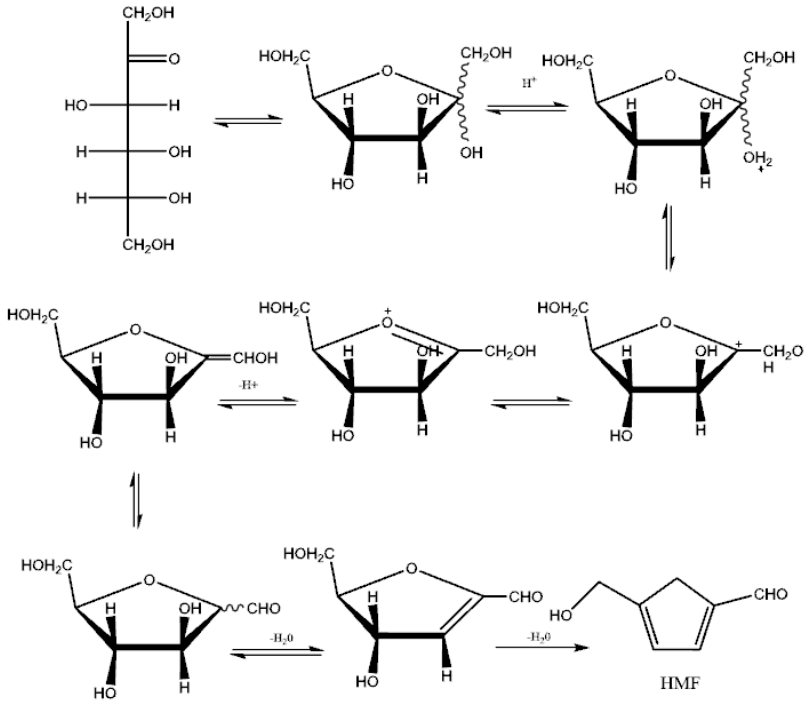


Figure 7.3 Proposed reaction scheme for the formation of HMF from fructose. Adapted from Antal *et al.*.[380]

Maillard reaction

The Maillard reaction is a complex reaction between the nucleophilic amino group of either amino acids or proteins and the carbonyl group of reducing sugars (Figure 7.4). The formation of HMF via the Maillard reaction can remove amino acids and proteins, which lowers the nutritional value of a food.

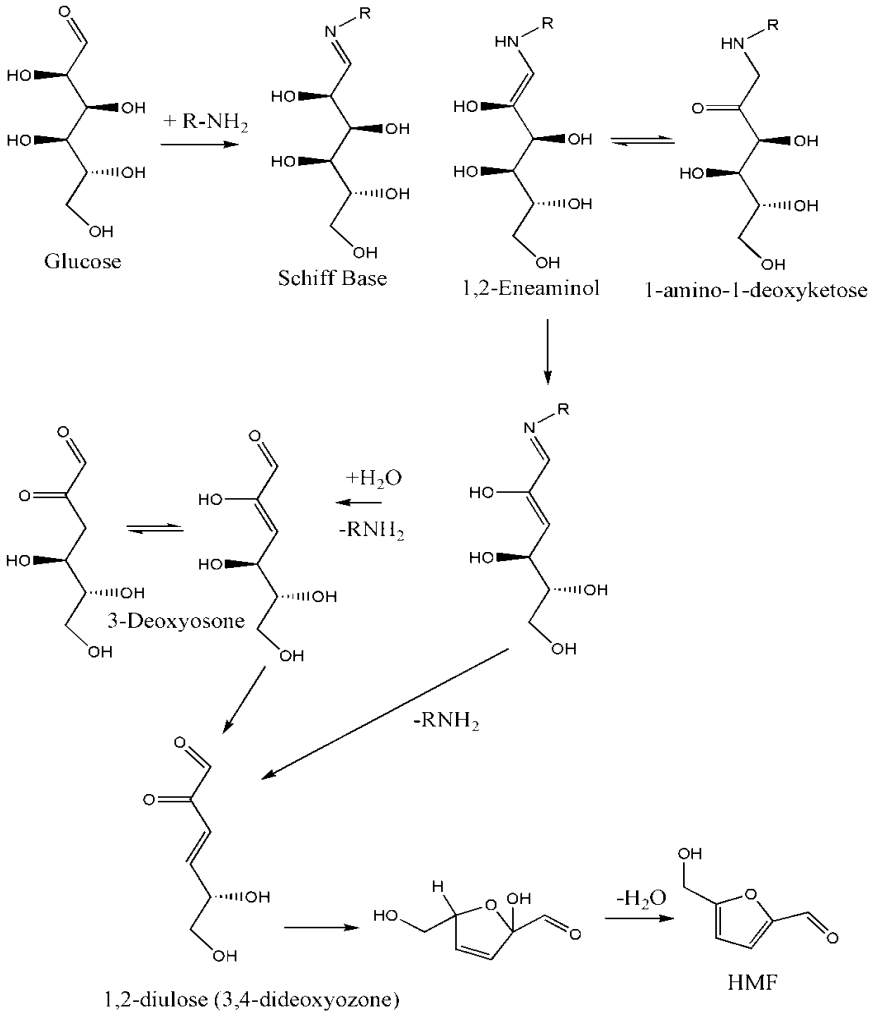


Figure 7.4 Formation of HMF from glucose via the Maillard reaction. Adapted from Zirbes *et al.*[383]

Factors influencing formation of HMF

The amount of HMF formed predominately depends on temperature and time. There are a number of other factors that can also influence the rate of HMF formation, such as pH, total and free acidity, mineral content and glucose:fructose ratio.[262-263, 384] Thus honeys of different floral origin behave differently. Fallico *et al.*[134] reported that the concentration of HMF in honey heated below 50 °C is mostly dependent on the time of heating, but is also related to its composition (e.g.

pH and acidity). Honeys with a lower pH have been reported to have a higher HMF formation.[136, 263] The presence of organic acids and low water activity have been reported to favour HMF production.[385] Water content has also been identified by Bulut and Kilic[386] as an important parameter in HMF formation via the Maillard reaction. They reported that for storage at 20 and 30 °C, honey with a low moisture content had a higher formation of HMF. However, this effect was not seen at 40 °C, which the authors suggest is due to compensation of the higher temperature.

There are reports that the botanical origin of honey also affects the formation of HMF.[195, 387 263] This most likely arises from differences in physical properties such as pH and total acidity.

High pressure processing (HPP) of honey was reported to have no influence on the increase in HMF. Studies were carried out at 100-600 MPa for 15-90 minutes at 25-30 °C [120] and 100-800 MPa for 15-120 minutes at 25 °C.[338]

Kinetics of formation

Most literature on HMF formation in honey is based around storage at ambient temperature over a long time to investigate shelf life, moderate temperatures (50-100 °C) and short heating period to investigate the effects of processing and packing or high temperatures (>100 °C) to examine overheating. Honey may be heated during processing to modify its tendency to crystallise, to delay crystallisation,[387] to facilitate filling of containers by reducing the viscosity or to destroy micro-organisms.

There are differing reports in the literature about the order of the reaction of HMF formation. Reports have given the formation of HMF in honey as zero-order, pseudo-first-order and first-order. Table 7.1 summarises reaction conditions and reaction order assignment for HMF formation from a selection of the literature. The differences in reaction order assignment most likely arise from the temperature at which the honey was treated and also the duration of heating.

Table 7.1 Summary of assigned reaction order for HMF formation under various conditions in the literature.

Temperature (°C)*	Time	Matrix	Reaction order assigned	Reference
25-85	14 days	Honey	Zero-order	Boonchiangma (2011)[388]
85-100	90 hours	Aqueous glucose systems	Zero-order	Carabasa-Giribet and Ibara-Ribas (2000)[389]
100	5-120 min	Fructose aqueous model systems	Zero-order	Ajandouz *et al.* 2001[390]
Ambient	18 months	Honey	Zero-order	Fallico *et al.* (2009)[377]
20-40	4 days	Honey	First-order	Bulut and Kilic (2009)[386]
20	28 months	Honey	First-order	Sancho *et al.*(1992)[391]
75-100	15-90 min	Honey	First-order	Turhan *et al.*(2008)[387]
50-100	6 days	Honey	Pseudo first-order	Fallico *et al.* (2004)[134]
100-160	90 seconds	Honey	Pseudo first- order	Tosi *et al.* (2002)[392]

*The authors held honey at a variety of temperatures within the range noted.

7.1.3 Degradation of HMF in honey

HMF is usually referred to as an end product in the acid-catalysed dehydration reactions of hexoses and Maillard reactions. HMF degradation in honey is usually very slow and often neglected in studies. However, the reported increase in HMF is a balance between the macroscopic formation and degradation reactions,[377] similar to the formation of MGO in mānuka honey. The activation energy of HMF formation is reported to be higher than for its degradation.[383,385, 393]

Fallico *et al.*[385] examined the degradation kinetics of HMF in honeys of different floral origins and various temperatures (25-50 °C). The authors reported that chestnut and citrus honeys have higher degradations rates of HMF compared to the formation rates, but the two rates were similar in multi-floral honeys.

Zhang and Weitz[394] studied formation of HMF from fructose and its consequent degradation to levulinic acid. They reported that in DMSO no degradation of HMF was seen. DMSO may bind with the water produced during HMF formation, thus preventing it from being involved in the rehydration of HMF to form levulinic acid. This mimics the situation that is occurring in honey. The authors reported that in a water matrix HMF degraded to levulinic acid.

7.1.4 Reactions of HMF with other compounds

The formation of HMF is well understood, but chemical reactivity of HMF is not very well studied. Most of the literature involves reactivity of HMF and amino acids in physiological conditions. These studies have shown reaction of HMF with amino acids and tissue proteins in the body. This is outside the scope of this research and will not be reviewed here.

Nikolov and Yaylayan (2011)[395] studied the reaction of HMF with lysine, glycine and proline. They stated that primary amino acids can only form Schiff bases (which are reversible) with HMF; whereas secondary amino acids can further undergo vinylogous Amadori rearrangement. Schiff base adducts of HMF with amino acids are prone to undergo decarboxylation through an oxazolindin-5-one intermediate to form two isomeric imminium ions – one which is conjugated and

can undergo vinylogous Amadori rearrangement, and the other is a non-conjugated ion that can be stabilised by dehydration.

7.1.5 Decomposition of glucose and fructose

The chemistry behind the reactions of common materials, such as glucose, is well studied under hydrothermal conditions.

Kabyemela *et al.*[396] studied the decomposition of glucose and fructose in sub-critical and super-critical water. They reported that glucose decomposes to fructose, glyceraldehyde, DHA and MGO, among other products, while fructose decomposes to glyceraldehyde, DHA and MGO. These reactions were conducted at temperatures between 300-400 ºC, pressures between 25-40 MPa and residence times between 0.02-2 seconds. These temperatures and pressures are much higher than that which honey would be subjected to during storage. However, this study is of interest as the elucidation of reaction pathways may provide insight into the reactions occurring in honey. The authors reported that more glyceraldehyde was formed compared to DHA when the initial products were examined; had the reactions been parallel, equal amounts of both would have been formed. They suggested that DHA most likely arises from isomerisation of glyceraldehyde. In the experimental conditions, both DHA and glyceraldehyde reacted further to form MGO, which in turn decomposed to acids.

Antal *et al.*[397] proposed a scheme for the dehydration of fructose to HMF (Figure 7.3) from experimental and theoretical investigation of the acid-catalysed kinetics of ketose and aldose reaction in liquid water at high temperatures (200-250ºC) and pressure (34.5 Mpa) with pH ranging from 2 to 7. The fructofuranosyl cation intermediate which is required in this mechanism is produced directly by the hydrolysis of sucrose and reacts to produce HMF in high yields. However, the fructose in honey is likely to be in the pyranose form so it is not so readily amenable to this mechanism and may explain the initial lag of HMF formation.

7.1.1 Toxicity of HMF

Toxicity of HMF is of much interest in the literature because HMF is considered a potential carcinogen to humans. The toxicity of HMF is out of the scope of this

research, hence this section will only be briefly covered. For more information the reader is directed to a number of studies, reviews and the references within.[172, 383, 173] Despite the concerns about the toxicity of HMF, controversial information has arisen in the literature in recent years reporting that HMF and its derivatives may have health benefits. This includes increasing the scavenging capacity of free radicals, improvement of nerve ischemia and hypoxia and decreasing the free radical damage.[398]

Dietary intake of HMF is much higher than the intake of other toxins in foods (in the order of mg/kg), therefore is frequently investigated. However, there are many other food sources that have higher concentrations of HMF than honey.[173 172, 399] Studies by various authors have estimated that bread and coffee are the foods which contribute the most to HMF exposure due to the amount consumed.[400-402] Janzowski *et al.*[403] cited an early study by Ulbricht *et al.* (1984), which estimated HMF intake was up to 150 mg/person/day (2.5 mg/kg body weight). But the review by Janzowski *et al.*[403] states this estimation is high due to more recent data which estimates HMF intake is between 30 to 60 mg/person/day (0.5 to 1 mg/kg body weight). Hence intake does not exceed the tolerable daily intake of 132 mg/person/day. Therefore one must keep in mind that the amount of honey eaten per day by a general person would not contribute extensively to HMF levels.

7.2 Experimental

Analysis of HMF was carried out using HPLC with PFBHA derivatisation. The storage trials are the same as those used for the analysis of DHA and MGO. Details of the HPLC method and method to make artificial honey for the storage trials can be found in chapter 2. Starting concentrations of each system can be found in Appendices D, E, G and I.

7.3 Results and discussion

There is little information in the literature about long term storage of honey in mildly warming temperatures. This information is valuable in order to find a temperature that best balances the gain of MGO while keeping the HMF concentration below 40 mg/kg. The following sections examine the order of the

reaction, the effect temperature and perturbants have on the reaction and formation of HMF in artificial honey and real honey.

7.3.1 Reaction order of HMF formation

The reaction order of the formation of HMF has been assigned as zero-, pseudo first- or first- order depending on the reaction conditions. Zero-order reactions have a rate that is independent of the concentration of the reactant(s). Increasing the concentration of the reactant will not speed up the reaction (i.e. the amount of product is proportional to time).

The integrated zero-order rate law is shown below

$$[A]_t = -kt + [A]_0 \quad (7\text{-}1)$$

where:

$[A]_t$ = species of interest at a given time

$[A]_0$ = species of interest at time 0

A reaction that obeys zero-order kinetics will give a straight line, with gradient $-k_{HMF}$, when [A] vs. time is plotted.

The integrated first-order rate law is shown below:

$$[A]_t = [A]_0 e^{-kt} \quad (7\text{-}2)$$

where:

$[A]_t$ = species of interest at a given time

$[A]_0$ = species of interest at time 0

A reaction that obeys first-order kinetics will give a straight line, with gradient $-k_{HMF}$, when ln[A] vs. time is plotted.

If the reaction is second-order in two reactants A and B, but one is present in large excess, the reaction rate will depend only on the concentration of the other reactant and the reaction is pseudo first-order.

To correctly assign the order of the reaction, over 80% of the reactant should have converted so that differences can be readily observed in the plots (Appendix C).

However, since the reactant fructose is in huge excess (800 g/kg) the reaction will not proceed to 80% completion. The percentage loss of DHA was therefore used to approximate how far the reaction had proceeded.

Examples of zero- and first- order plots for the formation of HMF in mānuka honey stored at 37 °C are shown in Figure 7.5. The zero-order plot is linear, whereas the first-order plot does not show linearity for the entire reaction, suggesting that at this temperature the sample obeys zero-order kinetics. This was seen for mānuka (samples 946, 953, 66, 78 and 84) and clover honeys (HB, AB and Hol) tested at 37 °C and also at 27 °C. However at 20 °C, there is no deviation in either the zero- or first- order plot (Figure 7.6) suggesting that at the lower temperature the reaction could be assigned as zero- or first- order, which may partially explain the variation of rate assignment in the literature.

At the start of the zero-order plot for mānuka honey there is a region up to approximately 40 days in which the data does not follow the linear trend and there appears to be a lag in the formation of HMF; this is known as an induction period. An induction period was also reported to occur in HMF formation by Chen *et al.*[398] and Carabasa-Giribet and Ibarz-Ribas[389] in rice wine* and glucose model systems respectively when heated at high temperatures. Zhang *et al.*[254] also noted an induction period in HMF formation in honey. An induction period is the initial slow phase of a chemical reaction which later accelerates. This may occur before a steady-state concentration† of reactants is reached.[405] As discussed in section 7.1.2, free fructose is slow at forming the fructofuranosyl cation compared to fructose from sucrose, which may be the cause of the induction period in honey. Furthermore, there are other intermediates which may need time to build up.

* Rice wine contains monosaccharides, disaccharides, trisaccharides and tetrasaccharides.[404]
† A steady state approximate can be applied if an intermediate is present at a low and constant concentration throughout (most of) the reaction.

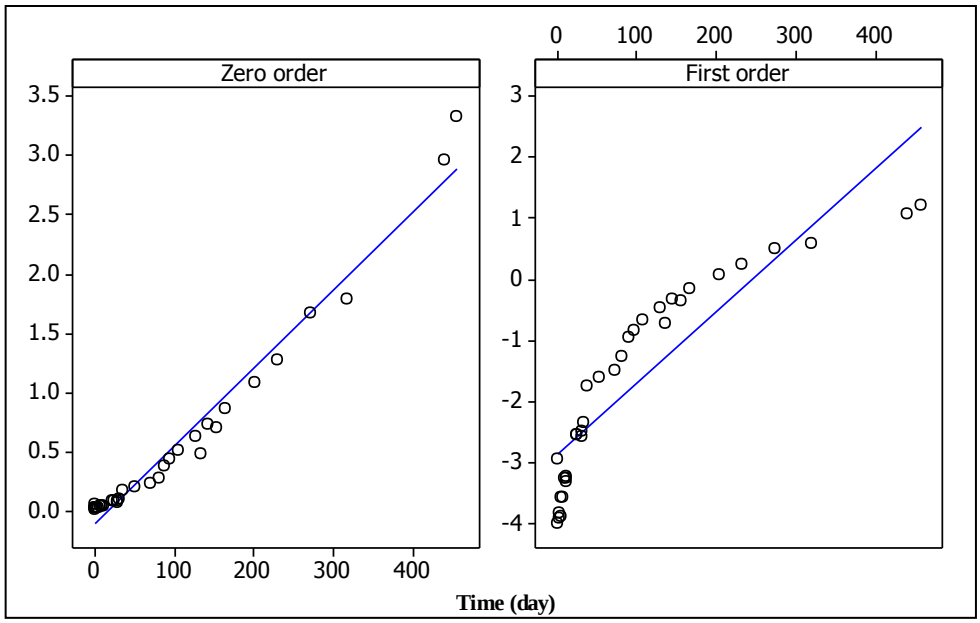


Figure 7.5 Zero-order plot ([HMF], mmol/kg, vs. time, left) and first-order plot (ln[HMF] vs. time right) for the formation of HMF in mānuka honey (sample 946) at 37 °C. The zero-order plot is linear and the first-order plot deviates from linearity suggesting that under the reaction conditions, HMF formation is zero-order.

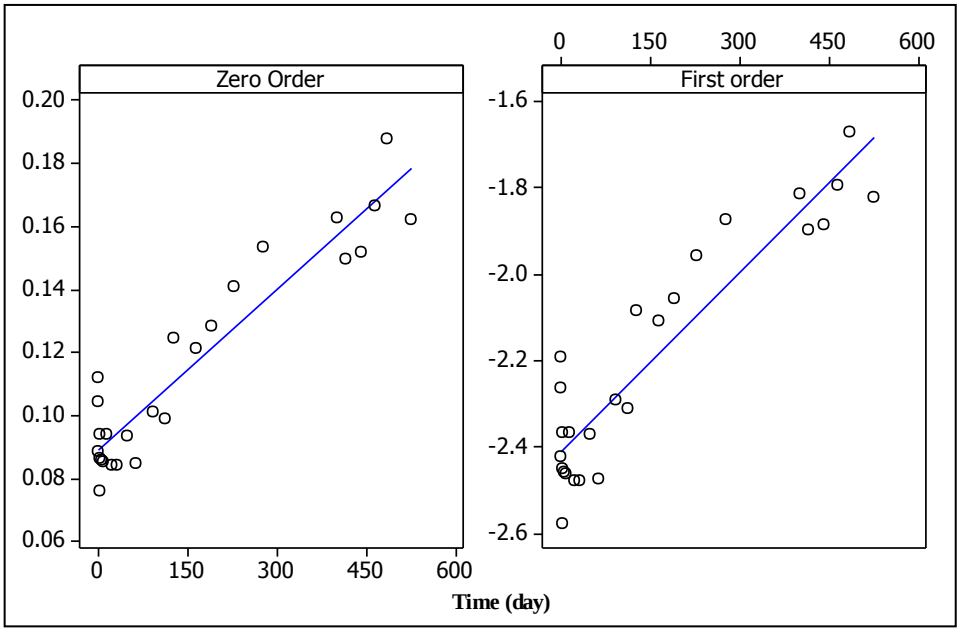


Figure 7.6 Zero-order plot ([HMF], mmol/kg, vs. time, left) and first-order plot (ln[HMF] vs. time right) for the formation of HMF in clover honey (Happy Bee) at 20 °C. After 600 days there is no large deviation in either plot, suggesting the reaction could be assigned as either reaction order.

Chen *et al.*[398] and Carabasa-Giribet and Ibarz-Ribas[389] referred to this as a short period; however, they were both heating at higher temperatures than used here. Chen *et al.*[398] stated that in rice wine after the induction period HMF formation could increase linearly with time (zero-order kinetics) or can increase exponentially with time (first-order kinetics) depending on the sample.[398] Carabasa-Giribet and Ibarz-Ribas[389] reported HMF formation in honey to be zero-order (85-100 °C for 80 hours). There is an initial lag in formation of HMF under the conditions tested in this research before the HMF vs. time graph obeys linearity; this is most noticeable at 37 °C as the reaction has proceeded further.

Samples stored at 37 °C obey linearity in the first-order plot (ln[HMF] vs. time) while they are in the induction period before changing to zero-order. This is up to approximately 40 days. Samples stored at lower temperatures appear to still be in the induction period which may explain why they can be assigned as either zero- or first-order reactions. Clover honey (doped with 2,000 mg/kg DHA) stored at 4, 20, 27 and 37 °C showed different behaviour at the various temperatures. Figure 7.7 shows Happy Bee clover honey (which is representative of the other clover honeys) at the four temperatures; the x and y scales are the same for all four graphs. The induction period is noticeable at the beginning of HMF formation in the sample at 37 °C; the sample stored at 27 °C appears to be passing through the induction period, but the samples at 20 and 4 °C are still in the induction period. This suggests that temperature and time strongly influence the reported order of HMF in honey matrices. If zero- and first-order rate constants for Happy Bee clover honey are calculated for the sample stored at 20 °C the reported rate constants are very different (0.0002 mmol/kg/day compared to 0.0014 mmol/day. This indicates the difficulty in comparing samples with the literature.

A set of mānuka honeys (samples 14, 25, 32 and 41) were stored at 27, 32 and 37 °C. The induction period is more noticeable in this set of samples, compared to the samples stored at 37 °C discussed above, because the experiment was of shorter duration and sampling was less frequent than the foregoing experiment. The zero- and first-order plots are shown in Figure 7.8. The deviation is more pronounced in the 37 °C samples compared to 32 and 27 °C, but these samples still show the induction period, compared to samples at 20 °C and below. In comparison to the aforementioned samples (Figure 7.5), deviation from linearity

in the first-order plot is not as noticeable, which may be due to a lower density of data points.

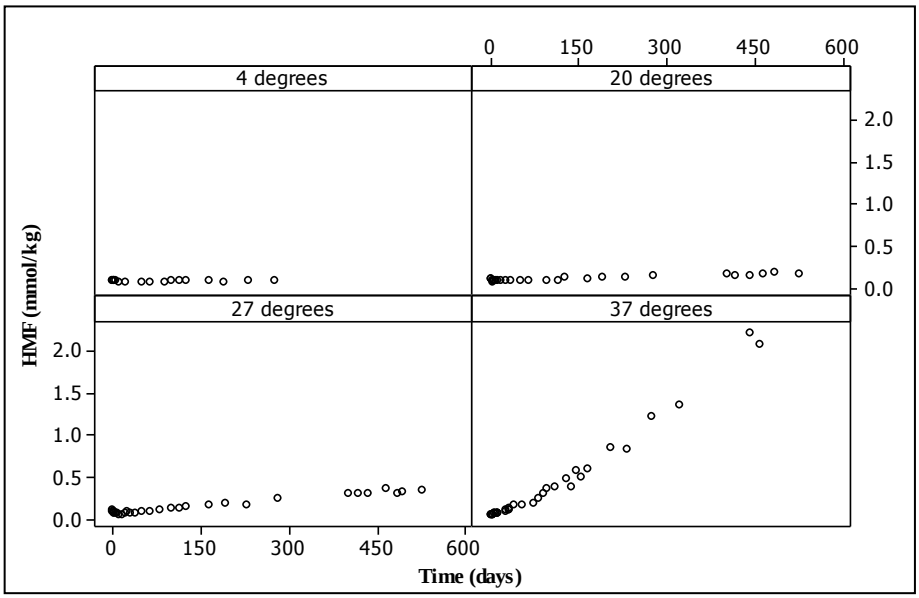


Figure 7.7 Formation of HMF (mmol/kg) vs. time for Happy Bee clover honey (doped with 2,000 mg/kg DHA) at 4, 20, 27 and 37 °C. The sample stored at 37 °C has passed through the induction period; the sample stored at 27 °C appears to be passing though the induction period; however, samples stored at 20 and 4 °C appear to still be in the induction period.

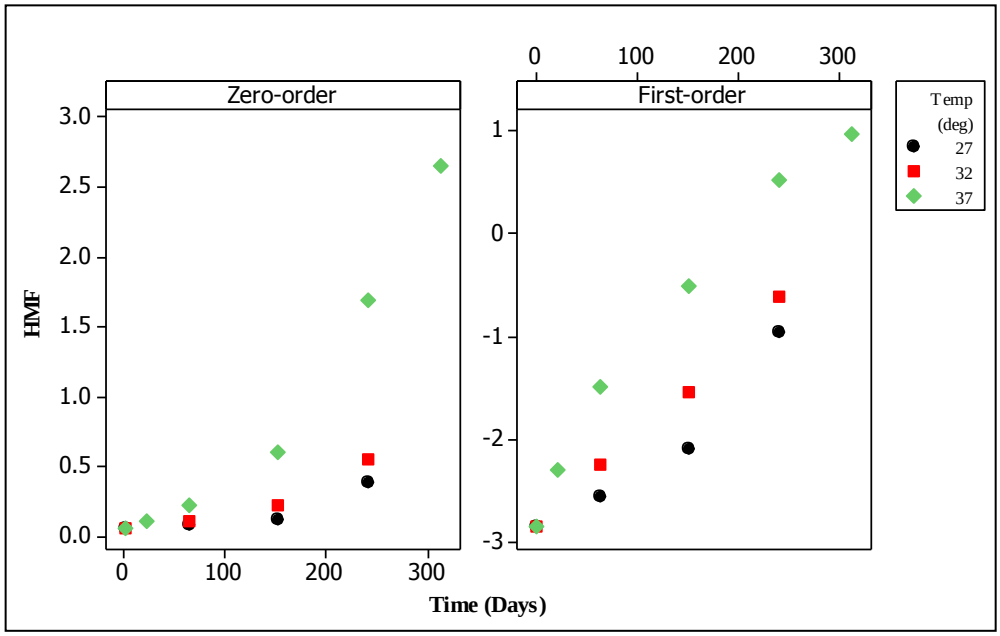


Figure 7.8 Determination of reaction order; plot for zero-order reaction ([HMF], mmol/kg, vs. time, left) and first-order (ln[HMF] vs. time, right) for mānuka honey samples at 27, 32 and 37 °C. The induction period is apparent in the zero-order plot.

Differences in assignment of the rate order within the literature are most likely due to the temperature and duration of heating. The above results show that depending on the frequency and length of sampling as well as the temperature, it is possible to assign either zero- or first-order to the formation of HMF. Plots of [HMF] vs. time may appear exponential (such as in Figure 7.8) because of the induction period. Therefore if they are not analysed for a long duration, the plot will not have enough points to make the induction region have an insignificant effect on the measured kinetics. The distribution of data points can also weigh the data in favour of the induction period or post-induction period (for example, Figure 7.5). Fallico *et al*.[377, 385] (18 months) and Boonchiangma *et al*.[388] (14 days) stored honey at low temperatures and assigned the formation of HMF as zero-order. However, in comparison, Sancho *et al.*[391] stored honey at 20 °C for 28 months and assigned the reaction as first-order.

Plots of [HMF] vs. time for honeys stored at 37 °C had very high correlations (>95%). This concurs with Khalil *et al*.[378] who reported a very strong correlation between HMF formation and time ($R^2 = 0.912$) for Malaysian honeys (n =9) and mānuka honey (n = 1) when stored between 25 and 30 °C for various lengths of time, which supports a zero-order reaction. For this study, honeys stored between 20 and 37 °C for a long period of time were treated as having a zero-order rate of HMF formation. Samples were prepared in duplicate for each system. The standard deviation in the rate constant for HMF formation in the duplicate samples was between 0.0000 and 0.0004 day^{-1} for 25 samples that were calculated. Therefore 0.0004 mmol/kg day^{-1} was used as the error in the rate constant for HMF formation for comparison of different systems.

7.3.2 Kinetics of HMF formation

7.3.2.1 Real honey matrices

Clover and mānuka honeys were stored between 4 and 37 °C for up to 500 days; rate constants and temperature dependence were examined. Initial concentrations of DHA for each sample can be found in Appendix G.

Clover honey

Three clover honeys (HB, AB and Hol) doped with 2,000 mg/kg or 10,000 mg/kg DHA were stored at 4, 20, 27 and 37 °C up to approximately 500 days. As expected, HMF concentration increased more rapidly with increasing temperature (Figure 7.9). Samples at 37 °C exceeded the recommended limit of 40 mg/kg at approximately 100 days, whereas samples held at 27 °C did not reach the limit until approximately 400 days (13 months). Juarez-Salomo and Valle-Vega[406] developed a model for prediction of HMF in honey stored over time and predicted that HMF will exceed the limit after 230 days when stored at 20 °C, which is earlier than that seen for the mānuka honey in this research. In comparison, Sancho *et al.*[391] reported that 97 honeys out of 115 (84%) stored at 20 °C exceeded 40 mg/kg at approximately 2-3 years. Escriche *et al.*[407] also reported that honey can exceed 40 mg/kg at low temperature if it is stored for a long time. This is an important point because mānuka honey is stored for an extended time and may be mildly warmed to increase the MGO content before it is sold, at which time it may sit on a store shelf for several or more months. Therefore it is desirable to keep the HMF low during development of MGO content. Storage at 4 °C halted the formation of HMF.

Zero-order rate constants for HMF formation in clover honeys were calculated for each sample at each storage temperature (Table 7.2). The average rate of HMF formation at 37 °C was the same for the Happy Bee sample with 2,000 and 10,000 mg/kg DHA added, suggesting that DHA does not contribute either positively or negatively to the formation of HMF. Samples stored at 20 and 27 °C were within experimental error (0.0002 mmol/kg/day and 0.0007 ± 0.0002 mmol/kg/day respectively), but an order of magnitude of difference was seen in the samples stored at 37 °C (0.005 ± 0.001 mmol/kg/day).

The rate constant increased by a factor of 6.7 from 27 to 37 °C in the clover honey samples. In contrast, Boonchiangma *et al.*[388] reported that the formation of HMF increased by a factor of two when the temperature was changed from 25 to 45 °C and factors of 30 and 1,500 were seen when the temperature was increased to 65 and 85 °C respectively.

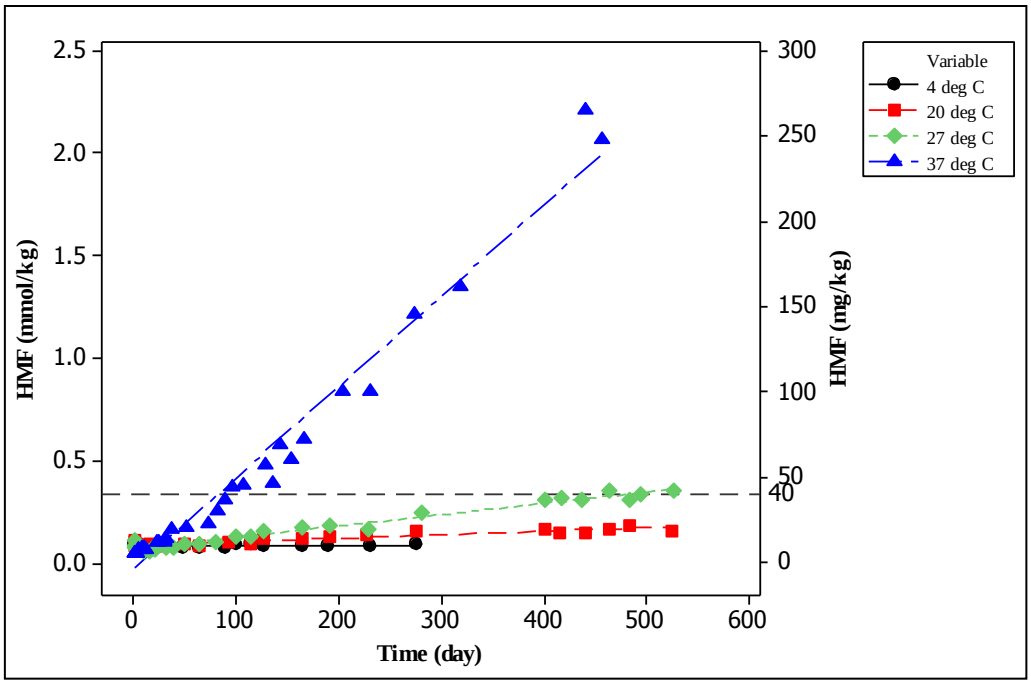


Figure 7.9 [HMF] vs. time for Happy Bee honey (doped with 2,000 mg/kg DHA) stored at 4, 20, 27 and 37 °C up to 500 days. The higher the storage temperature, the more rapidly HMF was formed. At 37 °C, the sample exceeded the recommended 40 mg/kg limit at 100 days, compared to 400 days when held at 27 °C. The same trend was observed for the other two clover honeys.

Table 7.2 Zero-order rate constants (mmol/kg/day) for the formation of HMF in clover honey samples.

Sample	Initial DHA (mg/kg)	20 °C	27 °C	37 °C
		Rate constant (mmol/kg/day)		
Happy Bee	2000	0.0002	0.0006	0.0047
Airborne	2000	0.0002	0.0007	-
Hollands	2000	0.0002	0.0009	-
Happy Bee	10000	-	-	0.0047
Airborne	10000	-	-	0.0049
Hollands	10000	-	-	0.0068
Average	2000	0.0002	0.0007 ± 0.0002	0.005 ± 0.001
Average	10000	-	-	0.0054
Average	All	-	-	0.005 ± 0.001

Fallico *et al.*[377] reported zero-order rate constants for HMF formation between 0.00007 and 0.0009 mmol/kg/day for various floral honeys stored at 25 °C. The upper end of these results is similar to that observed in the current research for

honeys stored at 27 °C. The authors reported that at 25 °C only two of the five floral honeys analysed exceeded 40 mg/kg HMF after 18 months.

Mānuka honeys

Six mānuka honeys were stored at 37 °C and tested frequently over time. Four mānuka honeys were stored at 27, 32 and 37 °C and were sampled less frequently up to 250 or 314 days; these will be referred to as Gibbs honeys for differentiation. The results confirm that as the temperature increases, the rate of HMF formation also increases.

A plot of HMF vs. time for Gibbs honey sample 14 stored at 27, 32 and 37 °C is shown in Figure 7.10 and is representative of the other three Gibbs honeys stored at these temperatures. Samples 946 and 953 were stored at 37 °C and the formation of HMF over time is shown in Figure 7.11. Honeys stored at 37 °C exceeded 40 mg/kg (marked on the graph) after 2.6 months for all samples. In comparison, samples heated at 27 and 32 °C did not exceed 40 mg/kg until after 5 months. The results show the importance of choosing a low storage temperature throughout the life of the honey since HMF will increase steadily over time even at low temperature (such as on the shelf in a shop).

The zero-order rate constants for the five mānuka honeys stored at 37 °C are summarised in Table 7.3. Sample 1404 was excluded from data analysis because it was only tested up to 128 days and had not completed the induction period, which affects the slope and hence reported rate constant. This is indicative of the problems associated with comparisons within the literature. This shows the importance of using a high density of points and an extended sampling time for this reaction.

The Gibbs honeys stored at 27, 32 and 37 °C do not have a high density of data points, which causes the induction period to have more of an influence on the reported rate constant. An example of this is shown for sample 25 stored at 37 °C in Figure 7.12. The first two points fall during the induction period; if they are included in the regression fit the calculated rate constant is 0.0071 mmol/kg/day, compared to 0.0083 mmol/kg/day if they are removed. Therefore, rate constants for the Gibbs samples were calculated excluding the first two data points. The

induction period did not influence the regression fit in the mānuka honey samples analysed more frequently due to the higher density of data points. Zero-order rate constants could only be calculated for samples stored at 37 °C in the Gibbs samples because not enough data points were collected for samples at the lower temperatures (Table 7.4).

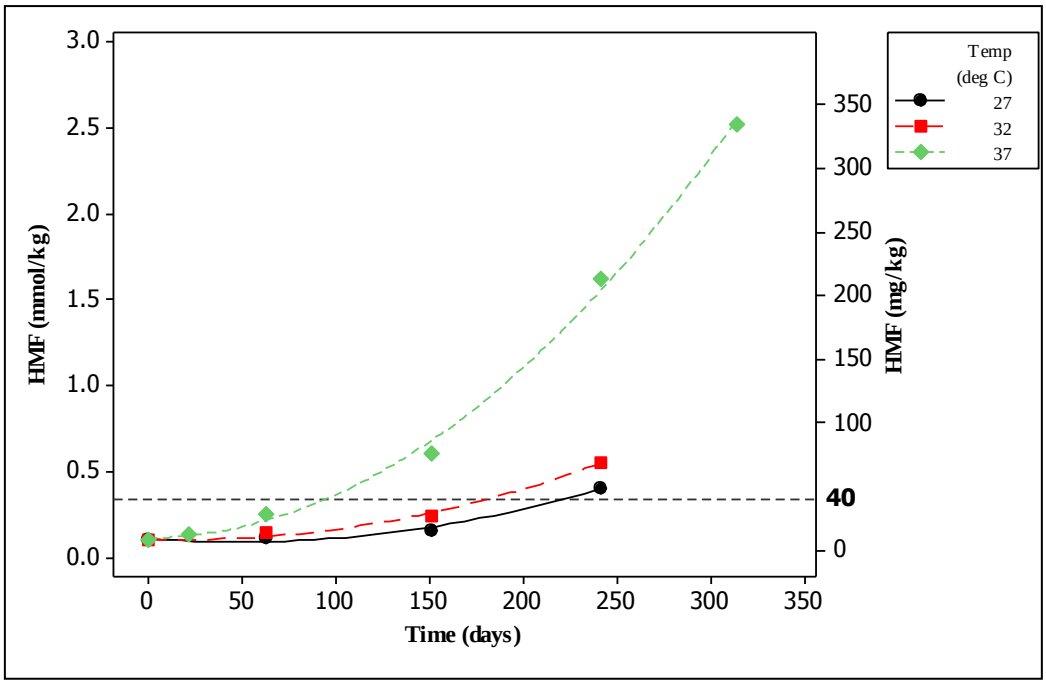


Figure 7.10 [HMF] vs. time at 27, 32 and 37 °C for mānuka honey sample 14, which is representative of the other three mānuka honeys stored under these conditions.

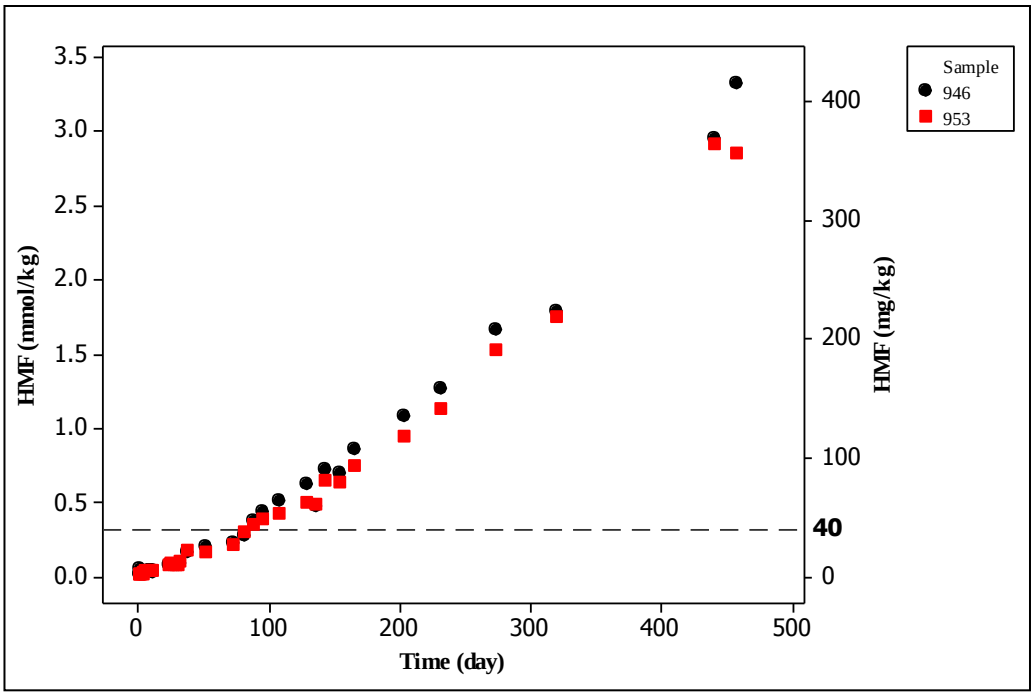


Figure 7.11 [HMF] vs. time for mānuka honey samples 946 and 953 stored at 37 °C.

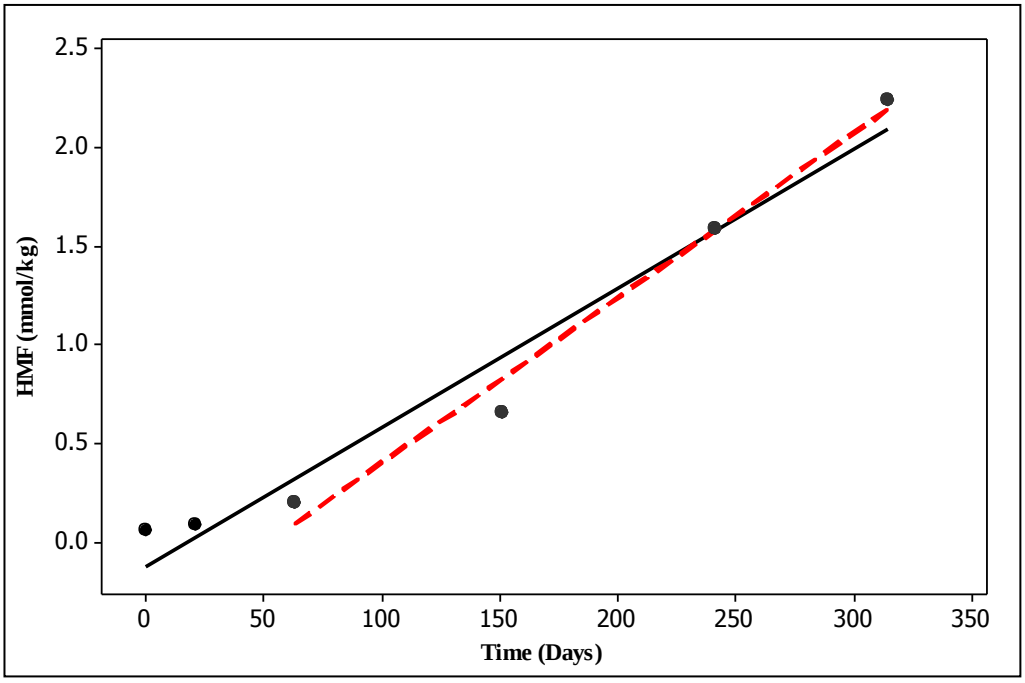


Figure 7.12 HMF (mmol/kg) vs. time for Gibbs honey sample 25 stored at 37 °C. The solid line shows the regression fit if the points in the induction period are included (rate constant = 0.0071 mmol/kg/day) and the dashed line shows the regression fit if the first two points are excluded (rate constant = 0.0083 mmol/kg/day).

Table 7.3 Zero-order rate constants for formation of HMF in mānuka honey stored at 37 °C for up to 500 days and tested frequently.

Sample	Rate constant (mmol/kg/day)
946	0.0067
953	0.0061
66	0.0043
78	0.0037
84	0.0053
Average	0.005 ± 0.001

Table 7.4 Zero-order rate constants for formation of HMF in mānuka honeys stored at 37 °C up to 314 days and tested periodically.

Sample	Rate constant (mmol/kg/day)
14	0.0099
25	0.0083
32	0.0080
41	0.0083
Average	0.0086 ± 0.0009

The average rate constant for the formation of HMF in the mānuka honey samples stored for up to 500 days is the same, within experimental error, as observed for the doped clover honey samples (0.005 ± 0.001 mmol/kg/day). This concurs with the suggestion made above that the presence of DHA does not contribute to the formation of HMF. Gibbs honeys (tested less frequently and stored up to 314 days) had a larger average rate constant compared to the other mānuka honeys (0.0086 ± 0.0009 compared to 0.005 ± 0.001 mmol/kg/day). However, due to a lower density of data points, this outcome should be treated with caution.

7.3.2.2 Artificial honey matrices

Artificial honeys that were doped with DHA and various perturbants (chapter 5) were analysed for HMF. The formation of HMF followed zero-order kinetics, similar to that seen in the real honey matrices (section 7.3.2). A summary of the rate constants for each sample at various temperatures is shown in Table 7.5. Initial concentrations of compounds in the matrix of individual samples can be found in Appendices D and E.

Table 7.5 Zero-order rate constants for formation of HMF at 20, 27 and 37 °C in artificial honey with various added perturbants and 2,000 mg/kg DHA.

Perturbant	20 °C	27 °C	37 °C
		mmol/kg/day	
Control	0.0002	0.0007	0.0096
Proline	0.0001	0.0005*	0.0023
Alanine	0.00003	0.0003	0.0057
Lysine	0.0001	0.0004	0.0038
Serine	†	0.0003	0.0028
Iron	-	-	0.0104
Proline + Alanine	†	0.0004	0.0042
Proline + Iron	-	-	0.0053
Alanine + Iron	-	-	0.0063
Proline + Alanine + Iron	-	-	0.0084

*This sample had large differences in the duplicate samples (0.0003 and 0.0007 mmol/kg/day), and was the largest difference out of all samples analysed.
† There was too much scatter to obtain a rate constant.

As expected, in every case the rate constant for HMF formation increased with increasing temperature. The sample containing alanine had a very small rate constant for HMF formation at 20 °C (0.00003 mmol/kg/day), which is a factor of 10 smaller than the other samples. The reason for this is unknown. Samples with various perturbants stored at 27 °C had similar rate constants for HMF formation (0.0003-0.0007 mmol/kg/day), which are within experimental error, but at 37 °C there was a large range of rate constants depending on the perturbant added (0.0023-0.0104 mmol/kg/day).

At all temperatures, addition of perturbants to the matrix decreased the rate constant of HMF formation compared to the control sample, except for iron; at 37 °C the rate constant for the control sample (0.0096 mmol/kg/day) and the sample perturbed by iron (0.0104 mmol/kg/day) were within experimental error. Proline and serine had the smallest rate constants for HMF formation at 37 °C (0.0023 and 0.0028 mmol/kg/day respectively). Cumulative effects were not observed when two or more perturbants were added together.

The real honey samples had a lower rate of formation of HMF compared to the control artificial honey system (Figure 7.13), which is likely to be due to a combination of the pH and other species in the matrix. The average rate of HMF formation in real honey samples was within experimental error to that of artificial honeys containing alanine, proline + alanine and proline + iron. Discussion on the

effect of amino acids and trace elements on HMF formation in artificial honey can be found in section 7.3.1.2.

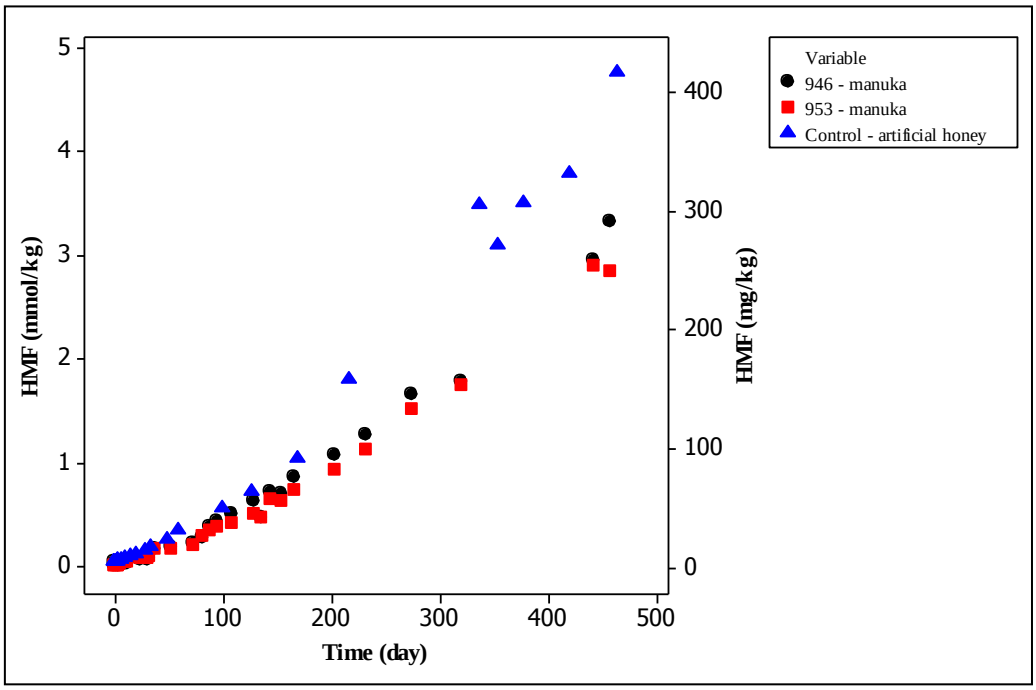


Figure 7.13 [HMF] vs. time in artificial honey (triangles) and mānuka honeys (squares and circles). The real honey matrices have a lower formation of HMF than the artificial honey (control).

7.3.1 Influence of selected compounds and physical properties on formation of HMF

7.3.1.1 Real honey matrices

The mānuka honeys used in the storage trials were analysed for a number of chemical and physical properties before storage (chapter 4). Correlation of various compounds with the rate constant for formation of HMF at 37 °C may identify compounds that influence its formation. However, it must be noted that there are a limited number of samples, hence trends should be treated cautiously. Individual concentrations for each sample can be found in section 6.3 in the discussion on influence of compounds on the loss of DHA and MGO and formation of MGO.

The strength of the correlation of k_{HMF} with some chemical and physical properties are shown in Table 7.6. There are strong positive correlations between free acidity and total acidity with k_{HMF}; a strong negative correlation between

lactone content and k_{HMF} was also observed (Figure 7.14). Fallico *et al.*[134] also reported a correlation of free acidity with HMF formation in Sulla honey; furthermore, they reported that a correlation between time, pH and free acidity with HMF formation was observed for orange blossom honey. The authors modelled the formation of HMF at 50 °C using these three variables with 96.47% accuracy, compared to only 76.02% if only time was considered. Khalil *et al.*[378] reported strong correlations of HMF with both free acidity and total acidity (R^2 = 0.786 and 0.763 respectively) and also reported a negative correlation of HMF and lactone (R^2 = 0.411), which concurs with the current research. In comparison, Escriche *et al.*[407] reported no clear correlation between HMF content and moisture content, total acidity or conductivity in honeys stored between 35 and 65 °C (3 hours to 28 days).

Table 7.6 Correlation of selected physical and chemical properties of mānuka honey with formation of HMF at 37 °C (n = 6).

Compound	Correlation	R^2 for k vs. compound (%)
Lactone	Negative	97
Free acidity	Positive	94
Total acidity	Positive	94
Proline	Positive	84
Total primary amino acids	Positive	74
% Water	Positive	34
pH	Negative	25
MGO	N/A	12
Total trace elements	N/A	10
DHA	N/A	0

There is a weak negative correlation (R^2 = 25%) between k_{HMF} and pH. There are many reports in the literature that note the same correlation.[134, 136, 263, 378-379] Fallico *et al.*[134] heated honey at 50 °C for 6 days; chestnut honey (pH 5.9) did not form HMF compared to other floral honeys with lower pH (3.4-3.7, n = 3). Khalil *et al.*[378] reported a negative correlation between pH and HMF (R^2 = 0.4) for Malaysian honeys (n = 9) and mānuka honey (n=1) stored at 25-30 °C for various lengths of time. A weak positive correlation between k_{HMF} and water content was observed (R^2 = 34%). However, Bulut and Kilic[386] reported the opposite trend in honey stored at 20 and 30 °C. The authors suggested that water may cause a dilution of the reaction. However, the majority of water will be H-bonded to sugar

in the matrix hence this may not be a likely explanation. The authors reported that at 40 °C water did not influence the reaction.

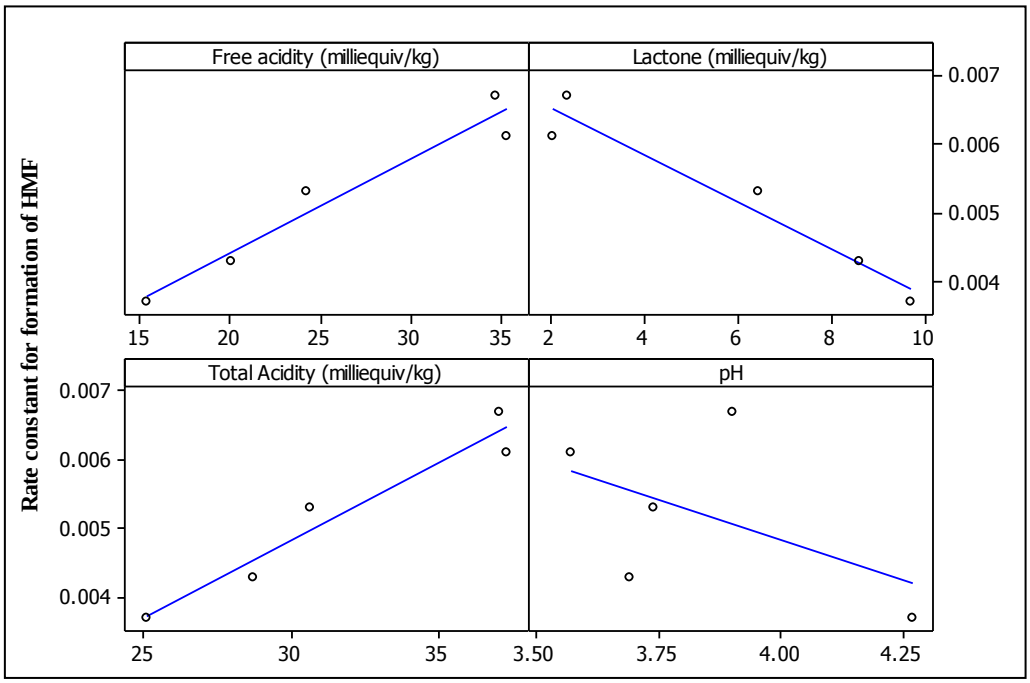


Figure 7.14 Rate constant for formation of HMF (mmol/kg/day) vs. free acidity (top left), lactone content (top right), total acidity (bottom left) and pH (bottom right).

There is no correlation between k_{HMF} and DHA in the six mānuka honey samples. A linear relationship is noted for four of the samples but samples 946 and 1404 do not follow the same trend. More samples need to be examined to see if the initial concentration of DHA has an influence on the formation of HMF or if this is coincidental.

Proline and total primary amino acids both have strong correlations with the formation of HMF (R^2 = 87 and 70% respectively); however, sample 84 was an outlier in both plots. Individual amino acids were plotted against k_{HMF} for the mānuka honeys. Most plots had a R^2 between 70 and 94%. Exceptions to this were glutamic acid (58%), phenylalanine (60%), lysine (67%), arginine (52%) and tryptophan (5%), which had lower correlations due to sample 84 being an outlier, similar to that seen when the total primary amino acids were plotted. A strong correlation was expected between k_{HMF} and amino acids because amino acids are involved in the Maillard reaction to form HMF. A discussion on the

influence of amino acids in artificial honey model systems can be found in section 7.3.1.2.

There was no correlation between the total trace elements and k_{HMF}. Vanadium had the highest correlation with k_{HMF} (R^2 = 76%); however, the reason for this is unknown. Anam and Dart[384] reported that Mn, Zn, Mg and Fe (II) added to honey (10 ppm) increased the formation of HMF (60-80 °C for 1 hour). The authors reported that the effect was larger at higher temperature. However, no correlations were seen in the current research (R^2 = <20%). Khalil *et al.*[378] cited White (1979)[132] who reported that the storage of honey in metallic containers may increase the metal content in the honey, which may in turn increase the level of HMF. However, currently, all honey drums are lined with food grade liners which would prevent this from occurring.

The strength of the correlation of k_{HMF} with selected phenolic compounds are shown in Table 7.7. There is a high correlation of the total phenolic compounds with k_{HMF} (R^2 = 84%). Phenyllactic acid and 4-methoxyphenyllactic acid have good individual correlations with k_{HMF} and a strong correlation when the sum of the two compounds is plotted against k_{HMF} (R^2 = 77%). The compounds are in high abundance in mānuka honey and can act as acids and may donate a proton to fructose so that it can form the fructofuranosyl cation, which is the first step in one pathway of HMF formation. Syringic acid and methyl syringate also have good individual correlations with k_{HMF} and a good correlation when the sum of the compounds is used (64%), but they are in low abundance in mānuka honey so will not have a large effect. Clover honey had a similar rate constant for HMF formation, but does not contain phenolic acids in large quantities. Therefore pH and/or total acidity may be largely responsible for HMF formation, but could possibly be estimated by the total phenolics in mānuka honey due to their high abundance. Furthermore, the presence of organic acids have been reported to favour HMF production.[385]

Leptosperin and luteolin do not have correlations with the formation of HMF; this is expected as they are not readily able to donate a proton. 2-Methoxybenzoic acid is the only phenolic compound studied that has a negative correlation with

formation of HMF (R^2 = 72%). The reason for this is unknown, but a negative correlation was also observed between it and the rate constant for DHA loss.

Table 7.7 Correlation of selected phenolic compounds with formation of HMF at 37 °C (n=6).

Compound	Correlation	R^2 for k vs. compound
Total phenolics	Positive	84
Syringic acid	Positive	77
Sum of phenyllactic acid and 4-methoxyphenyllactic acid	Positive	77
2-methoxybenzoic acid	Negative	72
Sum of syringic acid and methyl syringate	Positive	64
4-methoxyphenyllactic acid	Positive	63
Methyl syringate	Positive	57
Phenyllactic acid	Positive	43
Leptosperin	N/A	3
Luteolin	N/A	3

7.3.1.2 Model systems

Model systems (artificial honey with various added perturbants) used to study the conversion of DHA to MGO (chapter 5) were also analysed for HMF (initial concentrations for each sample can be found in Appendix D, E and I). These simplified systems allow effects of certain compounds (amino acids and trace elements) on HMF formation to be investigated. It must be noted that some trends may be different to the literature due to an influence of DHA and MGO in the system, which may also be reacting with compounds that could influence the formation of HMF.

Effect of DHA, MGO and initial HMF on HMF formation

Studies of HMF formation in honey have been reported in the literature on honey that does not contain DHA or MGO. The current research analysed the effect of DHA and MGO on HMF formation. The control samples used in chapter 5 (containing DHA or MGO) and DHA and MGO samples with added HMF (40 mg/kg, 0.32 mmol/kg) were stored at 20, 27 and 37 °C. However, true control systems for this experiment (samples that did not contain DHA or MGO) were not analysed, hence it is not possible to isolate their effect. The initial concentrations

of compounds in the samples can be found in Appendix I. A summary of the zero-order rate constants for HMF formation for each sample are shown in Table 7.8.

Table 7.8 Zero-order rate constants (mmol/kg/day) for the formation of HMF in artificial honey samples with DHA/MGO and with or without HMF.

Sample	20 °C	27 °C	37 °C
	Rate constant (mmol/kg/day)		
DHA-control	0.0002*	0.0007*	0.0096
MGO-control	0.0003*	0.0008*	0.0015*
DHA + HMF	Negative trend†	Negative trend†	0.0033*
MGO + HMF	0.0008*	Negative trend†	0.0014*

*These samples were only analysed up to 100 days, hence the reported rate constant may be influenced by the induction period.
†Samples with a negative trend should be treated with caution due to the scatter and limited amount of data points.

As expected, as the temperature increased, so did the rate constant. Control samples with either DHA or MGO had the same rate constant at 20 and 27 °C, within experimental error, but at 37 °C the DHA-control had a much higher rate constant of HMF formation (0.0096 mmol/kg/day) compared to the MGO-control sample (0.0015 mmol/kg/day). At 37 °C, the sample containing both DHA and HMF had a smaller rate constant (0.0033 mmol/kg/day) than the sample containing only DHA. These two differences may be due to the difference in sampling time; the DHA-control sample stored at 37 °C was analysed up to 500 days where as the other samples stored at 37 °C were only analysed up to 100 days and the rate constant may be affected by the induction period.

Anomalous results were seen for samples containing DHA and HMF at 20 and 27 °C; a loss of HMF was seen over time instead of an increase (Figure 7.15). This was also observed for the MGO-HMF sample at 27 °C. There are limited data points and the samples have some scatter, therefore this outcome should be treated with caution. In contrast, Tosi *et al.*[392] reported that the initial HMF concentration did not affect the kinetics of HMF formation; however, they analysed honeys with naturally occurring concentrations of HMF (3.9-26.6 mg/kg) for 14-60 seconds at 100-160 °C. Further work should be carried out for samples with naturally occurring levels of HMF stored at ambient temperatures for long periods of time.

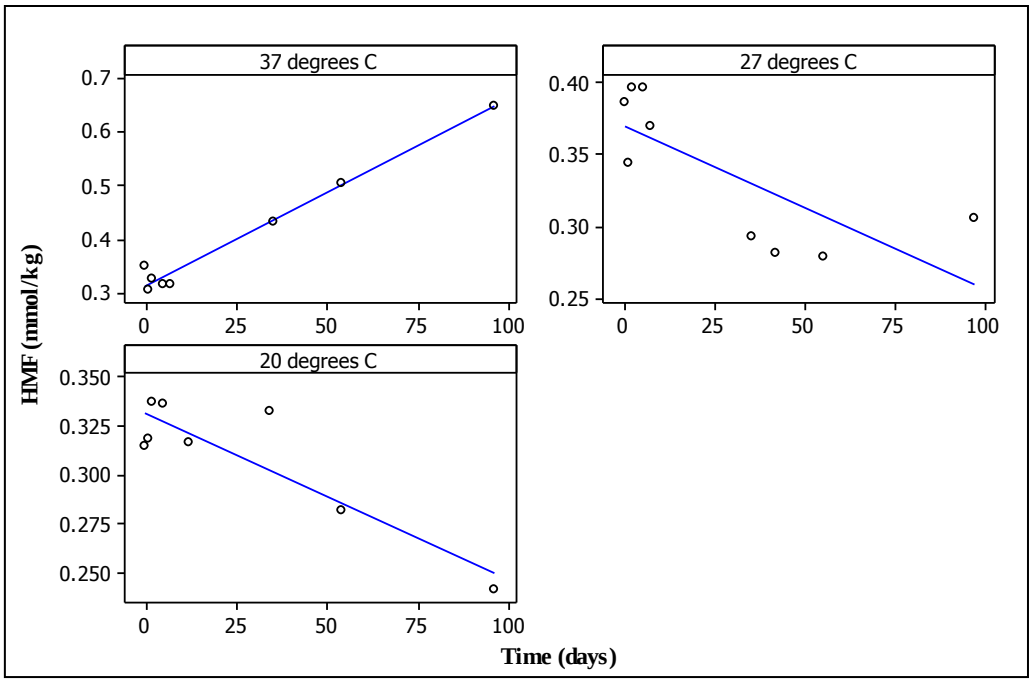


Figure 7.15 Artificial honey with naturally occurring levels of DHA and HMF (2,000 and 40 mg/kg respectively) stored at 20, 27 and 37 °C. HMF is formed at 37 °C, but is lost at the lower two temperatures.

The rate constant for formation of HMF in the MGO-control sample also increased with temperature; there was not a large increase at 37 °C as was seen for the DHA-HMF sample, but the sample was only analysed for 100 days, which as discussed earlier may affect the reported rate constant. Furthermore, rate constants for the formation of HMF at 20 and 37 °C were within experimental error for the MGO-HMF sample, but this was also only analysed up to 100 days, hence results should be treated with caution. The MGO-HMF sample at 27 °C showed a negative slope, similar to the DHA-HMF sample at 27 °C.

Effect of amino acids of HMF formation

The addition of amino acids into artificial honey (in similar concentrations to that found in real honey) reduced the rate of formation of HMF (Figure 7.16). The slower formation of HMF was unexpected because amino acids provide a second pathway for the formation of HMF (Maillard reaction in addition to caramelisation). A similar effect was reported by Pichler *et al.*,[379] who studied HMF formation in sugar model systems set up to mimic honey. The authors reported that the addition of amino acids at concentrations found in natural honey

slightly lowered the concentration of HMF, compared to systems without amino acids. They reported that a honey solution (pH 4.2) had 87 ppm HMF without amino acids and 76 ppm HMF with amino acids after heating at 85 °C for 7 hours; at pH 3.96 the control had 93 ppm HMF and the system with amino acids has 79 ppm HMF. In contrast, Gogus *et al.*[370] reported an increase of HMF in systems with sugars and amino acids (2.0 mol/L glucose, 2.0 mol/L fructose, 0.015 mol/L amino acid, pH 3.5) compared to the control when heated at 55, 65 and 75 °C. Pichler *et al.*[379] reported that as pH increases, HMF production decreases and the system study by Gogus *et al* had a lower pH than the current research, or research carried out by Pichler *et al.*,[379] which may influence the reaction. Furthermore, Pichler *et al.*[379] reported that production of HMF is positively correlated with sugar concentrations up to 50-60%, but at higher sugar concentrations not as much HMF was formed. Therefore the findings of increased HMF with increased amino acid concentration by Gogus *et al.*[370] may have been partially influenced by the sugar concentration (2.0 mol/L of both glucose and fructose).

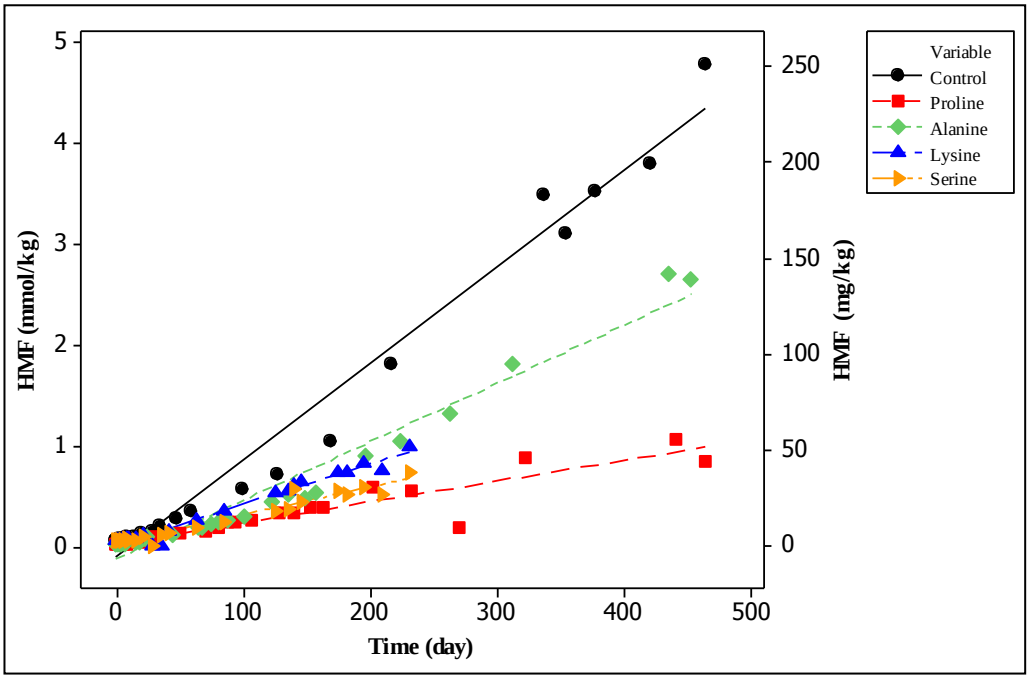


Figure 7.16 [HMF] vs. time for artificial honeys with various added amino acids. The amino acids reduce the amount of HMF that was formed compared to the control sample.

In the current study of model systems, proline reduced the formation of HMF the most, followed by serine, lysine then alanine. However, in the real honey samples there was a positive correlation between proline and k_{HMF}. Gogus *et al.*[370] reported

that proline had the least effect on HMF formation because amino acids with hydrophobic side chains react slower than amino acids with other side chains. The authors noted a cumulative effect when more than one amino acid was added to the sugar system, equal to the sum of the individual systems. However, as previously noted, no cumulative effect was seen with proline and alanine in the current study (Figure 7.17).

Carabasa-Giribet and Ibaraz-Ribas[389] studied HMF formation in model systems with various amino acids and reported that in increasing order, glutamic acid, asparagine and aspartic acid increased the rate of HMF formation. Glutamic acid and aspartic acid both have acidic R groups and asparagine has an amide terminating the R group. Hence it is expected that glutamic acid and aspartic acid would behave in a similar manner.

Artificial honey stored at 37 °C that had different concentrations of proline (800 and 400 mg/kg) displayed no difference in the formation of HMF, within experimental error in the two systems (Figure 7.18); rate constants were 0.0017 and 0.0021 mmol/kg/day respectively. The lack of difference may be due to the high DHA content (10,000 mg/kg DHA), which may have bound to proline, thus preventing it from affecting the formation of HMF.

It is possible that mānuka honey and other floral honeys go through different routes for HMF formation, due to possible binding of DHA and amino acids in mānuka honey, which would affect formation of HMF via the Maillard reaction leaving only the route through the fructofuranosyl cation. Boonchiangma *et al*.[388] reported that when amino acids in the honey were consumed the Maillard reaction abruptly stops. The authors also reported that degradation of amino acids followed first-order kinetics. However, the current study did not analyse any honeys that did not contain DHA, hence this cannot be assessed. Further work should be carried out to assess if the presence of DHA and MGO have an indirect effect on the formation of HMF, by binding with amino acids.

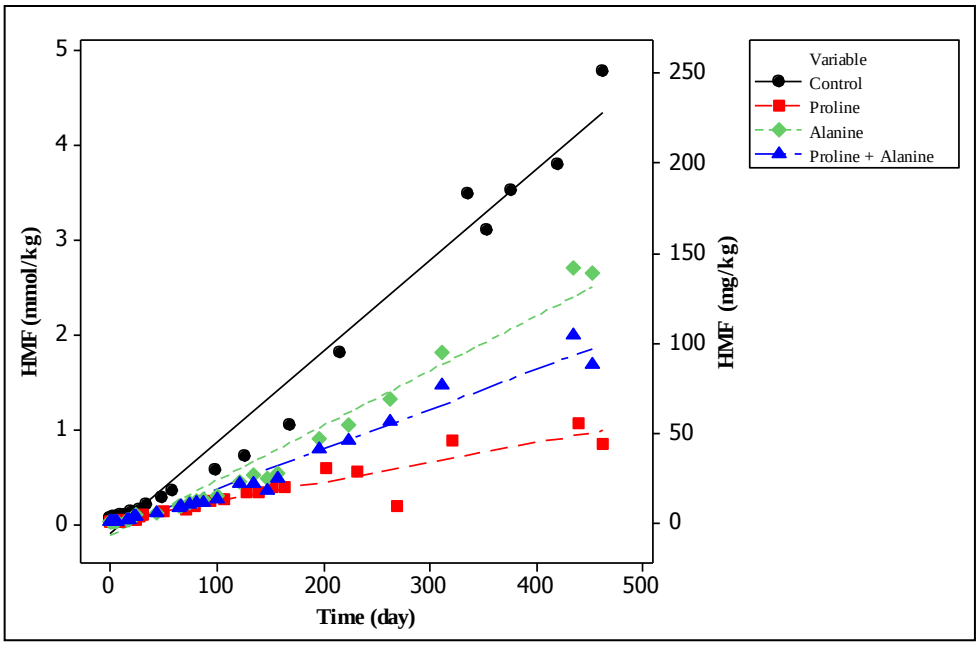


Figure 7.17 [HMF] vs. time for artificial honey systems with amino acids. No cumulative effect was observed with proline and alanine on the formation of HMF. The samples have 2,000 mg/kg DHA.

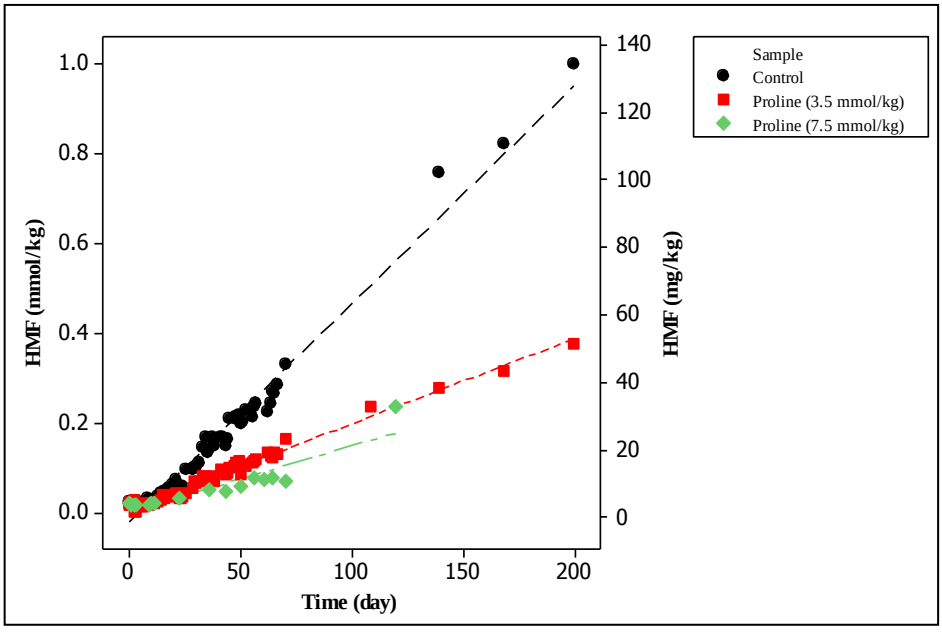


Figure 7.18 Formation of HMF (mmol/kg) with respect to proline concentration, stored at 37 °C. Control sample (circles) had the highest formation. Artificial honey perturbed by proline (3.5 mmol/kg, squares) had the same rate constant, within experimental error, for HMF formation as the sample perturbed by extra proline (7.5 mmol/kg, diamonds). The samples had an initial starting concentration of 10,000 mg/kg DHA.

The induction period of HMF formation in the model system depended on the amino acid. For samples stored at 37 °C, there was a small induction period for the control sample and samples perturbed by alanine and both proline and alanine, but it appears that the sample perturbed by proline is still in the induction period after 400 days (Figure 7.19). This may arise from DHA binding to all of the proline so that the only route available is through the fructofuranosyl cation, compared to the sample perturbed with alanine which may still have free alanine available for HMF formation to occur via the Maillard reaction.

Carabasa-Giribet and Ibar-Ribas[389] studied model systems of glucose and amino acids heated between 85-100 °C to examine the effect of amino acids on HMF formation (15, 30 or 45% glucose and pH 3.1-4.4). The authors reported that the induction period was dependent on the amino acid, concentration of glucose and temperature. The induction time was longer for solutions containing asparagine compared to those containing aspartic acid and glutamic acid. The authors also noted that as the temperature increased, the induction time became shorter. The current study also showed a short induction period (approximately 40 days) for the artificial honey sample perturbed with alanine that was stored at 37 °C compared to the samples stored at 20 and 27 °C which have not passed the induction period after approximately 200 days. The samples at the lower temperatures were not analysed for an extended period of time to observe when the induction period ended (Figure 7.20). However, they may take on a similar trend to the clover honey samples stored at various temperatures (section 7.3.1).

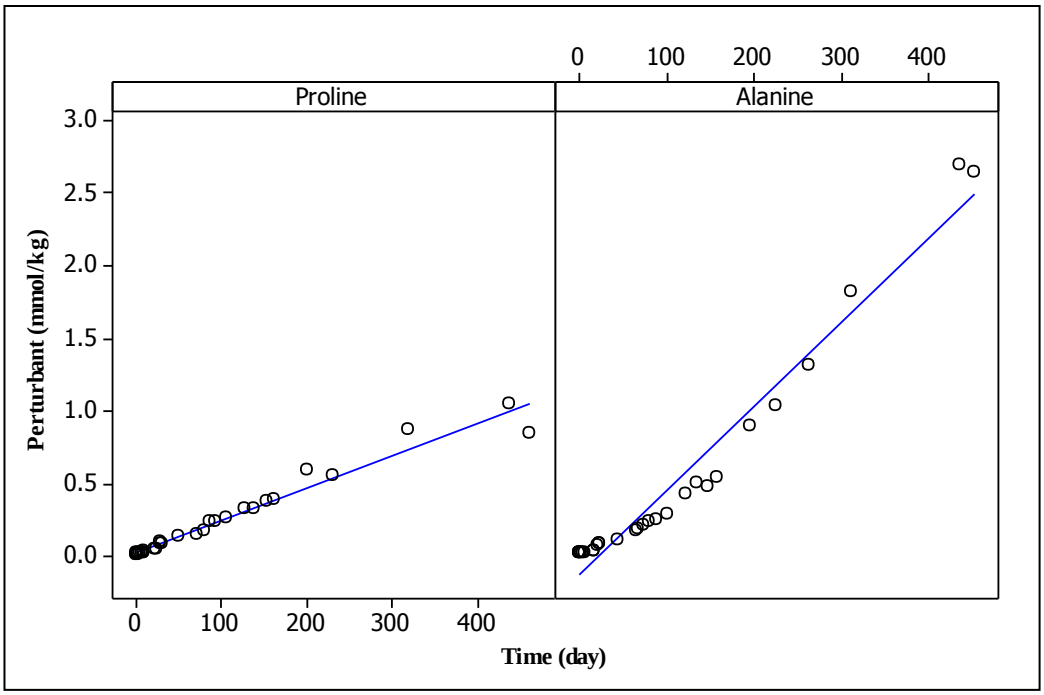


Figure 7.19 The sample perturbed by proline is still in the induction period after 400 days (left), but the sample perturbed by alanine is post-induction period (right). Samples were stored at 37 °C.

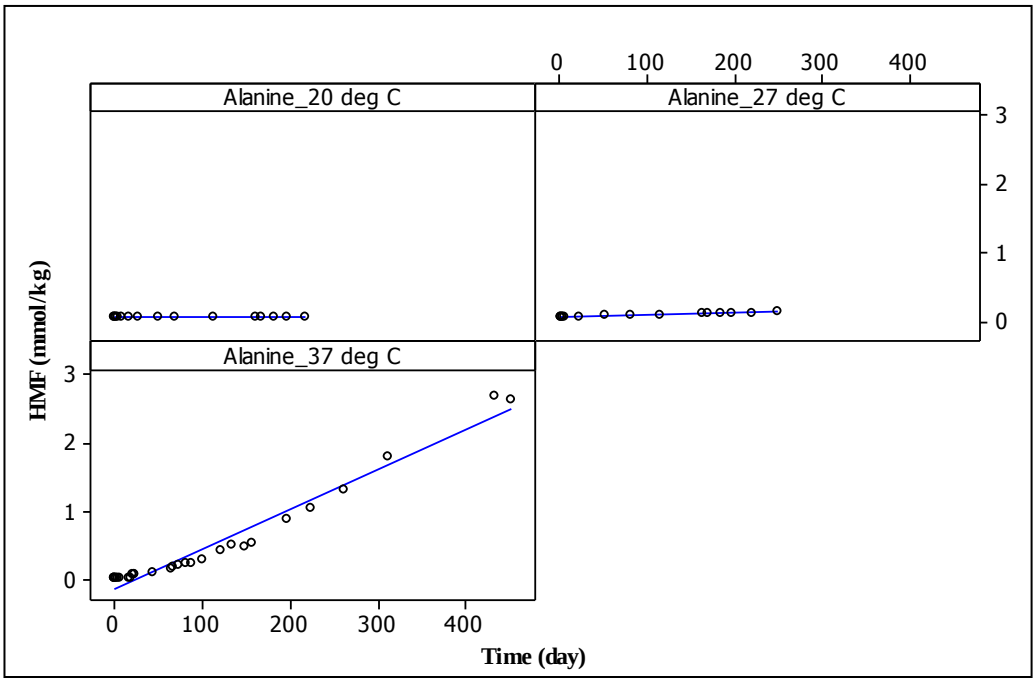


Figure 7.20 Formation of HMF in artificial honey perturbed by alanine at 20, 27 and 37 °C. The sample stored at 37 °C has passed the induction period after approximately 40 days compared to the samples at the lower temperatures which are still in the induction period after 200 days.

Effect of selected elements on HMF formation.

An artificial honey system perturbed by iron (800 mg/kg) was stored at 37 °C. Samples perturbed by iron + proline and iron + proline + alanine were also investigated (Figure 7.21). Iron had no influence on the formation of HMF compared to the control sample. In contrast, Anam and Dart[384] reported that addition of (in increasing order of effect) 10 ppm Mn, Zn, Mg or Fe(II) to honey accelerated the formation of HMF (60-80 °C for 1 hour). A larger effect was observed at the higher temperature. The difference between the two studies may be due to the temperature at which the samples were stored.

HMF is formed from sugars by an acid-catalysed reaction in which a proton draws electrons to itself and thus weakens the bond to be broken. Anam and Dart[384] suggested that metal ions in the honey are able to carry out the same function more efficiently because they carry more than a single charge, allowing them to coordinate to several donor atoms, compared to the proton which can only coordinate to one. Thus both acid and metal ion catalysis can occur in the honey. They reported that the concentration of the added metal ions in their experiment was higher than the H^+ in the honey, hence their catalytic effect outweighed that of H^+.

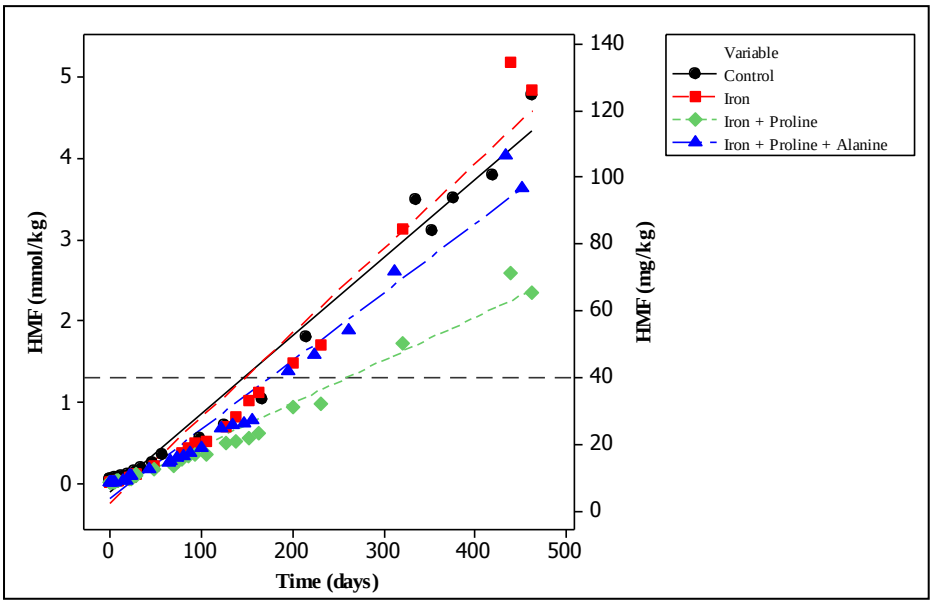


Figure 7.21 [HMF] vs. time for artificial honey with various perturbants. Iron does not contribute to the formation of HMF. An induction period is observed for samples containing iron.

A sample perturbed by potassium phosphate and alanine showed slightly less retardation on HMF formation than alanine alone (Figure 7.22). Turhan *et al.*[387] reported that potassium content contributed to the increase in HMF in honeys, hence potassium cancelled out the affect by alanine in this research.

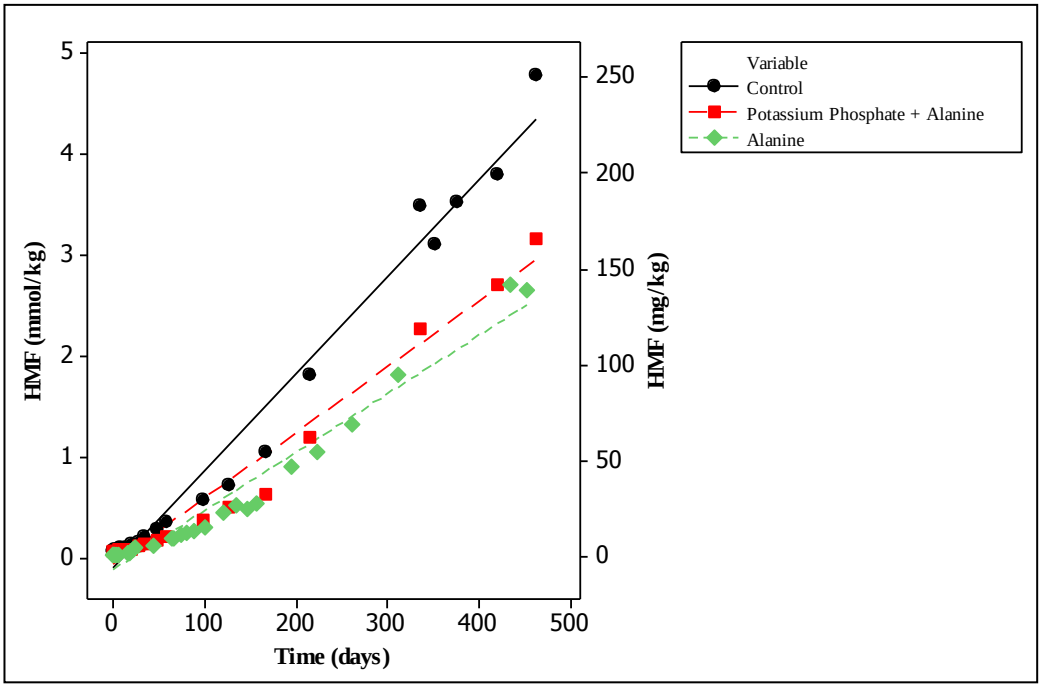


Figure 7.22 HMF vs. time for artificial honey samples perturbed with potassium phosphate and alanine, or alanine alone.

7.4 Conclusion

Knowledge of HMF formation in mānuka honey is important because mānuka honey is often stored for long periods of time (ambient or mildly warmed) to increase the NPA before it is sold. Therefore the HMF content of a mānuka honey may be slightly higher when it is placed in a shop compared to a honey from another floral source. The results show that HMF continues to increase over time, even at 20 °C, and can exceed the recommended limit of 40 mg/kg after an extended period of time (13 months) at this temperature.

A zero-order reaction was used to describe the formation of HMF in honeys stored between 20 and 37 °C for long periods of time. The induction period influenced the reported rate constant when only a few data points that were far apart where used or when the reaction had not been followed for long enough.

This shows the importance of using a high density of points and sampling for an extended period of time when these reactions are examined for the first time in order to examine the overall behaviour of the reaction. In some instances, such as at the lower temperatures, a first-order reaction may also have fitted the data.

Amino acids added to artificial honey lowered the rate constant compared to the control sample, but iron did not have an effect on HMF formation. Cumulative effects were not observed.

There was a strong positive correlation between the k_{HMF} and the total acidity of the sample. High positive correlations were also observed between k_{HMF} and proline, total primary amino acids, phenyllactic acid and 4-methoxyphenyllactic acid, suggesting that the donation of a proton helps catalyse the reaction – presumably in the first step of the reaction to form the fructofuranosyl cation.

8 Simulation of DHA conversion to MGO and independent HMF formation

This chapter describes the building of a model to simulate the conversion of DHA to MGO so that the final concentration of MGO can be predicted. The model is based upon the work carried out in chapter 5 on the artificial honey system with known perturbants.

The model was then used to simulate the conversion of DHA to MGO in real honey matrices. The model addresses the need for identification of other compounds in mānuka honey that contribute to the loss of MGO at later times in the reaction.

This chapter also builds a model to simulate the formation of HMF and is based on the work carried out in chapter 7. The formation of HMF was compared to real honeys. This information will help honey producers make informed decisions on how long they can store their honey at a certain temperature before exceeding a given HMF level that they deem acceptable before selling the honey.

8.1 Building the programme for simulation of DHA to MGO at 37 °C – finding a plausible model

In order to carry out a simulation of a complex set of chemical reactions, all, or at least the most important chemical reactions involving each chemical species, must be considered. This allows the appropriate differential equations, necessary to describe the dynamics of the system, to be written down. An example of how the Matlab program was built is given in Appendix J.

The storage trials provided insight into the reactions occurring in the matrix and have been discussed in detail in chapter 5. The full range of possible products formed are not known, but whether or not perturbants react in the artificial honey matrix helps to build an understanding of what is occurring in the matrix; in turn this allows a kinetic model to be proposed.

Important observations from the storage trials are:

- The control sample does not show a 1:1 conversion of DHA to MGO, possibly from involvement of DHA and MGO in irreversible side reactions.
- The MGO concentration in the MGO-control sample is stable for more than 1 year, indicating it is not binding/reacting with itself or the artificial honey matrix.
- DHA was not completely consumed in all but one real honey analysed, suggesting it may be in an equilibrium with other compounds.
- The DHA-alanine and DHA-proline samples showed an initial fast loss of DHA possibly due to limited monomeric DHA available for reaction.
- The DHA-alanine sample consumed the same amount of DHA as the control sample (and the rate constant was the same), but more MGO was formed, indicating that alanine is able to prevent the irreversible loss of DHA in side reactions.
- The MGO-alanine sample did not show a loss of MGO.
- The DHA-proline system had a slower rate constant for DHA disappearance than the control sample, after an initial fast decrease in DHA.

- The rate constant for MGO appearance in the DHA-proline system was smaller than the control sample, yet the [MGO] vs. time plot for this sample showed approximately the same amount of MGO was formed compared to the control sample.
- The MGO-proline system showed a loss of MGO over time.

Proposing a mechanistic chemical model is constrained by chemical observations; a model with a set of rate parameters may fit the data, but may not have exact relevance to the system chemically. Models in this research were constructed based on the results from the storage trials. In cases where products are unknown, educated assumptions were made with the help of the literature. However, as already discussed, the honey matrix is likely to behave differently from aqueous solutions generally discussed in the literature. Three models were proposed; however, there were inconsistencies with the first two proposals, as explained below, which led to the use of Model 3 for predicting the loss of DHA and formation of MGO in honey matrices.

Modelling has an iterative nature and the data may not always fit perfectly. It needs to be decided how close the fit should be to be deemed satisfactory. Rate constants can be continually adjusted, which makes it difficult to decide on an end point. However, one should stop when altering the model either causes a decreased fit compared to the experimental rate, or when there are diminishing returns from further changes. The 2013 inter-laboratory comparison programme (ILCP, Appendix B) reported the standard deviation of MGO measurement between laboratories range from 24-119 mg/kg[*] for six mānuka honeys ranging from 186 to 815 mg/kg MGO indicating that there is variation in the reported measurement of MGO. In the ensuing discussion, if the difference between the simulation and the experimental data is less than the upper end of the standard deviation range (119 mg/kg, 0.32 mmol/kg), the simulation will be described as a good fit.

Furthermore, simulation on the basis of models not only tells us what we know but also what we do not know, focussing further work on where the problems are.

[*] Two laboratories had Z-scores larger than 2, and were removed to calculate the standard deviation. If these samples were retained, the upper range was 164 mg/kg.

The work presented here is the first attempt to model the conversion of DHA to MGO in aging mānuka honey. The model will evolve beyond this thesis as more data is accumulated and further insight is gained.

8.1.1 Model 1

Model 1 was the first attempt to bring together the wealth of information, which had been gathered from the storage trials, together into one model. Retrospectively, this initial model is overly complicated and contains unnecessary features intended to better fit the model to the experimental data. Even so, certain aspects of this model are discussed here for the reader to gain an understanding of why particular reactions were disregarded in the final model.

Initially, an attempt to simulate the DHA-control system was undertaken to account for the base rate without influence from any perturbing species. This model did not include the DHA dimer because originally its importance in controlling the amount of DHA available for reaction was not recognised. Protonation of DHA was proposed as the rate determining step in conversion to MGO, due to an assumed limited supply of available H^+ ions. It was suggested that, once protonated, DHA could lose water and convert to MGO (Figure 8.1). While this model worked for the control sample, it failed when perturbing species were added. One problem was that the MGO simulation did not slow down to mirror the deviation from linearity at later times in the experimental data. To account for this, the reversible equation $H^+ \rightleftharpoons H\ bound$ was incorporated into the model. Addition of this equation solved the discrepancy; however, this may not be chemically sound. Furthermore, when perturbing species were added to the model, the loss of DHA in a side reaction with the perturbing species was initially very rapid due to the large pool of available DHA. This quickly depleted the DHA and only a small amount of MGO was formed.

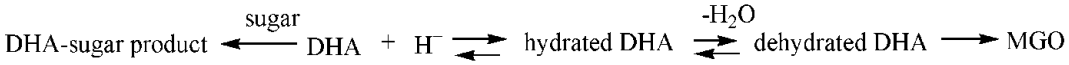


Figure 8.1 Proposed reactions for the control system in Model 1.

An equation is required to account for the loss of DHA in side reactions. Because there is only DHA and sugars in the matrix, it was initially proposed that DHA was binding to sugars and this was added to the model (as seen in Figure 8.1).

Model 1 was overly complicated. If two reaction pathways leading to the same product were proposed, both were added to the model. For example, the reaction of DHA and alanine was proposed to form both a single bonded adduct and a double bonded adduct (imine), both of which could react to give MGO and return alanine to the system (Figure 8.2).

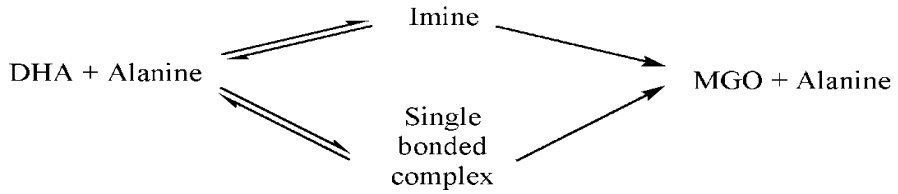


Figure 8.2 Proposed reactions of DHA with alanine.

The intermediate species were not measured experimentally, so the proportion of reactants following each pathway (and even whether both reactions occur) is unknown. In later models, a single pathway was used to incorporate all possible side reactions of DHA and alanine; the rate constant was chosen accordingly to encompass the effect of the various pathways. The possible routes are discussed in the text in sections 8.1.2 and 8.1.3.

Additionally, two compounds (e.g. DHA + proline) may produce multiple side products, whose identities and amounts could not be measured with the methods available. Therefore, one pathway was added to the model and the rate constant chosen encompassed the effect of all the possible reactions.

Despite the short comings of Model 1, rate constants were found that gave a good fit with the experimental data. However, the requirement for extra equations (such as free and bound H^+) to help fit the data suggests that this model is ill fitting for the system. Hence a second model was proposed which mitigates the complexity of Model 1.

8.1.2 Model 2

Model 2 is based on Model 1, but was refined to give a better fit and is a more chemically sound model. The overview of Model 2 is shown in Figure 8.3.

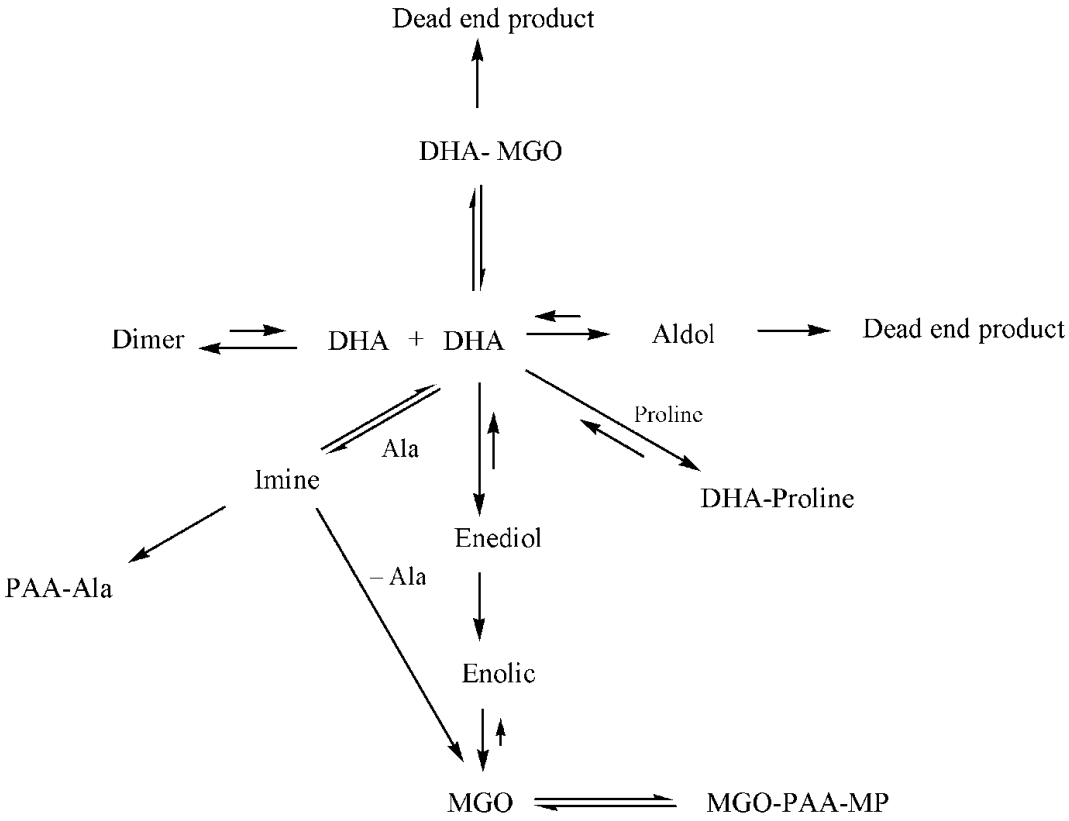


Figure 8.3 Proposed kinetics Model 2 based on the experimental results of the storage trials. PAA = primary amino acid, Ala = alanine, MP = Maillard product, Aldol = product of 2 DHA.

The first key difference is that Model 2 incorporates the DHA dimer. This alleviates the need for the free and bound H^+ equations because the dimer ties up a large proportion of the DHA. With this addition, the simulation shows the same curve for MGO formation that is observed in mānuka honey. The concentration of H^+ has been ignored for all reactions and its influence was absorbed into the rate constants. There is only a small amount of free DHA in the system at any time, which introduces competition between different species for the limited supply of DHA. The dissociation of the DHA dimer to free DHA is likely to be the rate determining step for the entire scheme. A large proportion of DHA cannot be quickly depleted by a perturbing species since most DHA is tied up as the dimer.

In this model, perturbing species (such as alanine) are able to shift the $Dimer \rightleftharpoons DHA$ reaction to the right because the species binds to a portion of DHA in a separate reaction ($DHA \rightleftharpoons DHA - perturbant$). This equilibrium can shift back to the left when free DHA is used up, allowing MGO to be formed, or a portion of DHA bound to the perturbing species can in some cases (such as when bound to alanine) undergo a different pathway (of catalytic nature) to form MGO.

In Model 2, the formation of MGO from DHA in the control route no longer goes through the protonated species because H^+ is no longer used in the model. Instead, the enediol route seen in the literature is used.[286] The rate determining step is the dissociation of the dimer, not protonation. This section of the model only changes slightly because there are the same number of steps in the newly proposed pathway as in Model 1 and only one reversible step is removed (enolic to enediol).

The loss of DHA into side reactions was also modified. It was thought that DHA is not likely to bind to sugars because sugars would be predominately in the cyclic form in the honey matrix, but instead reacts with itself, as discussed in chapter 5. These reactions may be reversible or irreversible. Reversible reactions were included in the model because it is thought that alanine is able to shift the DHA equilibrium, back towards free DHA; this conversion is supported by the fact that the loss of DHA in this sample is no greater than the control, even though more MGO is produced. A likely reaction between two DHA is an aldol-like condensation.

Fitting Model 2 to the experimental data was a difficult and time-consuming task because of the relatively large number of parameters to be determined. Altering one rate constant influences other reactions because the concentration of a reaction or product is affected due to linking of many species and reactions through the common DHA. A set of rate constants that provides a good fit with the control data, does not necessarily fit when a perturbing species is added. For example, a set of parameters that worked well for the control did not work when alanine was added to the system; whilst the MGO data fitted, too much DHA was consumed, indicating that too much DHA was lost in side reactions. This suggested that the rate constant for loss of DHA into side products was too large. However, adjusting this rate constant to give a good fit for the system with alanine

did not also work for the control system because not enough DHA was lost. Rate constants for reactions connected to these reactions were altered in order to find a set of values that were a compromise. For example, the rate constant for DHA + alanine → imine was increased in order to shift the equilibrium of DHA + DHA ⇌ side product back towards free DHA. However, a set of rate constants common to both systems was not achieved. The lack of fit for the loss of DHA indicates that Model 2 needed some modification.

8.1.3 Model 3

Model 3 accounts for most of the experimental observations in artificial honey systems stored at 37 °C. Many reactions in Model 3 are the same as in Model 2. The main difference is in the fate of the side product formed from the reaction of 2 DHA; this has been termed aldol and accounts for any product from this reaction. In Model 2, two DHA form a reversible product which could convert to an irreversible product to account for some DHA being permanently lost in side reactions. As discussed in section 8.1.2 this allowed the equilibrium of the reversible reaction to be shifted back towards free DHA when alanine was added. However, too much DHA was still lost. Therefore in Model 3 it is proposed that in the control system the reaction of 2 DHA to form the 'aldol' is irreversible. But when alanine is added to the system, it provides a route in which the aldol can be broken into two DHA then converted to MGO. This maintains the correct loss of DHA. This is discussed in more detail in section 8.2.2.

An overview of Model 3 is shown in Figure 8.4. The reactions have been drawn out in two unconnected schemes to reduce the complexity which arises from the stoichiometry differences i.e. the release of the 2 DHA from the dimer, and the reaction of only 1 DHA converting to MGO. However, it must be remembered that all reactions are interconnected by DHA. The simulation and experimental data fit well for the systems studied. The simulation follows the same trends that DHA and MGO exhibit (deviating from linearity at later times in the reaction) for the various systems. This model is discussed below in section 8.2 in a 'building block' manner, similar to that seen in chapter 5 so that the influence of individual perturbants and their involvement in reaction pathways can be discussed.

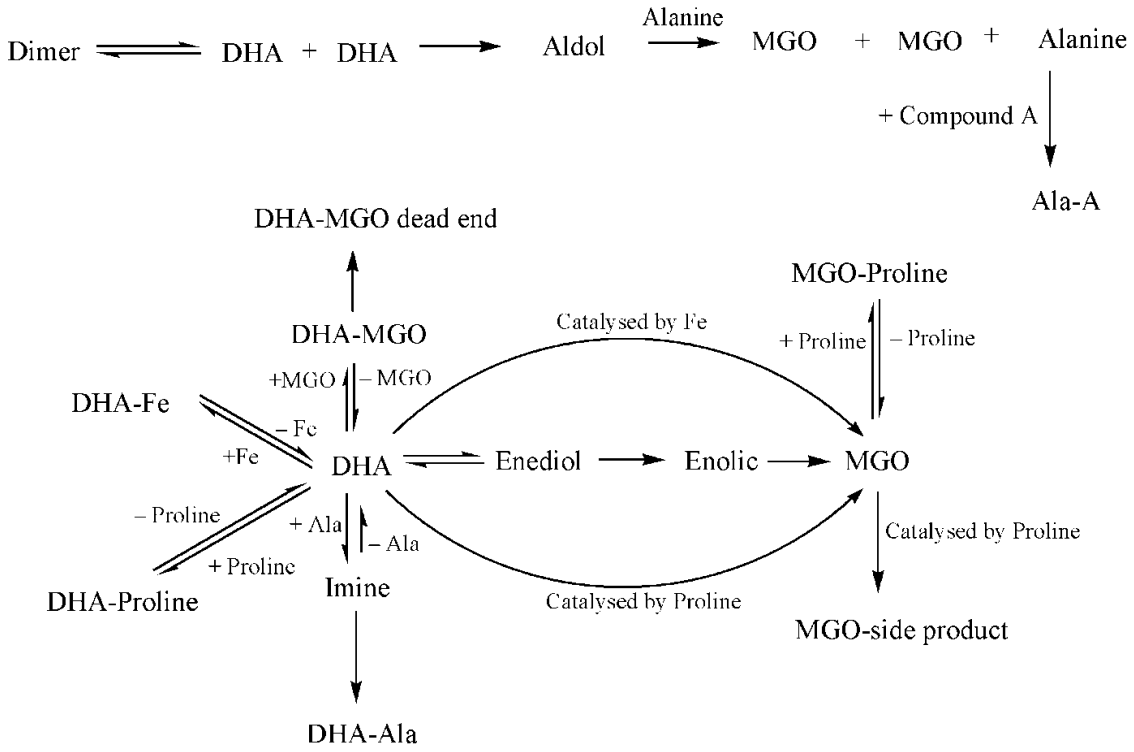


Figure 8.4 Proposed chemical reactions for the conversion of DHA to MGO and their side reactions in Model 3. Reactions that state that they are catalysed by a perturbant do not consume the perturbant, whereas reactions with a plus sign show a perturbant that is binding to either DHA or MGO. All reactions are linked; however, the complexity that occurs from the stoichiometry has been removed by separating it out into two schemes. The term 'aldol' refers to any product of 2 DHA reacting.

The side products of DHA and MGO reactions were not measured experimentally. These products and their individual proportions are unknown; hence collective terms were used to group reactions with similar dependence of reaction rate on particular species concentrations.

8.2 Building a Matlab programme to model experimental results at 37 °C using Model 3

This section follows the same general outline as in chapter 5, using a building block approach so that the reactions in each system (control and perturbed) can be discussed individually. Initially, the simplest chemical system was modelled (control sample), followed by addition of various perturbing species to the model yielding an increasingly complex system. The main reactions in which each perturbant was involved were added to the model and rate constants were adjusted so that the model fitted the experimental data. Once this was achieved, two perturbants were considered in the model and the programme was further adjusted iteratively to fine tune the model and give a better fit to the experimental data. As previously mentioned, one reaction (such as 2 DHA reacting to a side product) may encompass a number of different products, which are assumed not to affect the core chemistry. The various compounds that could form have been talked about in detail in chapter 5 and will only briefly be discussed in this section.

A summary of the final chemical equations and their rate constants for Model 3 is given in Table 8.1. This model was used to predict DHA and MGO concentrations over time in artificial honey matrices. The model was expanded to predict changes in real honeys (see section 8.3).

Table 8.1 Summary of chemical equations and the estimated rate parameters for Model 3 for simulation of the conversion of DHA to MGO in artificial honey stored at 37 °C.

Chemical equations	Model	Rate constants (day^{-1})
Dimer → 2 DHA	Control	0.0500
2 DHA → Dimer	Control	8.0000
DHA → Enediol	Control	0.0550
Enediol →DHA	Control	0.0090
Enediol → Enolic	Control	0.0400
Enolic → MGO	Control	0.5000
2 DHA → Aldol	Control	0.4500
DHA + MGO → DHA-MGO product	Control	0.0060
DHA-MGO product → DHA + MGO	Control	0.0060
DHA-MGO product → Dead end DHA-MGO product	Control	0.0010
DHA + Alanine → Imine	Alanine	0.0200
Imine → DHA + Alanine	Alanine	0.0300
Imine → Maillard-like product	Alanine	0.0010
Aldol + Alanine → 2 MGO + Alanine	Alanine	0.0200
Alanine + Compound A → Alanine-A product	Alanine	0.0020
DHA + Proline → DHA-Proline product	Proline	0.0100
DHA-Proline product → DHA + Proline	Proline	0.0100
DHA + Proline → MGO + Proline	Proline	0.0030
MGO + Proline → MGO-proline product	Proline	0.0003
MGO-Proline product → MGO + Proline	Proline	0.0003
MGO + Proline → MGO side product + Proline	Proline	0.0003
DHA + Iron → DHA-Iron product	Iron	0.0500
DHA-Iron product → DHA + Iron	Iron	0.0500
DHA + Iron → MGO + Iron	Iron	0.0500

8.2.1 DHA-control

The DHA-control system contained only DHA in an artificial honey matrix to investigate the conversion of DHA to MGO without any perturbing species. A small amount of gluconic acid was added to the matrix to adjust the pH to reflect

that of natural honey. Although there is a chance that gluconic acid has a catalytic effect, the necessity to have the correct pH overrides this. As pointed out in chapter 5, reactions occurring with DHA depend upon the pH of the matrix. All samples contained some gluconic acid, hence it is expected that if there is a rate enhancement from it, this will not be significant because it is incorporated into the control rate of conversion. It is assumed that this is the base rate of DHA to MGO conversion that will occur in all samples.

The initial concentrations of all of the species involved are required in the model. The DHA concentration is experimentally known; however, the proportion of DHA dimer and free DHA is unknown. As reported in chapter 5, it appeared that alanine and proline were initially reacting in a 1:1 reaction with DHA, when individually added to systems with 2,000 mg/kg DHA, but may have been coincidence. Instead of a 1:1 reaction, this may have been the amount of monomeric DHA available for reaction. Furthermore, when both alanine and proline were added to a system, a fast initial loss of DHA corresponding to the amount of both perturbants added was not observed, suggesting that only a certain proportion of DHA was available as the momomer. An initial ratio of 75:25 for dimer:monomer was used on the assumption that the honey matrix is closer to a solid state than aqueous solution, and this ratio is close to what has been observed in the storage trials chapter 5. Therefore if 22 mmol/kg DHA is added to the system, 5.5 mmol/kg would be in the monomeric form and 16.5 mmol/kg would be in dimeric form.

The equilibrium constant chosen for the reaction $2\ DHA \rightleftharpoons dimer$ was 160, which means most of the DHA is tied up as the dimer and cannot react until it dissociates. It is assumed that this reaction has reached equilibrium in the system early on. Therefore most of the DHA should be present as the dimer and only a small portion is free to enter into other reactions. This creates competition between reactions and rate constants need to be carefully chosen to reflect this. A study on the equilibrium between the dimer and free DHA in a honey matrix is being carried out separately to this research.

The sum of concentrations of simulated DHA, DHA dimer and enediol were used for comparison to the experimental DHA concentration because all three species

are detected as DHA by the analytical method. When analysed by HPLC, the dimer converts to the monomer during sample preparation (2 hours in aqueous solution). This is confirmed by the initial theoretical concentration of DHA added to the system matching the reported initial concentration at the start of the reaction. The sum of the simulated concentrations of the enolic form of MGO and MGO were used to compare with the experimental MGO concentration because it is assumed that the enolic form of MGO will rapidly convert to MGO and the reaction from enediol to the enolic form of MGO is irreversible.

As discussed in chapter 5, there is not a 1:1 conversion of DHA to MGO. The MGO-control system showed no loss of MGO by reaction with itself or the matrix. Therefore only loss of DHA by reaction with itself or MGO were considered. The model was adjusted so that the dimer slowly converted to a dead end product. However, this caused too much DHA to be lost because the pool of dimer was large. Furthermore, from a chemical point of view, this is unlikely to occur because the dimer is assumed to be quite stable. The final model requires the dimer to dissociate so that two DHA may react together to form an irreversible product. If this reaction is reversible in the model, then over a long time all of the DHA is converted to MGO (a sink) which is not seen experimentally. It appears that there are 2 routes that act as DHA sinks; one leads to MGO, the other is one or more reactions of DHA to produce side products.

As discussed in chapter 5 a number of products from the reaction of 2 DHA may potentially form, including those reported by Popoff *et al*.[332] or from aldol condensation. Some of these products probably cannot react further, such as cyclic compounds. On the other hand, a portion of these may be stable in the control system, but be able to react further with the addition of certain compounds (possibly catalytic) which provide an alternative reaction pathway (see section 8.2.2). As previously mentioned, reactants that can produce multiple products by the same rate law have been encompassed under one reaction pathway in the model. It is probable that only a portion of these side products will be able to react further when a perturbant is added, but the rate constant will reflect this.

Figure 8.5 shows the chemical reactions that are included in the model for the control system. The rate constants defined for the control sample were used as the base rate for all subsequent models with added perturbants.

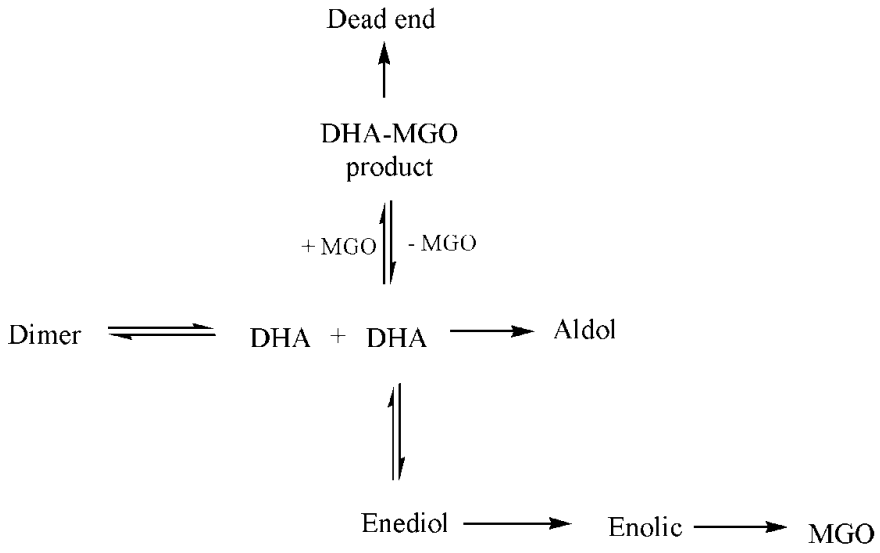


Figure 8.5 Scheme of the chemical reactions in Model 3 that are occurring in the control sample. Note that in the control system the aldol is acting as a sink of DHA that cannot convert into MGO.

Figure 8.6 and Figure 8.7 show the change in DHA and MGO concentrations respectively; replicates A and B of the experimental results are shown with the simulated results. The graph is plotted in mmol/kg for ease of determining the reaction of each mole. However, honey producers use mg/kg or NPA to assess the potential of the honey, hence mg/kg is inserted as a secondary axis on the right hand side (refer to appendix A for a table of conversion for MGO to NPA). 1 mmol/kg DHA is equal to 90.08 mg/kg; 1 mmol/kg MGO is equal to 72.06 mg/kg.

The change in DHA concentration of the simulation mirrors the experimental data well early in the reaction. However, at later times too much DHA is lost. The simulation fits the experimental data well for the change in MGO concentration. The simulation is slightly too low at the beginning of the reaction; however, this is only marginal. As previously mentioned, fitting the simulation with experimental data is a time-consuming process, often with diminishing returns when the simulation fits close to the experimental data. Therefore there may be some deviation in the simulated data from the experimental data. However, this discrepancy is not expected to be large.

The largest difference between the simulation and measured concentration of MGO in the DHA-control sample is at approximately 216 days. The measured concentration is 176 mg/kg compared to 141 mg/kg for the simulated data, which is only a difference of 35 mg/kg and is at the low end of the deviation reported by the ILCP.

The conversion between MGO and NPA is not linear, therefore the range of concentrations between NPA values is not equal; for example, the difference is larger between NPA 10 and 15 than it is between 5 and 10. Therefore over time if the deviation of the simulated result is far from the experimental data, the error is smaller when converted to NPA. For example, if the prediction falls short by 2 mmol/kg, this is equivalent to 144.12 mg/kg (1.32 mmol/kg). The difference between NPA 5 and 10 is 129 mg/kg. Therefore if the MGO value is low this may be the difference between assuming the sample will reach a NPA 5 or 10. However, at the high end of the scale, due to the non-linearity of NPA to MGO conversion, the difference between a NPA 20 and 25 is 388 mg/kg MGO, which lessens the error of predicting the wrong NPA end point. By January 2016, NPA will no longer be able to be displayed on jars of honey. However, the industry and consumers are familiar with the NPA rating and this helps to give a perspective of the quality of the fit of the model.

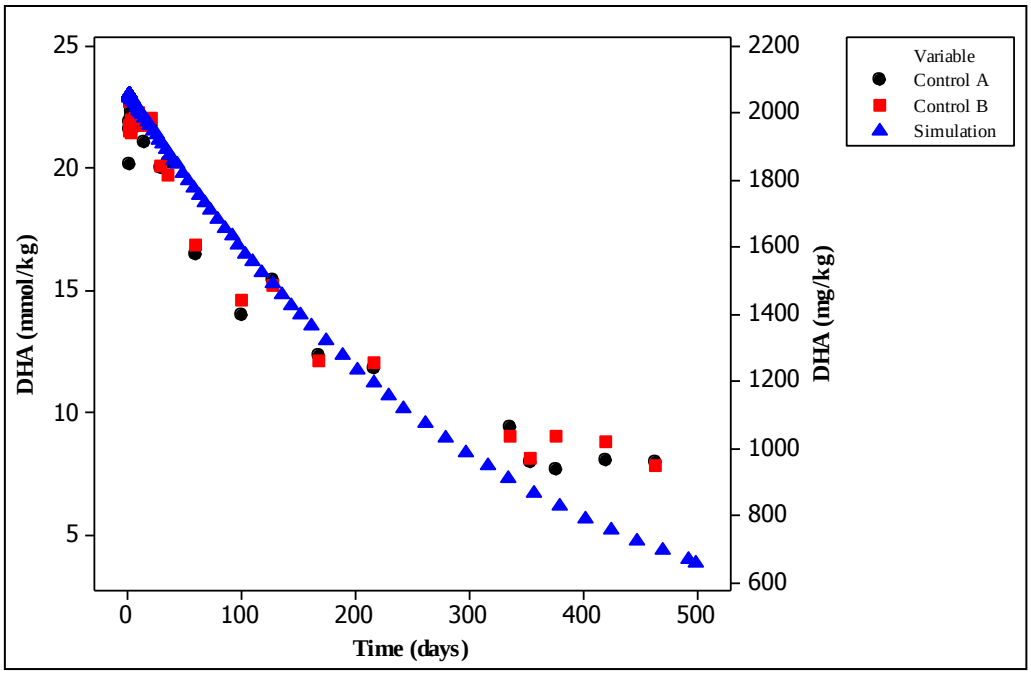


Figure 8.6 Experimental (circles and squares) and simulated (triangles) data for DHA loss in the control experiment with 2,000 mg/kg DHA added. The simulated data fits the experimental data well at the beginning of the reaction, but is slightly too fast at later times.

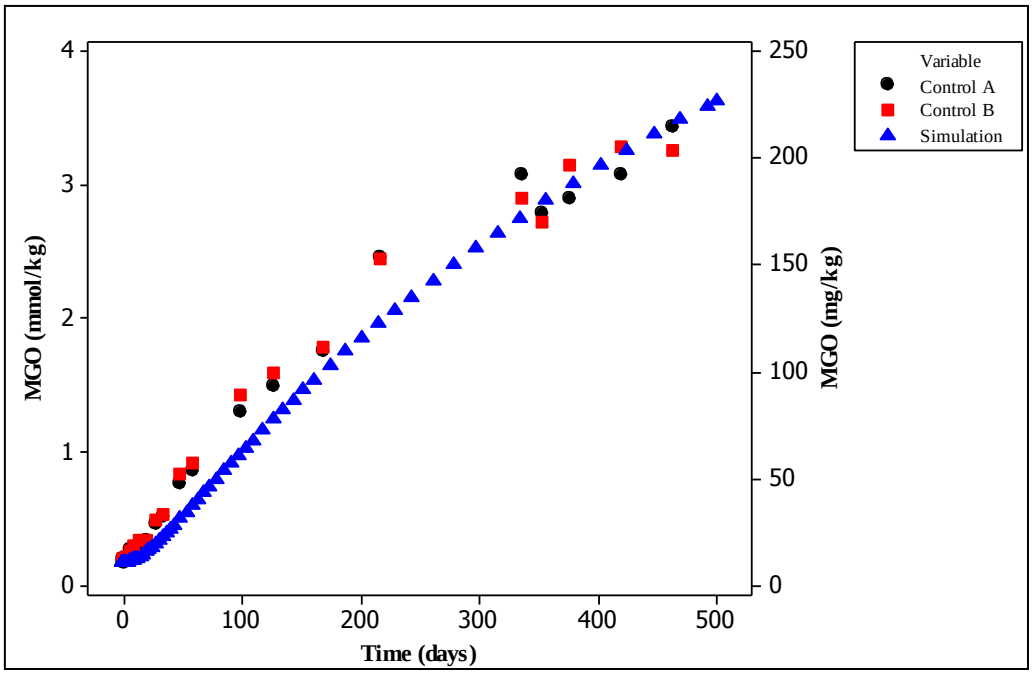


Figure 8.7 Experimental (circles and squares) and simulated (triangles) data for MGO gain in the control sample with 2,000 mg/kg DHA added. The simulated data is slightly slow at the beginning of the reaction but fits the experimental data well at later times.

Efficiency, as first described in chapter 5, represents the proportion of DHA that has been converted to MGO. The percentage efficiency of the simulated reaction is the same as the experimental data (average of 18%), suggesting that this model gives a good approximation on what reactions are occurring in the control sample and the proportion of DHA that is converting to MGO.

The model does not work with 10,000 mg/kg DHA (approximately 5 times more initial DHA than that observed naturally in mānuka honey). In the simulation, too much DHA is consumed and not enough MGO is produced compared to the experimental data. It is influenced by the higher concentration of monomeric DHA. Furthermore, it may be due to a postulated second-order reaction of 2 DHA going to a side product (aldol). When the concentration of DHA is doubled, the rate of this route is four times faster due to the square function in the rate equation – i.e. $\frac{d[DHA]}{dt} = k[DHA]^2$. The second-order reaction will accelerate differently to the first-order reaction, which is not influenced by the initial concentration of DHA. The reaction of 2 DHA to a side product can proceed by a first-order mechanism; this can occur if one DHA needs to convert to the enediol before reacting with another DHA. Since, the initial concentration of DHA is much higher than concentrations seen naturally, the model was not adjusted to fit the 10,000 mg/kg data. However, it is expected that rate constants and equilibrium constants could be adjusted in order to fit the simulation to the experimental data for this initial high level of DHA.

8.2.2 System perturbed with alanine

The loss of DHA in the DHA-alanine system was the same as in the control sample, but more MGO was formed. This indicates that alanine may be able to convert DHA to MGO before the DHA is able to convert to the aldol, which is a dead end product in the control sample. Alternatively, alanine could provide a new pathway for the aldol to form MGO (likely to be dissociation of the aldol into 2 DHA, then conversion to MGO). As mentioned in Model 2 (section 8.1.2), alanine was not able to shift the equilibrium between 2 DHA and the side product back towards DHA enough to recover the amount of DHA seen experimentally; too much DHA was always consumed in the simulation compared to the

experimental data. Therefore it is proposed that alanine is able to provide an alternative pathway for the side product (aldol) to dissociate into 2 DHA and form MGO that was not available in the control system. Addition of this equation to the model allowed the simulated data to mirror the experimental data, without losing excess DHA. Details on this route to MGO requires knowledge about which compound alanine is altering and how the alteration occurs; however, this is not presently known. It is possible that alanine is able to convert the product of 2 DHA (aldol) back into 2 free DHA by H^+ donation and then convert the DHA to MGO. In the model only a single step from the side product to MGO was included which encompassed the probable multistep reaction.

In chapter 5 the initial fast loss of DHA in the DHA-alanine system was discussed; it was thought to be due to the binding of available DHA with alanine, which prevented alanine from dissociating the dimer as quickly. Therefore an equation was added for the reversible reaction of DHA and alanine to form an imine, and subsequent irreversible formation of a product. These are Maillard-type reactions and were discussed in chapter 5. This reversible binding of alanine was required to reduce the amount of free alanine so that the conversion of the aldol into MGO by catalysis of alanine was not too fast. In addition, this also helped produce the deviation from linearity seen experimentally.

Rate constants were adjusted in order to find a set that mirrored the experimental data. The initial binding of DHA to alanine is fast as can be seen on the [DHA] vs. t plot (Figure 8.8). The severity of this loss of DHA can be changed by adjusting the rate constants for the forward and reverse reactions to the imine, while keeping the equilibrium constant, K, the same. The equilibrium constant chosen was 5. If the rate constants are large; for example, if $K_{forward} = 1$ and $K_{reverse} = 0.2$, then there is a short sharp decline, but if the magnitude is smaller (e.g. 0.5 and 0.1 respectively) the initial decline is not as sharp and occurs over a longer time period. Alternatively, changing the equilibrium constant will also affect the initial loss of DHA.

A reversible reaction between MGO and alanine was added to the model, which helped to control the production of MGO. Addition of this reaction slowed the overall production of MGO. The simulation fitted the experimental results well

with this set of reactions in the model. The calculated percentage efficiency was ~60%, which is in line with the experimental results. The comparison of this simulation with the experimental data for loss of DHA and gain of MGO are shown in Figure 8.8. Both the loss of DHA and gain of MGO have a close fit between simulated and experimental data. However, this reaction does not fit with all of the experimental observations; the MGO-alanine system (chapter 5) showed no irreversible reaction between MGO and alanine over 500 days. The lack of reaction experimentally is in contrast to the literature.[292] However, a reversible reaction (initial stage of the Maillard reaction) could occur, but MGO would still be analysed as free in the HPLC analysis, hence it would not affect the concentration output of MGO. However, this current model does not account for the MGO seen in the HPLC analysis because the product of the reaction of MGO and alanine was not added to the total MGO concentration to compare to the experimental data.

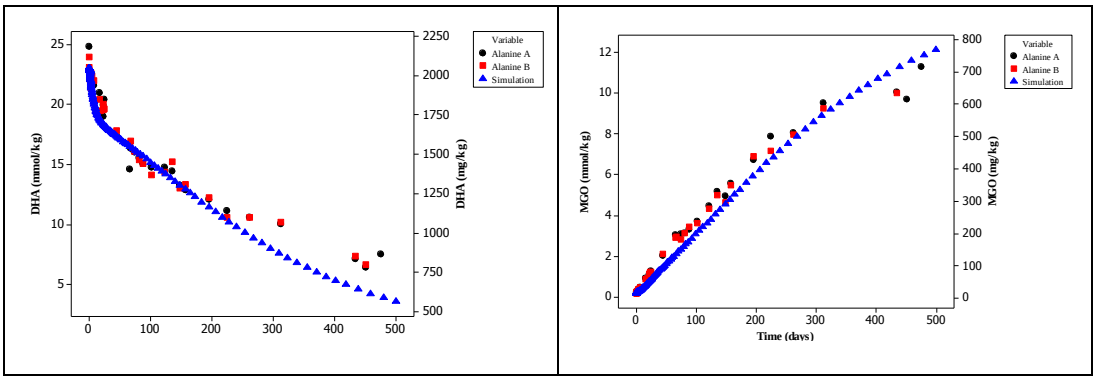


Figure 8.8 Experimental (circles and squares) and simulated (triangles) data for DHA loss (left) and MGO gain (right) in the alanine perturbed experiment with 2,000 mg/kg DHA added. The simulated data fits the experimental data well, but this simulation included equations for reaction between MGO and alanine.

When the reversible reaction between MGO and alanine was removed from the model the simulated loss of DHA was too great (presumably from DHA reacting with alanine) and the formation of MGO was too high (because excess MGO is no longer removed in a side reaction). The rate constant for $Aldol + alanine \rightarrow 2\ MGO + alanine$ was made smaller (0.1 cf. 0.5) which brought the simulation closer in line. However, there was some initial deviation in formation of MGO in the simulation, which most likely arises from a lag period where the aldol concentration needs to build up before a substantial amount of MGO can be

formed. Furthermore at longer times, too much MGO was formed, possibly due to the altered amount of alanine in the system. The rate constant for $DHA + alanine \rightarrow DHA - alanine - product$ cannot be increased because too much DHA will be lost. The results indicate that alanine is also lost in another pathway. As mentioned in chapter 5, alanine is less reactive than other amino acids in a honey matrix, but will form Maillard products;[163, 354] this will account for the loss of alanine in a separate pathway hence an equation was added to remove alanine in a side reaction. The compound with which alanine is reacting has been termed 'A' and was arbitrarily given an initial starting concentration of 10 mmol/kg.[*] With the addition of this equation, the model matches the experimental observations. The simulated data for DHA loss and MGO gain fitted the experimental data well (Figure 8.9 and Figure 8.10), but a little too much DHA may be lost at later times; this may be due to the influence of the equations from the control system, which also showed a slight excess of DHA consumed at later times.

The calculated efficiency of this reaction was ~64% at the end of the simulated time, which is slightly higher than seen experimentally. The simulated data lost slightly more DHA than the experimental data.

[*] 'A' is likely to be acyclic sugars. Due to the high concentration of sugars in the honey even a small percentage that exist in acyclic form would be significant.

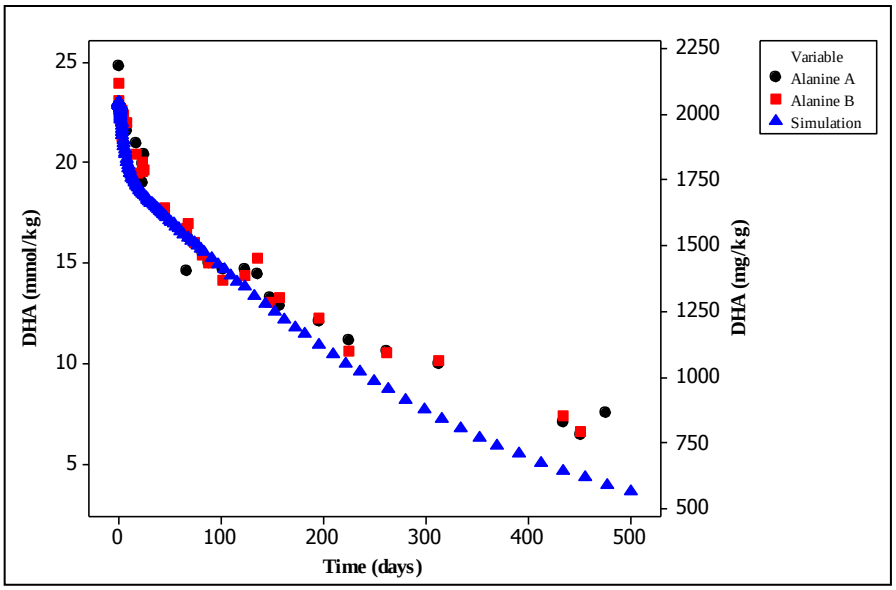


Figure 8.9 Experimental (circles and squares) and simulated (triangles) data for DHA loss in the alanine perturbed experiment with 2,000 mg/kg DHA added. The simulated data fits the experimental data well. This simulation included an equation to remove alanine independently.

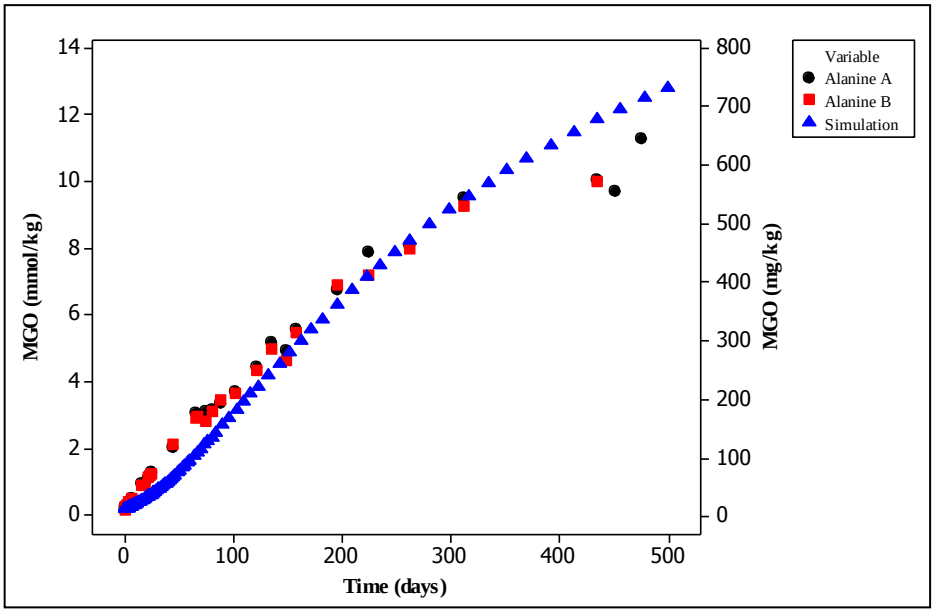


Figure 8.10 Experimental (circles and squares) and simulated (triangles) data for gain of MGO in the alanine perturbed experiment with 2,000 mg/kg DHA added. The simulated data fits the experimental data well. This simulation included an equation to remove alanine independently.

Figure 8.11 shows the scheme of reactions that were added to Model 3 when the system is perturbed by alanine. This is in addition to the equations for the control rate. Note that the reaction of 2 DHA to 'product of 2 DHA' is part of the control system but is shown here to clarify the route this product can take when alanine is added to the system.

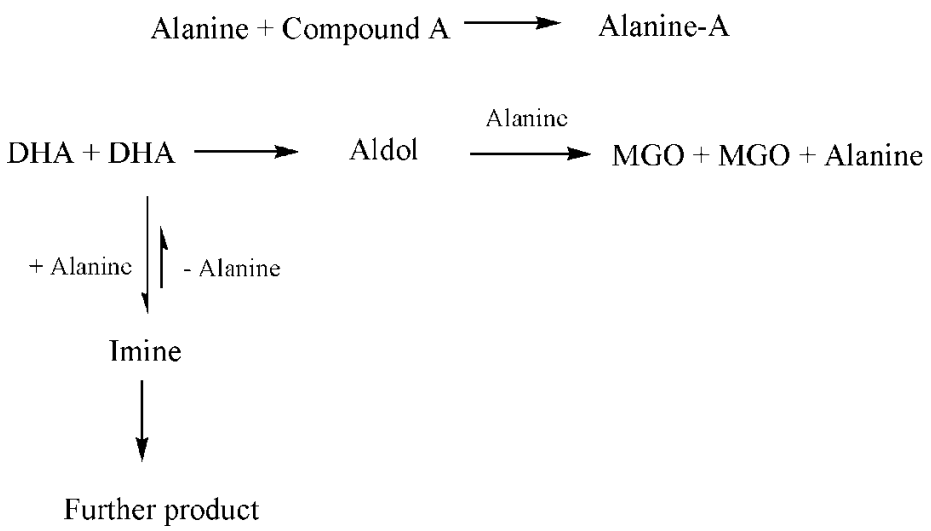


Figure 8.11 Scheme of chemical reactions added to Model 3 to simulate loss of DHA and gain of MGO when the system is perturbed by alanine.

The DHA-alanine system had 430 mg/kg (4.8 mmol/kg) alanine, which is higher than both the alanine and total amino acid concentration found in the six mānuka honeys analysed in chapter 4; the average alanine concentration was 12.5 ± 6.1 mg/kg (0.14 mmol/kg) and the total primary amino acid concentration was 154.9 ± 76.5 mg/kg (0.79-1.98 mmol/kg[*]). Alanine was originally used to represent all primary amino acids in the honey matrix, but the storage trials with lysine and serine showed that not all primary amino acids have the same effect on the system. For example, the reaction of the primary amino acid with 'A' to form a side product will be faster for lysine than it is for alanine, since lysine is the most reactive amino acid. Therefore the difference in rate constants between amino acids either needs to be absorbed into the rate constants for reactions with alanine, or groups of amino acids that react in the same manner need to be identified and equations added to the model for each group.

[*] The concentration of total amino acids in mmol/kg is worked out by converting each amino acid from mg/kg to mmol/kg then calculating the sum of all amino acids.

The concentration of primary amino acids between different mānuka honeys is not expected to vary by orders of magnitude. Therefore once a set of rate constants has been chosen, they should work for a large number of samples. Consequently, individual amino acid concentrations would not have to be analysed for each honey run in the prediction model. Doubling the initial concentration of alanine from 4.8 to 9.6 mmol/kg in Model 3 caused a large initial decline in DHA due to the formation of the imine, which is not likely to be observed in commercial samples because there are multiple compounds that can dissociate the dimer. The equilibrium constant could be altered, if required, so that the initial loss of DHA was similar to real honey samples. After the fast initial loss, the loss of DHA is similar to that seen experimentally. Furthermore, when the concentration of alanine is increased, the formation of MGO is only slightly enhanced. Unfortunately, no experiments were conducted with different starting concentrations of alanine in artificial honey, so it is unknown if the fast initial decrease of DHA is likely to occur, and whether an increase in alanine has an effect on MGO. Further experiments could be carried out with various starting concentrations of alanine and the rate constants for the forward and reverse reactions to form imine can be adjusted if necessary. For further discussion on alteration of the concentration of perturbants in the simulation, see section 8.4.

When real honeys were compared to the simulation, the fit was not close because the concentration of primary amino acids is only small. Alanine has been proposed to recover DHA from the 'aldol' and convert it to MGO, hence if there is only a small concentration of alanine available not as much DHA can be recovered. It appears unidentified compounds also play a role in converting DHA to MGO as well as removing MGO in the mānuka honey matrix (see section 8.3 for a discussion on this).

8.2.3 System perturbed with proline

The loss of DHA in the DHA-proline system had an initial fast loss of DHA followed by the secondary reaction which was slower than the control; this made it difficult to get a close fit with the simulation. Proline did not have a rate enhancing effect on the appearance of MGO. Therefore only the reversible equation $DHA + proline \rightleftharpoons DHA - proline - complex$ was added to the model

for samples perturbed by proline. The simulated data fitted the experimental loss of DHA at early times, but at later times too much DHA was consumed. Moreover, the simulated concentration of MGO was too low compared to the experimental data, which suggests that there is some enhanced effect from proline, even though it is not reflected in the overall rate constant for formation of MGO. While the experimental rate constant does not reflect the small rate enhancement of MGO formation from proline, the [MGO] vs. t graph for the DHA-proline system shows this enhancement when compared to the control system (Figure 8.12). The discrepancy in MGO formation possibly arises due to an effect from the calculated rate constant for disappearance of DHA being smaller than expected, which in turn influences the rate constant of MGO formation (because k is required in the equation). An equation for $DHA + proline \rightarrow MGO + proline$ was added to the model. The loss of simulated DHA was too great and it did not fit with the experimental data at later times (Figure 8.13), but the simulated MGO data mirrored the experimental data (Figure 8.14). Further refinement was carried out in section 8.2.6.

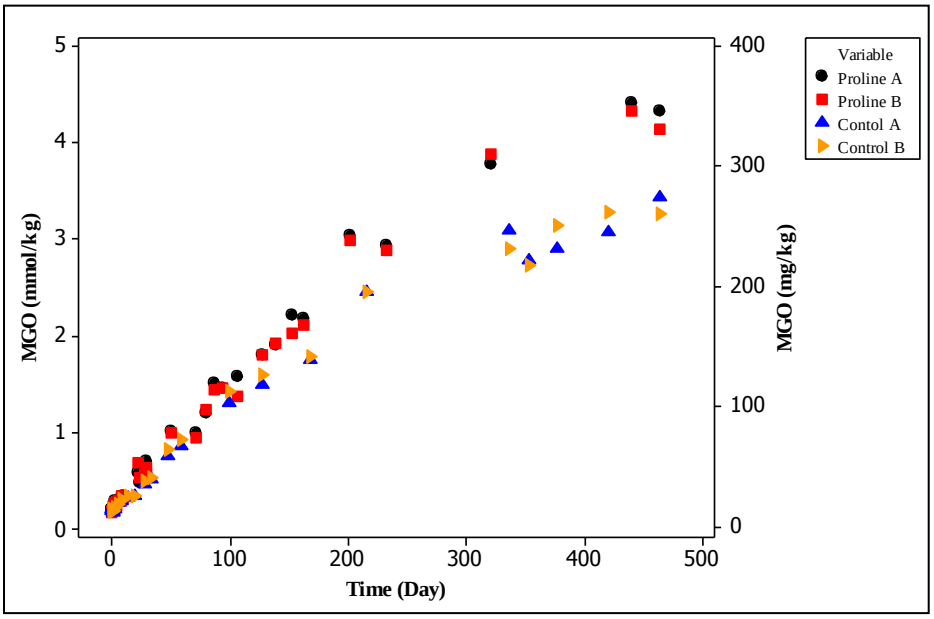


Figure 8.12 Comparison of gain of MGO for control system and system perturbed by proline. At longer times, more MGO is formed in the proline system than in the control system.

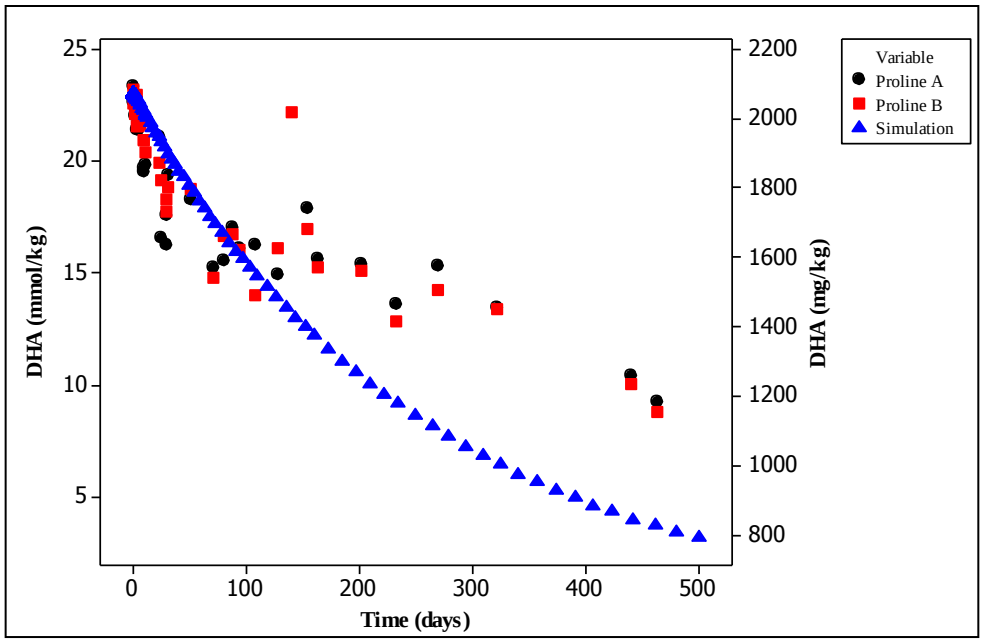


Figure 8.13 Experimental (circles and squares) and simulated (triangles) data for loss of DHA in the proline perturbed experiment with 2,000 mg/kg DHA added. The simulated data fits the experimental data well at the beginning, but deviates significantly at longer times.

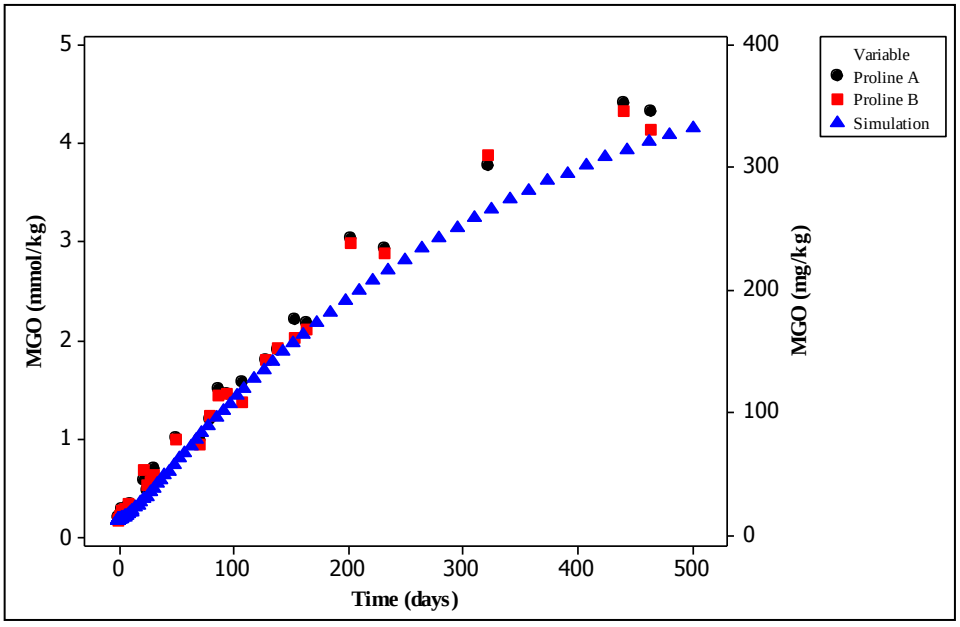


Figure 8.14 Experimental (circles and squares) and simulated (triangles) data for formation of MGO in the proline perturbed experiment with 2,000 mg/kg DHA added. The simulated data fits the experimental data well.

The calculated efficiency for the model was ~19%, which was slightly less than calculated experimentally (26%). However, this will be due to too much DHA being consumed in a side reaction in the simulated model.

As seen in chapter 5, the rate constant for DHA loss in systems with proline and one or more other perturbants was not slower than the control system due to the ability of the other perturbants to dissociate the dimer. Furthermore a competing reaction between proline and the other perturbant for the free DHA would mean not as much DHA could initially bind to proline, hence some proline could catalyse a side reaction of MGO. Therefore the final set of chemical equations and rate constants were not altered in order to mirror the DHA in the DHA-proline system. They were refined in later sections (sections 8.2.6, 8.2.7 and 8.2.8). The chemical reactions included in the model for perturbation by proline are shown in Figure 8.15.

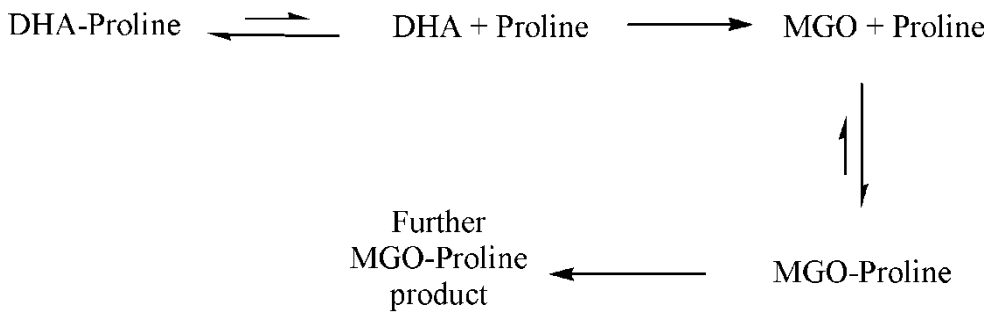


Figure 8.15 Chemical reactions added to the simulation to account for the loss of DHA and gain of MGO in a system perturbed by proline.

8.2.4 System perturbed with iron

The simulation of the DHA-iron system was the easiest to fit to the experimental data. The reversible reaction of DHA and iron and a reaction of the DHA-iron complex to MGO + iron were added to the simulation (Figure 8.16). These three reactions allowed the simulation to fit the experimental data well (Figure 8.17 and Figure 8.18). The calculated efficiency of the simulated reaction was ~37%, compared to 30 ± 10% for the experimental data, indicating that iron cannot recover DHA from the 'aldol' side product in a similar way to alanine (efficiency

~64%). Iron does not have the ability to donate a proton, hence would not be able to catalyse the release of 2 DHA from the 'aldol'.

The concentration of iron used in the model system (870 mg/kg, 3 mmol/kg) is much higher than what is naturally observed in real honey, hence the influence from iron would be minimal. However, this was simulated to test the folklore that iron is able to accelerate the conversion of DHA to MGO.

Figure 8.16 Chemical equations added to the simulation to account for the loss of DHA and gain of MGO in a system perturbed by iron.

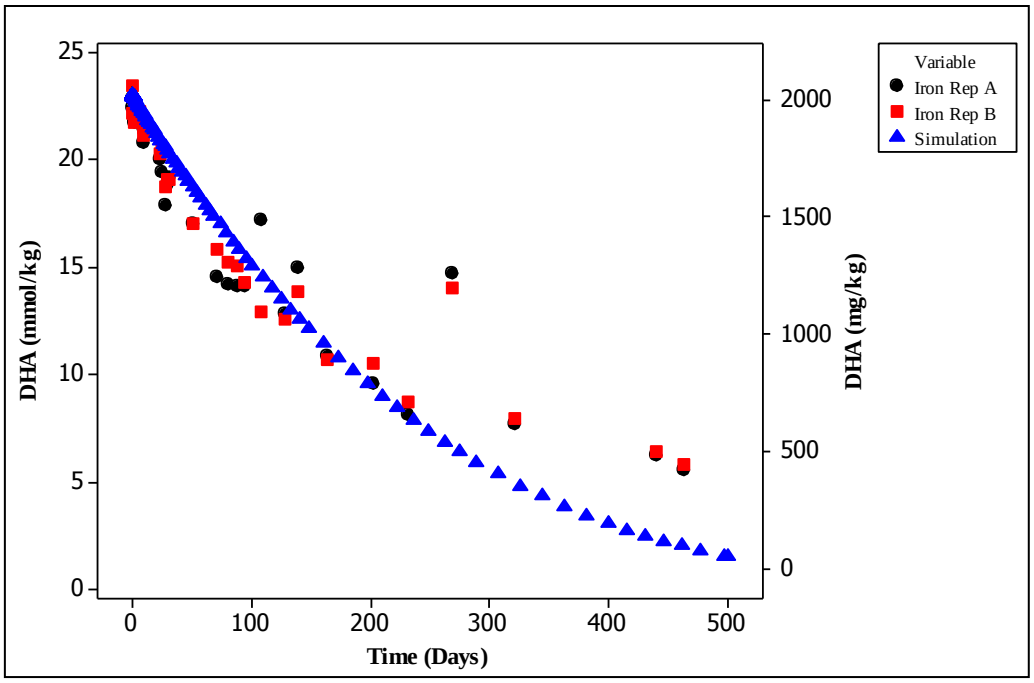


Figure 8.17 Experimental (circles and squares) and simulated (triangles) data for loss of DHA in the system perturbed by iron. The simulated data fits the experimental data well.

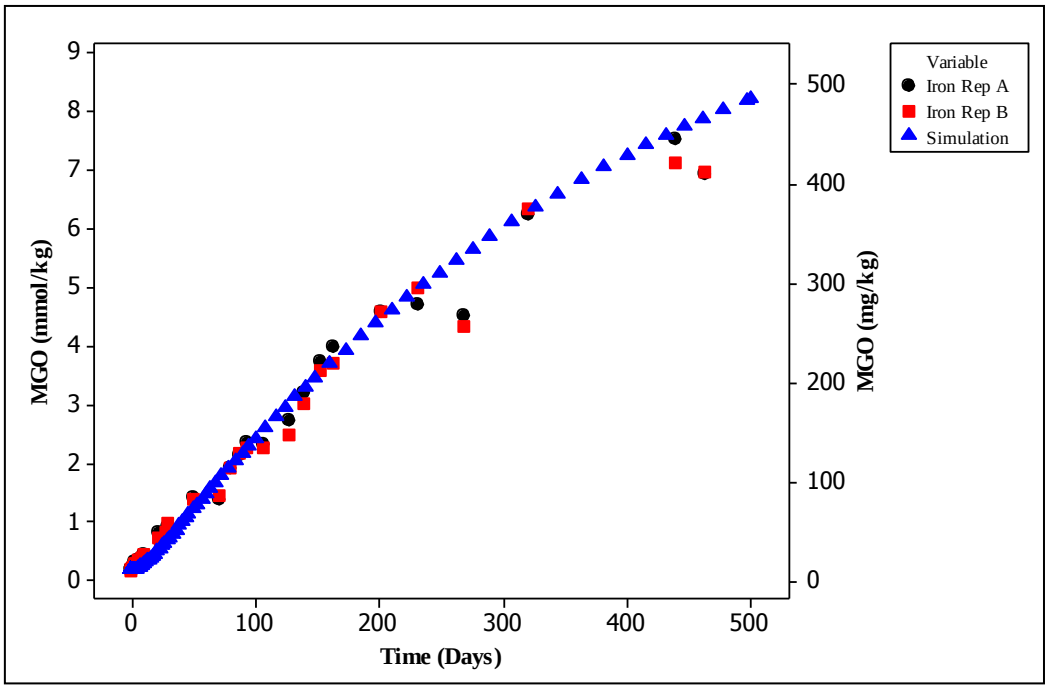


Figure 8.18 Experimental (circles and squares) and simulated (triangles) data for gain of MGO in the system perturbed by iron. The simulated data fits the experimental data well.

8.2.5 System perturbed with alanine and iron

The equations for both alanine and iron were added together, along with the control equations, to simulate the system that had both perturbants added to it. The simulation fitted the experimental data well for DHA and was only slightly too slow for the formation of MGO (Figure 8.19). No interaction between alanine and iron is present that affects the loss of DHA or gain of MGO; therefore no extra equations are required in the model.

Data for the alanine-iron system was not collected for as long as other samples, so it is unknown whether the MGO data will deviate substantially at later times, as seen for the alanine-proline system (section 8.2.6). But it is thought that a substantial deviation arises predominately when proline is present in a system This is discussed in detail in section 8.2.6.

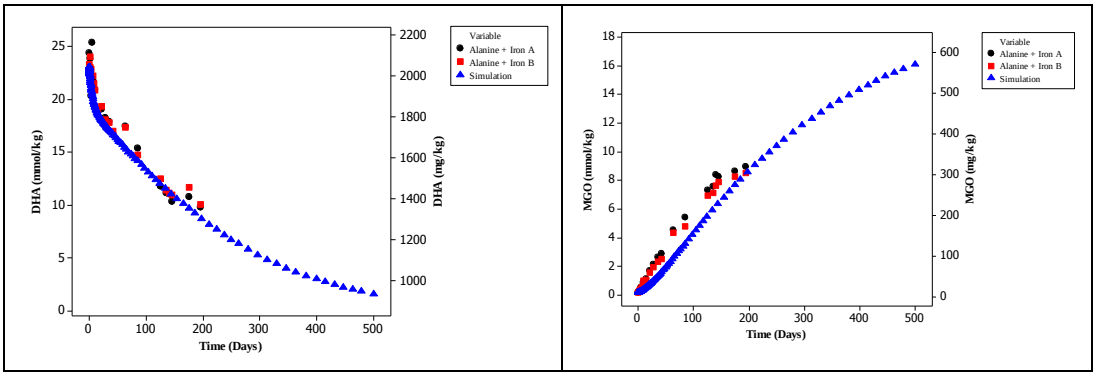


Figure 8.19 Experimental (circles and squares) and simulated (triangles) data for loss of DHA (left) and gain of MGO (right) in the system perturbed by alanine and iron with 2,000 mg/kg DHA added. The simulated data fits the experimental data well.

8.2.6 System perturbed with alanine and proline

The chemical equations for reactions involving alanine and proline were run in the model together with the control chemical equations. It is expected that if there is no interaction between alanine and proline then the simulated data should match the experimental data, as seen for alanine and iron (section 8.2.5). The DHA simulated curve mirrors the experimental data (Figure 8.20). The uncharacteristic shape of the [DHA] vs. t graph in the sample perturbed by only proline (section 8.2.3) is not seen in the system perturbed by both proline and alanine – the loss of DHA behaves as expected. This may be due to both alanine and proline

competing for the limited amount of monomeric DHA and the availability of alanine to dissociate the dimer.

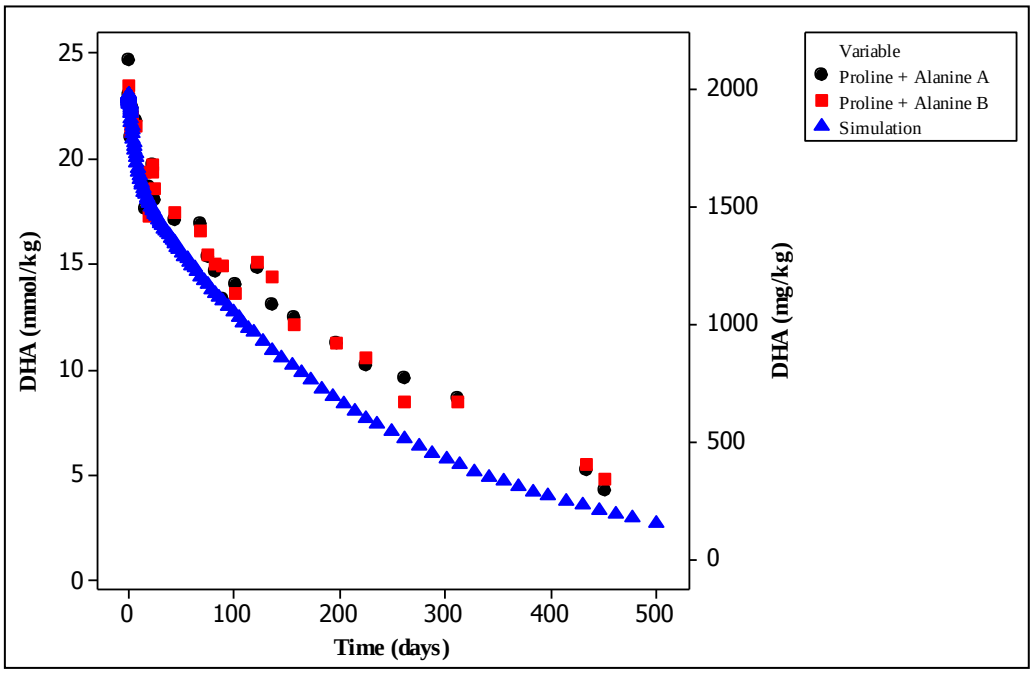


Figure 8.20 Experimental (circles and squares) and simulated (triangles) data for loss of DHA in the alanine-proline system with 2,000 mg/kg DHA added. The simulated data fits the experimental data reasonably well.

The initial part of the simulated MGO curve fits the experimental data well, but at later time the experimental MGO passes through a maximum and begins to decline which is not reproduced in the simulated data. The model is thus not accounting for one or more routes of MGO loss. The loss of MGO is not likely to be from reaction with alanine, or a reaction catalysed by alanine because the DHA-alanine system did not show a large loss of MGO at later times.

The loss of MGO may be due to proline reacting irreversibly with MGO and/or proline catalysing an irreversible reaction of MGO with no loss of proline. As previously mentioned, proline is known for its catalytic ability,[335-336, 408] hence it can continue removing MGO without being consumed in the reaction (although it may be consumed by involvement in an unrelated reaction e.g. binding to MGO or DHA). This concurs with more MGO being consumed from the MGO-proline system than the starting concentration of proline. Equations for reversible and irreversible reactions of MGO and proline were previously added to the model

(section 8.2.3). However, in the system perturbed by only proline, loss of MGO at longer times was not as noticeable. This may be due to the slow formation of MGO. In comparison, the system which also contains alanine has another pathway for formation of MGO (aldol + alanine → 2 MGO + alanine); hence the concentration of MGO increases more rapidly at later times the loss by a pathway involving proline is more noticeable.

If the rate constant for the reversible binding of MGO with proline is increased in the simulation then the simulated MGO data begins to decline, making it closer to the experimental data at intermediate times. However, it still deviates at later times in the reaction, indicating that the model still does not take into account all reactions that are affecting the observed loss of MGO. Therefore the conversion of MGO into an irreversible side product by catalysis of proline was also added to the model ($MGO + Proline \rightarrow MGO - side\ product + proline$). Rate constants for both reactions were varied and values were found for which the simulation showed the same decline in MGO at later times. (Figure 8.21). This plot has error bars shown on it (0.32 mmol/kg as determined by the ILCP) because it is the set of data that was used as the starting point to model real honey samples (section 8.3). The simulated data fits the experimental data well.

However, with these equations added too much MGO is lost in the simulated DHA-proline system and also in the simulated DHA-proline-iron system compared to the experimental data. This is because MGO cannot be recovered from the aldol if alanine is not present. Hence model systems without alanine lose too much MGO over time due to the catalytic removal of MGO by proline. Real honey matrices will contain both alanine and proline; therefore the equation for removal of MGO by proline catalysis will be used for simulation of real honey samples.

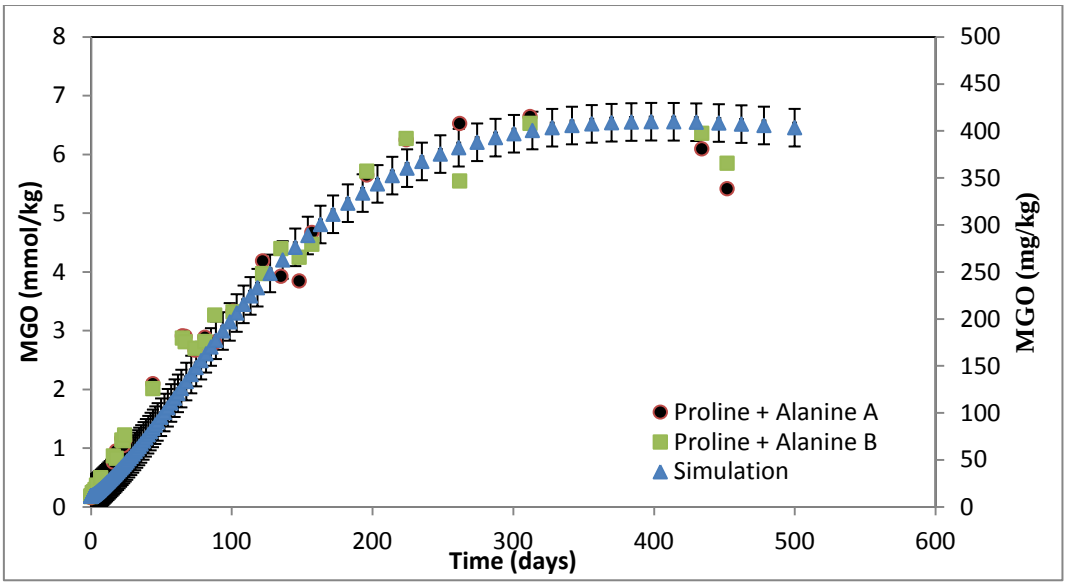


Figure 8.21 Experimental (circles and squares) and simulated gain of MGO (triangles) in system perturbed by alanine and proline. An equation for the removal of MGO catalysed by proline was added into the model to help simulate the curve at later times in the reaction. Error bars (0.32 mmol/kg, 119 mg/kg) relate to the error reported in the 2013 ILCP. The simulated data fits the experimental data well.

8.2.7 System perturbed with proline and iron

The rate equations for all chemical reactions involving proline and iron, and the set of control equations were used to simulate the system with both perturbants. The simulation matched the observed results for the loss of DHA (Figure 8.22).

The simulation was initially run without the equations for catalysis of proline to form a side product of MGO – the simulated data for MGO fits the experimental data well early in the reaction. However, at later times, experimentally MGO reaches a maximum and begins to level out. It is expected that it will eventually begin to decline, but data was not collected for long enough to see this occur. This decline is not reflected in the simulated data (Figure 8.22). When the catalysis equation (proline + MGO → MGO side product + proline) was added to the simulation with the same rate constant as used for the proline-alanine system too much MGO was lost. As discussed in section 8.2.6, this is most likely due to the ability of alanine to recover DHA from a dead end pathway. In the DHA-proline-iron system this recovery is not available as no alanine is present.

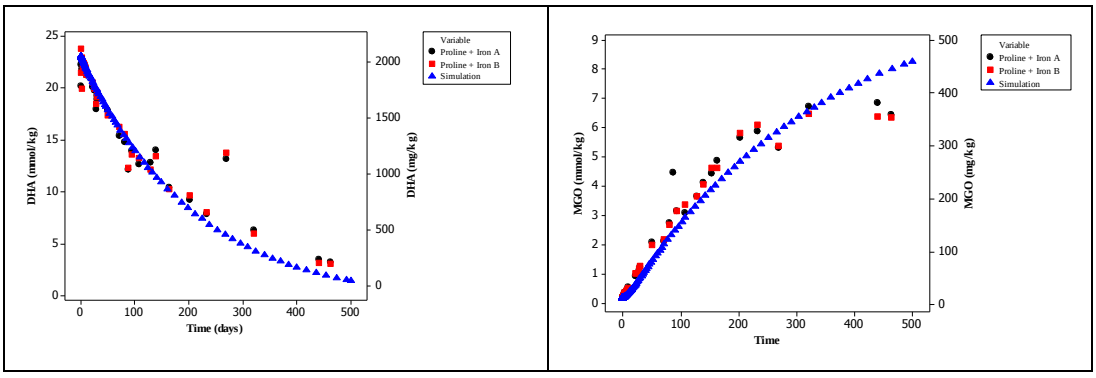


Figure 8.22 Experimental (circles and squares) and simulated (triangles) data for loss of DHA (left) and gain of MGO (right) in the system perturbed by proline and iron with 2,000 mg/kg DHA added. The simulated data fits the experimental data well, compared to the sample with only proline.

8.2.8 System perturbed with proline, alanine and iron

The proline-alanine-iron system was the only system studied that had three perturbants added. The rate constants for each of the individual systems were added together. The simulated data matched the experimental data well for the loss of DHA (Figure 8.23).

When the simulation was run without the equation for removal of MGO by catalysis of proline, the gain of MGO did not match because the simulation did not lose MGO at later time periods in the same way as the experimental data. When this equation was added (with the same rate constant used for the proline-alanine system), the simulated MGO curve fitted much better (Figure 8.24). The simulated curve began to decline , but still did not curve as much as the experimental data. The last two experimental points are lower than the simulated data; at this late stage in the reaction the samples are drier than they were at the start of the reaction and the lower measured MGO may be due to this. For this reason, and because the concentration of iron is added in much higher levels than that observed naturally in honey, the iterative process of fitting this system was not carried out.

At the beginning of the reaction, the simulation is slow. For example, at 65 days the measured concentration of MGO is 293 mg/kg compared to only 177 mg/kg for the simulated data, which is a difference of 116 mg/kg. Later in the reaction

the simulated data is closer to the experimental (88 mg/kg MGO difference at 135 days). Both of these are less than the upper end of the standard deviation reported in the 2013 ILCP, hence the fit is deemed as acceptable for the portion of the graph before MGO declines.

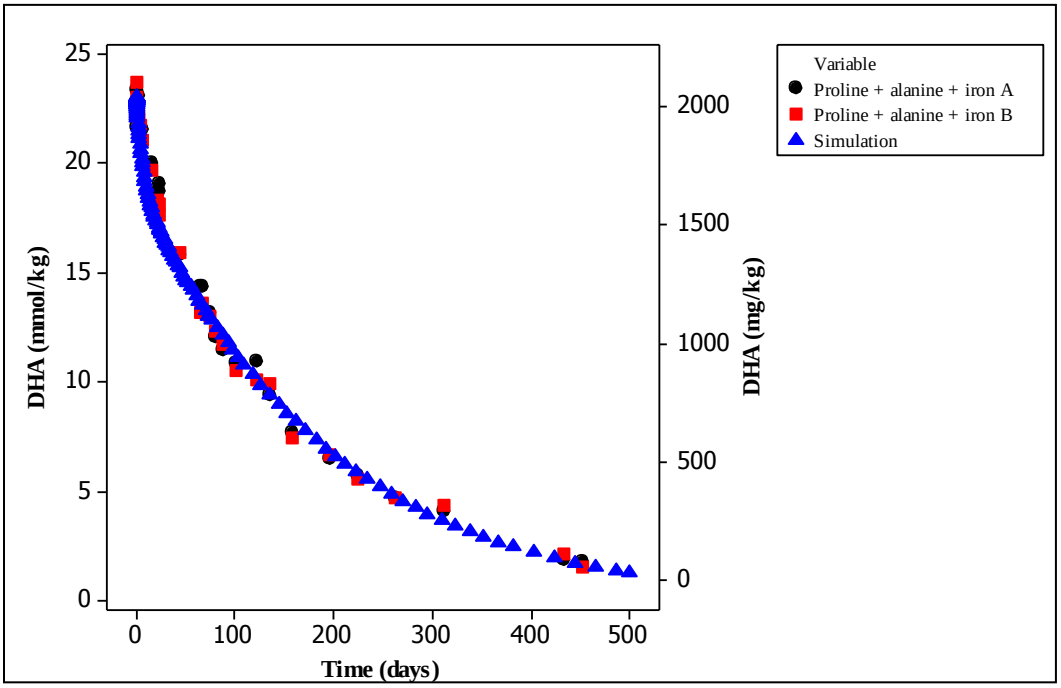


Figure 8.23 Experimental (circles and squares) and simulated (triangles) data for loss of DHA in the system perturbed by proline, alanine and iron with 2,000 mg/kg DHA added. The simulated data fits the experimental data well.

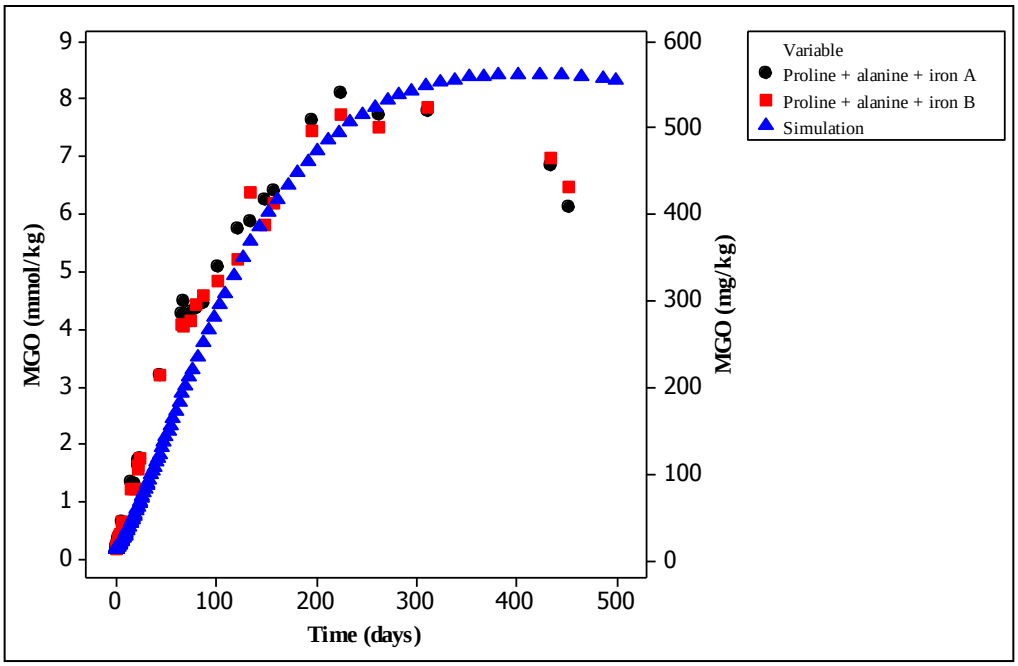


Figure 8.24 Experimental (circles and squares) and simulated (triangles) data for MGO formation in the proline-alanine-iron system. The simulation includes an equation for the removal of MGO into a side product by catalysis of proline; this curves the formation of MGO at later times, similar to the experimental data. However, this still does not account for the large curve in the experimental data at later times.

8.3 Simulation of real honey matrices at 37 °C

As discussed in chapter 5, the proline-alanine-iron system had the closest rate constants for loss of DHA and gain of MGO compared to the real honeys. Therefore the rate constants used for the simulation for this system were used as a starting point to simulate the loss of DHA and gain of MGO in real honey matrices. Initial concentrations of DHA (75% as dimer and 25% as monomer), MGO, proline, total primary amino acids and iron (as measured in chapter 4) for the samples were included in the model. However, as previously mentioned not all amino acids behave in the same manner, so incorporation of total primary amino acids may over or under estimate their catalytic ability. In addition, the total concentration of amino acids in real honey was lower than that used in the model systems, hence in real honey the concentration may not be high enough to have a significant effect upon the conversion of DHA to MGO. The model is deemed to fit well if the difference between the experimental and simulated data is less than the upper end of the standard deviation of the 2013 ILCP (24-119 mg/kg); this

was also used for comparison of the artificial honey systems with the experimental data.

For a first attempt at predicting the change in DHA and MGO doped clover honey (Happy Bee) was compared with the simulation. Table 8.2 summarises the initial concentrations entered into the simulation for the sample. Happy Bee honey was the only clover honey doped with 2,000 mg/kg DHA that was stored at 37 °C, hence was the only sample that could be tested. The simulation for the clover sample was slightly too slow for the loss of DHA, but had the same general shape; the simulated gain of MGO was much too slow (Figure 8.25).

Table 8.2 Initial starting concentrations (mmol/kg) entered into the simulation for Happy Bee clover honey.

	Starting concentration (mmol/kg)
Dimeric DHA*	9.33
Monomeric DHA*	6.23
Proline	3.66
Primary amino acid	0.81

*The dimer:monomer ratio was estimated as 75:25, as discussed in chapter 5.

† Dimeric DHA contains two DHA monomers

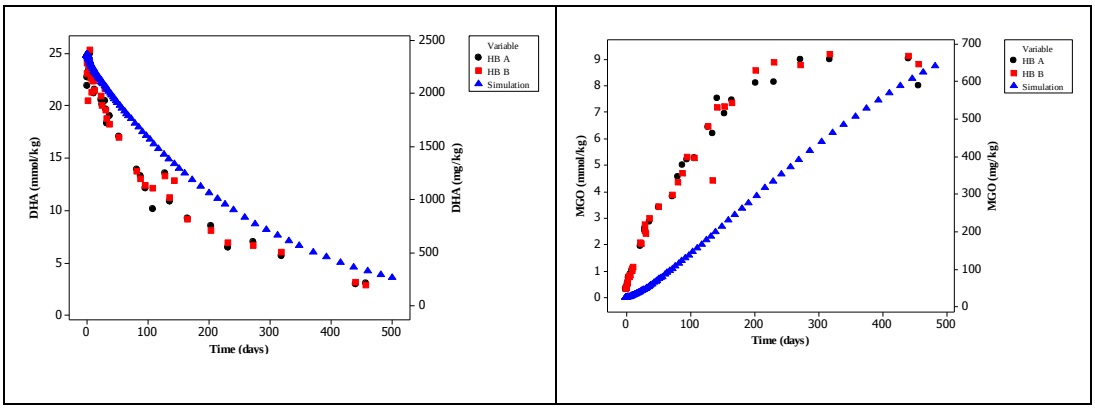


Figure 8.25 Experimental (circles and squares) and simulated (triangles) data for loss of DHA (left) and gain of MGO (right) in Happy Bee honey doped with DHA. The simulated data does not remove enough DHA or gain enough MGO, suggesting other compounds influencing the conversion of DHA to MGO are not yet accounted for.

Mānuka honey samples were compared to the simulated data; the initial concentrations for each honey are shown in Table 8.3. As with the clover honey sample, the simulated results did not fit the experimental data for any of the

honeys using the same rate constants as the DHA-proline-alanine-iron system. Examples of the loss of DHA and gain of MGO are shown in Figure 8.26 for sample 953. The simulated loss of DHA showed the same general curve as the experimental data, but the loss was too slow because not enough DHA was converting to MGO. These results indicate that there is a baseline effect in all real honey matrices that was not accounted for in the artificial honey system.

Table 8.3 Initial starting concentrations (mmol/kg) entered into the simulation for mānuka honeys.

Sample	Dimeric DHA*	Monomeric DHA	MGO	Proline	PAA
	mmol/kg				
946	10.80	7.20	0.76	7.03	1.98
953	16.44	10.96	2.89	6.49	1.63
66	13.75	9.17	3.86	4.97	1.01
78	10.88	7.25	2.34	3.40	0.62
84	15.00	10.00	4.33	4.78	0.80
1404	8.25	5.50	1.27	3.22	0.79

* Dimeric DHA contains 2 DHA

Iron was used as a perturbant in the artificial honey trials at very high levels (800 mg/kg), but in real honey only very low levels are reported (0.7 ± 0.5 mg/kg in the database honeys), hence iron will not have a large influence on the conversion of DHA to MGO and equations involving iron have been left out of future simulations of real honey samples. As mentioned, the real honeys had lower reported concentrations of amino acids than used in the artificial honeys, hence the effect on the conversion from the amino acids is not as large in the real honeys. Section 6.3 identified that the initial concentration of primary amino acids was insufficient to predict the appearance of MGO. The low concentration of primary amino acids will have a smaller contribution on converting the side product (aldol) to MGO, which is apparent by the lack of fit of the model (Figure 8.26). Hence one or more perturbants have not yet been identified that have an effect on the conversion of DHA to MGO. Furthermore, in mānuka honeys the simulated MGO curve did not show enough deviation at later times, indicating that one or more perturbants which have not yet been identified are also removing MGO from the system.

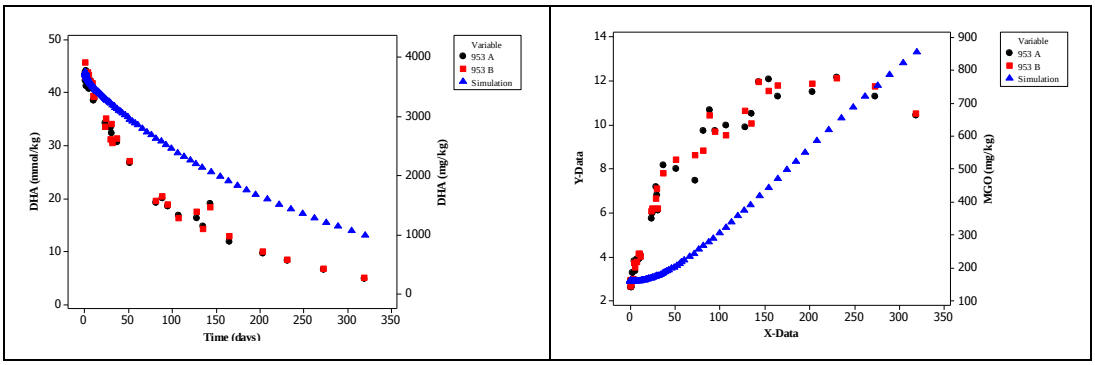


Figure 8.26 Experimental (circles and squares) and simulated (triangles) data for loss of DHA (left) and gain of MGO (right) in sample 953 doped with DHA. The simulated data shows the same general curve for DHA loss but is too slow because not enough MGO was formed. The simulated data for MGO formation is much too slow at the beginning and fails to deviate as much as the experimental data at later times. This suggests that other perturbants are critical in the conversion of DHA to MGO in mānuka honey and also the removal of MGO.

Chapter 6 identified phenolic acids as having a strong correlation with the rate constant for the loss of DHA; however, clover honey contains a very low level of phenolic acids which did not account for all of the MGO that was formed in the Happy Bee sample when a simulation containing equations for catalytic conversion of DHA to MGO by phenolic acids was compared to experimental data. This suggests that the conversion may be partially due to other compounds with acid functional groups, such as organic acids which would be able to donate a proton to the dimer and alter the equilibrium toward the monomer, or donate a proton to the monomer for conversion of DHA, which would also alter the equilibrium. Total acidity can be used to estimate the organic acids in honey. Total acidity of the honeys was measured for the database of honeys and ranged from 23.81-43.13 milliequivalents/kg. If we assume that this is largely made up of gluconic acid, it will be 0.12-0.22 mmol/kg. Equations were added to the simulation for the catalytic conversion of DHA to MGO by total acidity (converted to mmol/kg using the molecular weight of gluconic acid). The simulated DHA matched the experimental data when a rate constant of 0.7 day^{-1} was chosen, In addition, the MGO was a better fit, but there was no deviation of MGO at later times, so a second equation was added to remove some total acidity over time (assumed to be due to the unavailability of the acid groups over time), which partially altered the curve, but still not enough (Figure 8.27). Only one

clover sample was analysed during this research so multiple samples could not be tried in the model to see if the total acidity was responsible; this could be addressed at a later time.

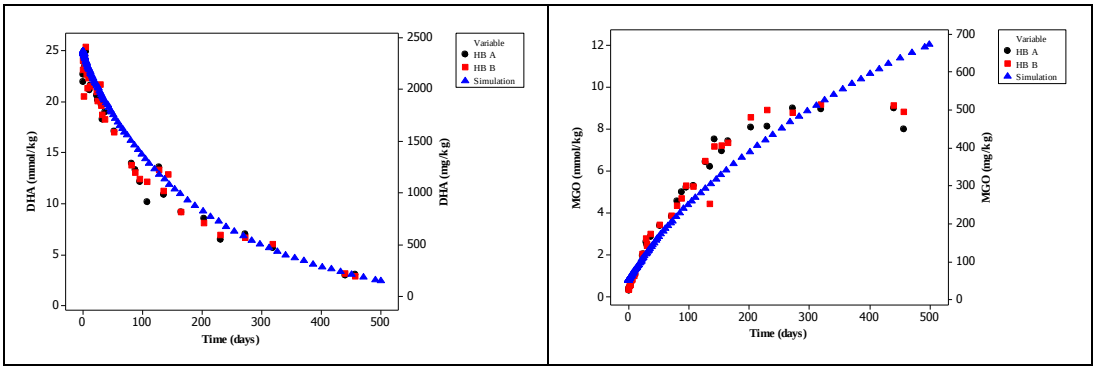


Figure 8.27 Experimental (circles and squares) and simulated (triangles) data for loss of DHA (left) and gain of MGO (right) in Happy Bee honey doped with DHA. The simulation has an equation for the reaction for catalysis of total acidity on the DHA conversion to MGO, which brings the simulation closer to the experimental data, except for later times for MGO.

To simulate the catalytic effect of phenolic acids on the reaction, the equation $DHA + phenolic\ acids \rightarrow MGO + phenolic\ acids$ was added to the model. If the concentration of total phenolic acids is high, more monomeric DHA will react which will shift the $Dimer \rightleftharpoons 2$ DHA equation to the right to balance the equilibrium, as proposed in chapter 6.* The total concentration of phenyllactic acid and 4-methoxyphenyllactic acid was used in the model. This gave a very good fit for DHA and also brought the MGO curve closer to the experimental data early in the reaction, but too much MGO was gained later in the reaction. Addition of syringic acid and 2-methyoxybenzoic acid to the total phenolic acids did not change the fit due to their low concentrations in the samples. The removal of phenolic acids (by conversion to a compound arbitrarily named Y) was also required as a way of slowing down the reaction over time. Phenolic acids may bind to other compounds in the honey either reversibly or irreversibly, but this has been incorporated as irreversible in the simulation; the chosen rate constant will reflect this.

The equation for the reaction of phenolic acids with DHA and the loss of phenolic acids to side product Z were not enough to fit the MGO data completely at later

* The model could also be adapted so that the phenolic acids dissociate the dimer.

times. Hence an equation for the removal of MGO was also added to the model. Reactions for the catalytic removal of MGO by a compound arbitrarily named Z ($MGO + Z \rightarrow MGO - side\ product + Z$) and also the removal of Z ($Z \rightarrow W$) were added to the model

Initially a reversible reaction between Z and MGO was also added to the model ($MGO + Z \rightleftharpoons MGO - Z$), but this did not strengthen the prediction; this was changed to an irreversible equation ($MGO + Z \rightarrow MGO - Z$), but this also did not strengthen the prediction. A closer fit was observed when both of these equations were removed from the model, suggesting that the equations are not required, need further adjusting, or the effect is incorporated into the catalytic equation. The reversible equation is unlikely to occur because MGO is a very reactive compound and generally reacts further to form irreversible products. Finding every pathway by which MGO reacts is likely to be unachievable, therefore the one equation for the catalytic conversion of MGO to a side product added to the model will encompass all reactions that occur.

The compound/s that remove MGO are in higher abundance in mānuka honey than in clover honey and a large decline of MGO at later times was not observed in clover honey matrices, but when the total concentration of phenolic acids was used for Z, varying results were observed with mānuka honeys. The simulation fitted the MGO experimental data reasonably well for sample 953, but too much MGO was gained for sample 946 and not enough for samples 66, 78 and 84 suggesting that the model needs refinement, or phenolic acids are not involved in the loss of MGO, or other compounds also influence the loss.

Compounds that remove MGO may be involved in Maillard-type reactions, hence compounds which have the presence of amine containing groups could be investigated. The protein content of honey samples has been reported to range from 0.058% to 0.786%, therefore MGO loss due to reaction with R groups of proteins in mānuka honey would occur at a slow rate and be minimal.[14] This suggests another compound is responsible for the removal of MGO, which may be a catalytic reaction to a further product.

The comparison of the simulated and experimental data for loss of DHA and gain of MGO can be seen in Figure 8.28 and Figure 8.29 respectively for sample 953. The simulated data for loss of DHA fits the experimental data well and the simulated MGO data fits the experimental data well at the beginning but is slightly too high at later times. The plot of MGO formation includes the error bars for this sample (0.32 mmol/kg as decided from the ILCP results) to show how close the simulation is. Error bars have been excluded from all other plots.

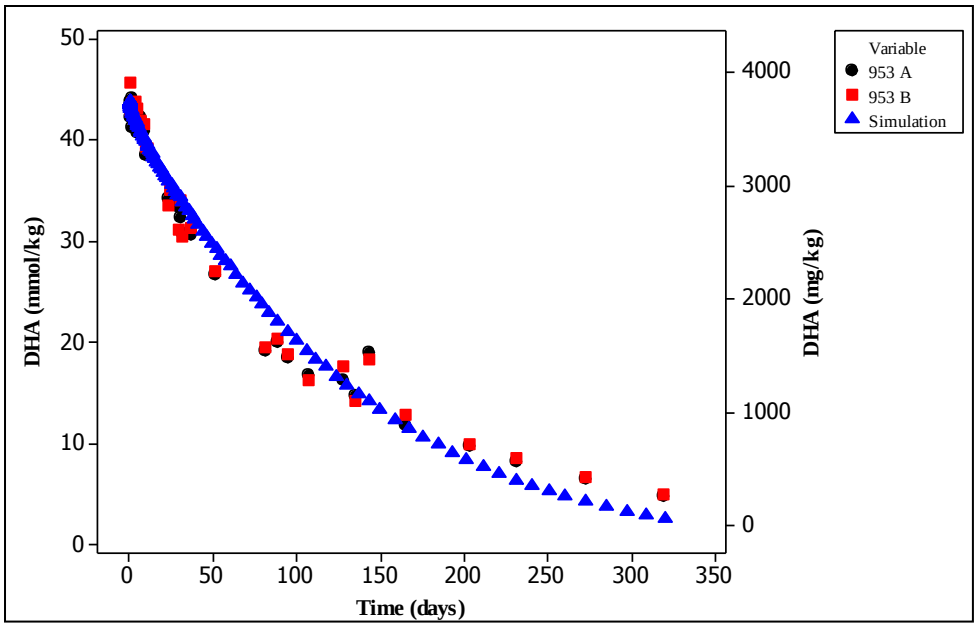


Figure 8.28 Experimental (circles and squares) and simulated (triangles) data for loss of DHA in mānuka honey 953. The simulated data fits the experimental data well.

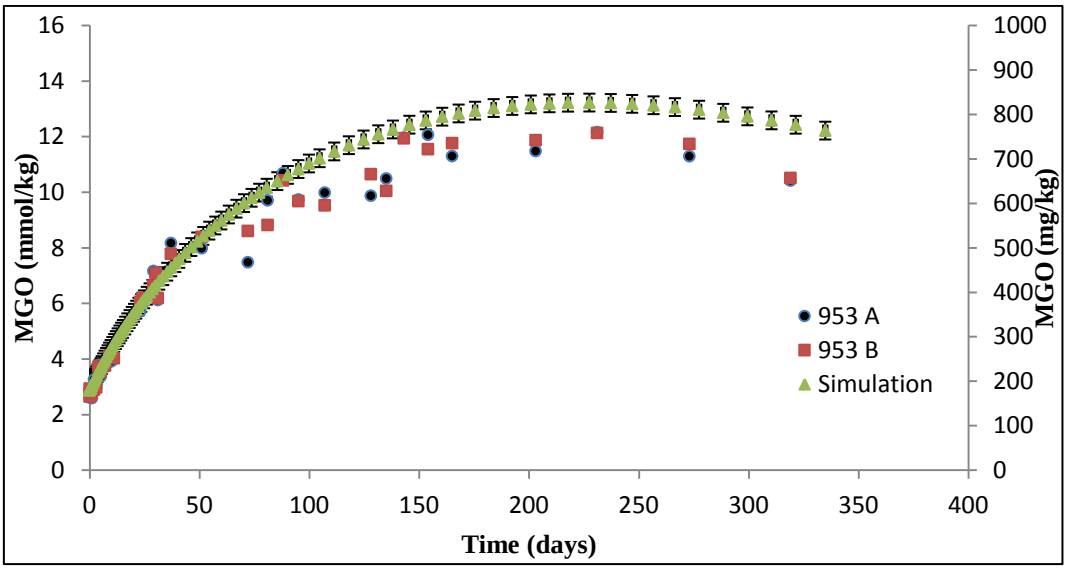


Figure 8.29 Experimental (circles and squares) and simulated (triangles) data for gain of MGO in mānuka honey 953. The error bars are set at ± 0.32 mmol/kg (119 mg/kg) as determined by the standard deviation in the 2013 ILCP. The simulated data fits the experimental data initially, however, slightly too much MGO is formed at later times in the simulated data.

The six mānuka honeys that were stored at 37 °C and were analysed for phenolic content were compared to the simulated data. The concentration of Z (perturbant that catalytically removes MGO) was chosen so that the best fit at later times was

achieved. This varied a lot between samples (1-20 mmol/kg). Further work is required to identify the compounds that influence the loss of MGO. A summary of the values used for each sample are shown in Table 8.4.

Table 8.4 Initial starting concentrations (mmol/kg) of total phenolic acids and Z.

	Total phenolic acids*	Z†
	mmol/kg	
946	9.63	20.00
953	6.36	6.36
66	6.01	3.00
78	3.70 (used 8)	5.50
84	6.93	3.00
1404	3.87	1.00‡

*Total phenolic acids is the sum of phenyllactic acid and 4-methoxyphenyllactic acid analysed from chapter 4.

†The compound/s responsible for Z is unknown and was chosen to give the best possible fit of MGO at later time periods.

‡Data was not collected for long enough in this sample to see a decline in MGO, hence it is unknown what curve this sample will follow.

Figure 8.30 shows the experimental vs. simulated data for DHA loss and MGO gain for samples 66, 78, 84 and 1404. The simulation fits the DHA experimental data well for all of the samples and, in most cases, increased the rate of MGO at early times in the reaction. However, sample 78 had a low concentration of total phenolic acids (3.70 mmol/kg) and not enough MGO was formed in the simulation (Z = 0); if a total of 8 mmol/kg total phenolic acids was used and if Z = 5.5, the simulation fitted the experimental data. This result also indicates there is a baseline effect in real honey matrices on the conversion of DHA to MGO that is unaccounted for in artificial honey. This may be due to organic acids, other than phenolic acids, but their effect is insignificant in mānuka honeys with high phenolic acid content. Hence the simulation works for mānuka honeys, but not for clover honey or mānuka honeys with low phenolic acid content. Therefore, future work could include adding organic acids to the simulation to see if these account for the difference; once this is added the rate constant for the reaction of DHA + phenolic acids would need to be reduced (because at the moment it is compensating for it).

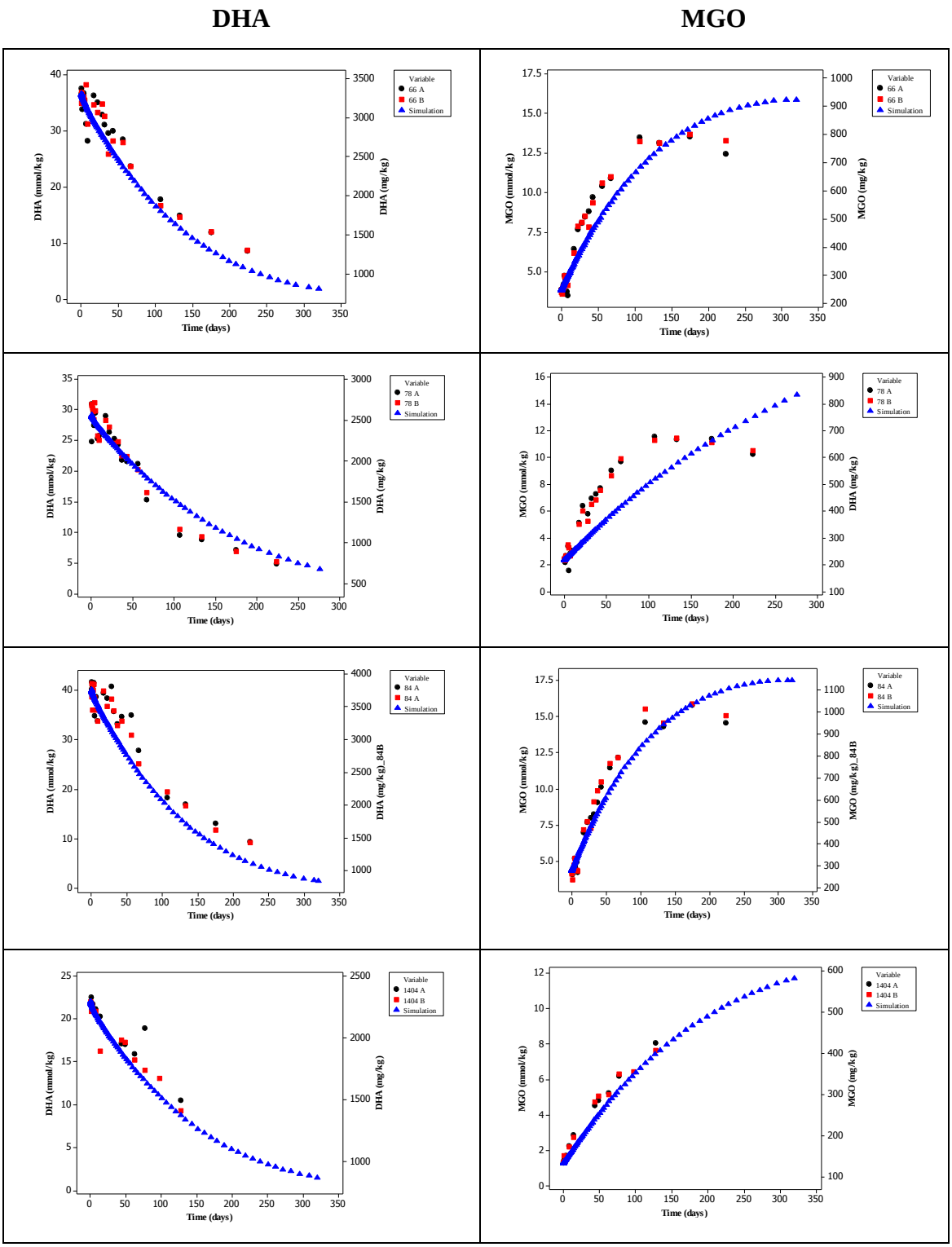


Figure 8.30 Experimental vs. simulated plots for DHA loss (left) and MGO gain (right) for samples 66, 78, 84 and 1404 (top to bottom). The simulation fits the experimental data well for most samples.

Sample 946 did not have a good fit for the loss of MGO (Figure 8.31). The total phenolic acids were much higher in this sample than other samples (9.63 mmol/kg), hence a lot of MGO was formed in the simulation and Z needed to be

large (20) to account for this. However, too much MGO was lost at later times. This sample has a high water content and fermented, which could be a cause of the poor fit.

The rate constant for the loss of MGO to a side product by the catalytic conversion of Z may need adjusting. However, until the identity and concentration of Z is known, it is not possible to incorporate a rate constant that will fit for all honey samples.

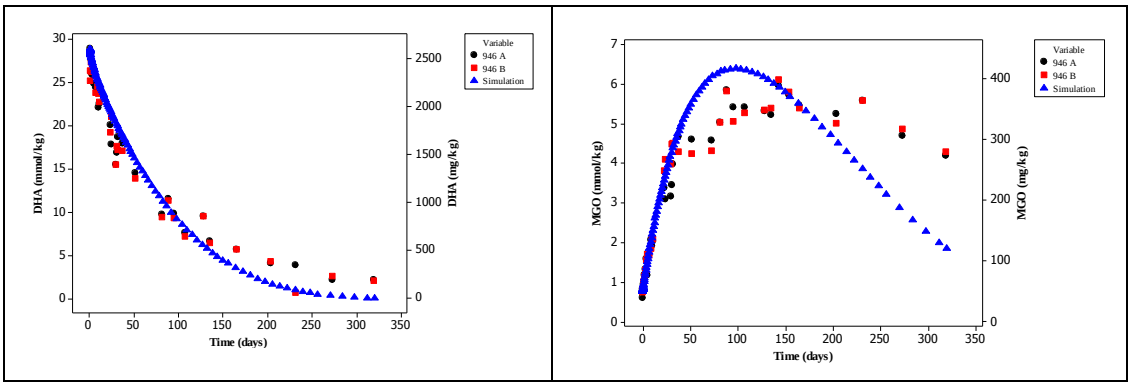


Figure 8.31 Experimental vs. simulated plots for DHA loss (left) and MGO gain (right) for sample 946. The simulation fits the DHA experimental data well, but does not fit the MGO data.

Four mānuka honey samples that were incubated at 37 °C and analysed 5 times over 320 days were also compared to the simulation. The initial concentrations are summarised in Table 8.5. The simulated data for each sample is shown in Figure 8.32. Total phenolic acids were estimated for samples 14 and 32 based on samples 25, 41 and 84 which were harvested from the same region.

The data fits relatively well at early times, but the MGO data deviates slightly at later times once the MGO begins to decline, which was also observed in the above samples (Figure 8.30). Further work is required to identify compounds that are affecting the loss of MGO so that the deviation in the MGO plot can be correctly simulated.

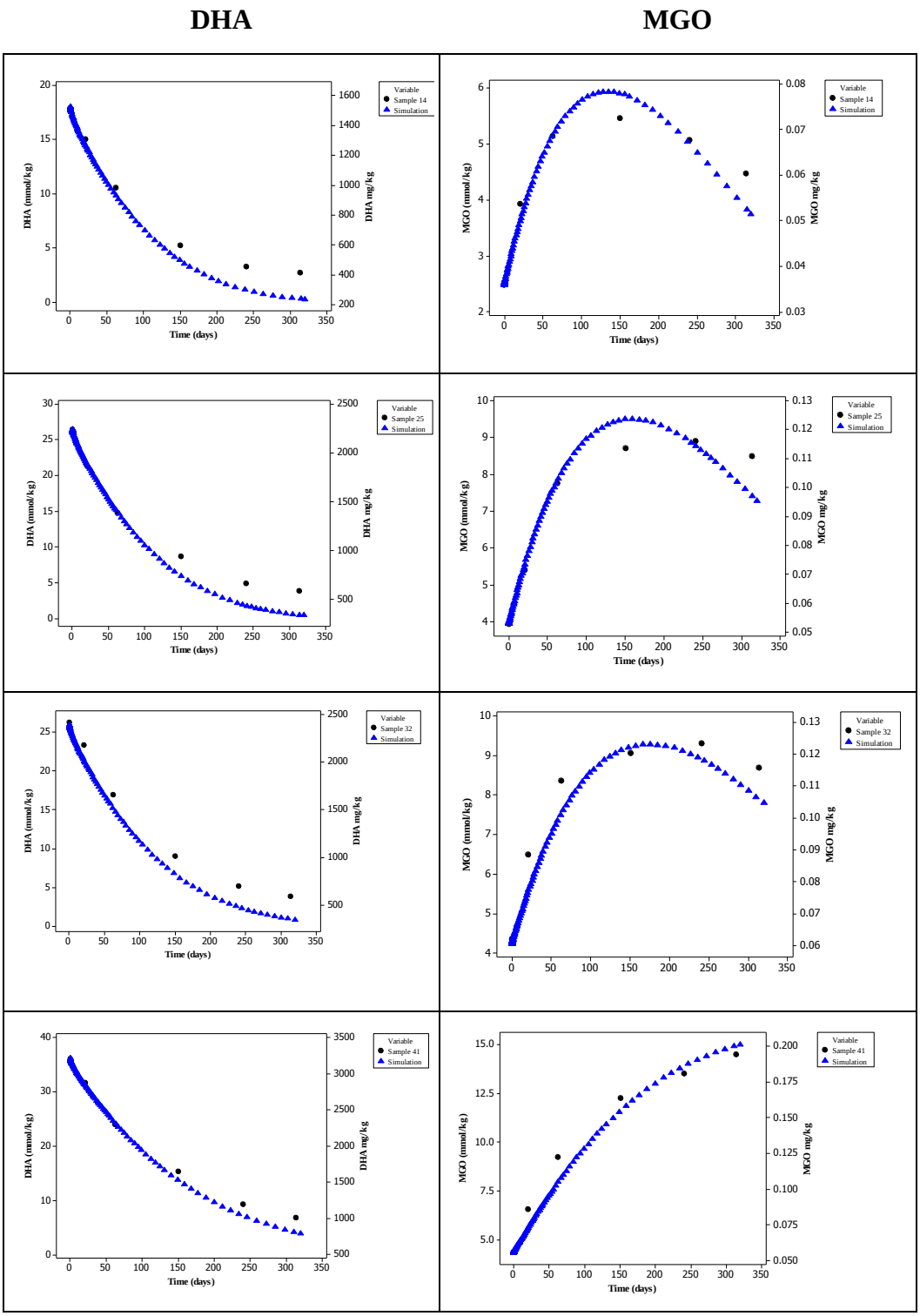


Figure 8.32 Simulated (triangles) and experimental (circles) data for mānuka honey samples 14, 25, 32 and 41 (top to bottom) stored at 37 °C. The data fits well at early times, but deviates slightly at later times due to the unknown compound influencing the loss of MGO.

Table 8.5 Initial starting concentrations (mmol/kg) entered into the simulation for four mānuka honeys.

	14	25	32	41
	mmol/kg			
Dimeric DHA	6.71	9.89	9.68	13.5
Monomeric DHA	4.48	6.60	6.57	9.00
MGO	2.48	3.94	4.25	4.38
Proline	3.50*	3.30	3.81	3.21
PAA*	1	1	1	1
Total phenolic acids	6†	6.96	6†	3.98
Z	10	6	7	5

* These values were estimated as they were not analysed.

†These values were estimated based on samples from similar regions.

8.4 Variations to the proposed model – influence of species

To investigate the influence of individual perturbants on the simulation of DHA and MGO, the concentration was changed while all other perturbants were held at a set concentration. This gave an insight into the robustness of the model and indicated how much of a change will occur if parameters are adjusted. If there is not a lot of variation, initial concentrations of some perturbants could be used for a wide range of samples so that compounds (for example, total primary amino acids) do not have to be tested for every sample that is to be run in the prediction tool.

It needs to be recognised that the system is complex, hence when concentrations are varied they will have an effect on other compounds; for example, two perturbants may compete for DHA, which may lead to different products. Table 8.6 summarised the starting concentrations for the following tests – only one perturbant was altered at a time.

Table 8.6 Initial starting concentrations of species used to assess the influence of each species.

Compound	Initial starting concentration (mmol/kg)
Monomeric DHA	6.1
Dimeric DHA	9.15
MGO	0.8
Proline	7.03
Alanine	1
Phenolic acids	0
Z	0

Alanine (primary amino acids)

The effect that alanine (representing all primary amino acids) had on the simulation was examined. Table 8.6 summarises the starting concentrations of each species. Samples analysed for primary amino acids during this research contained between 0.62 and 1.98 mmol/kg total primary amino acids, hence changes in the alanine concentration in the simulation was varied to reflect this (0, 0.25, 0.5, 1, 1.5, 2 and 3 mmol/kg). There was no substantial change in the loss of DHA. The gain of MGO was affected by the concentration of alanine (Figure 8.33); when no alanine is present in the system the maximum for MGO is reached early due to DHA converting to the 'aldol' side product. When alanine is present in the system it provides a pathway for the aldol to convert back to two DHA monomers and then on to MGO. Hence, the more alanine in the sample, the more MGO that is formed. However, the simulation is trending towards saturation when the concentration of total primary acids are high because only a certain amount of aldol is formed. The plot of MGO vs. time shows that there is a difference in the concentrations of MGO within this variation of alanine.

Total phenolics (6 mmol/kg) and Z (5 mmol/kg), at levels similar to that found in real honey, were included in a second simulation experiment for varying concentrations of alanine. There is no difference in the initial formation of MGO, but the maximum concentration is influenced by the amount of alanine present. The MGO vs. time plot (Figure 8.34) shows that with less alanine, the concentration of MGO begins to decline sooner. For example, if alanine is set to 0 mmol/kg, the maximum MGO is just under 7 mmol/kg (just under NPA 15), compared to the simulation with 1 mmol/kg alanine which has a maximum MGO concentration of 8 (just over NPA 15). This difference is approximately 144 mg/kg MGO and is just larger than the upper limit of the standard deviation

reported in the 2013 ILCP (24-119 mg/kg) which is used to estimate how well the simulation fits with the experimental data.

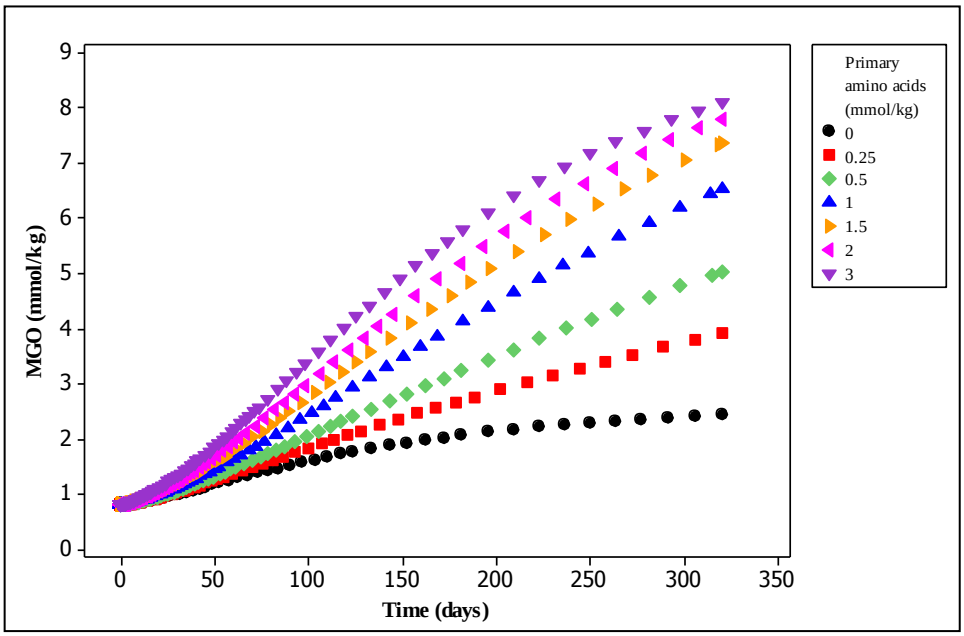


Figure 8.33 Simulated data for gain of MGO with various amounts of alanine. The maximum amount of MGO produced increased with increasing concentration of alanine (total phenolics and Z set to 0 mmol/kg).

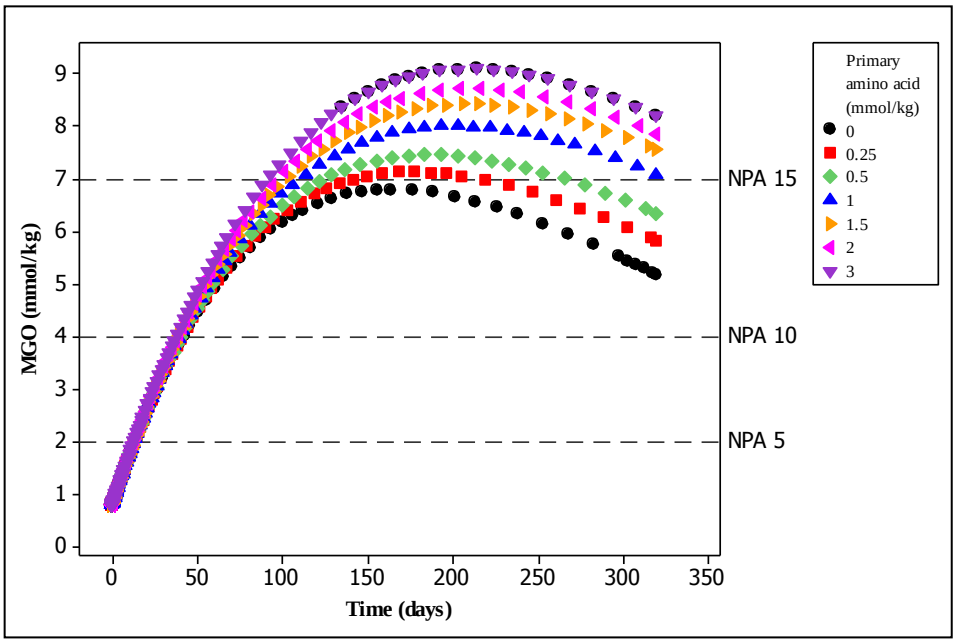


Figure 8.34 Simulated data for gain of MGO with various amounts of primary amino acid. The maximum amount of MGO produced increased with increasing concentration of alanine (total phenolics = 6 and Z = 5 mmol/kg).

Proline

Table 8.6 summarises the starting concentrations of each species. Proline is found at higher concentrations in honey than primary amino acids are. The 6 honeys used in the storage trial had a range from 3.23 to 7.03 mmol/kg proline, hence values that encompassed this range were compared. As with the simulation with various alanine concentrations, altering proline did not cause a change in the loss of DHA, but the higher the proline concentration, the lower the maximum of MGO formed. (Figure 8.35). Real honey samples are likely to have 4 mmol/kg (460 mg/kg) proline or more. Varying the proline concentration from 4 to 8 mmol/kg (460-920 mg/kg), which is within the variation seen in real honey samples, has a large effect on the maximum of MGO (approximately 4 mmol/kg MGO; this is approximately 288 mg/kg which is over double the standard deviation of measurement in the 2013 ILCP. Hence the initial concentration of proline in honey has a large influence on the final MGO concentration.

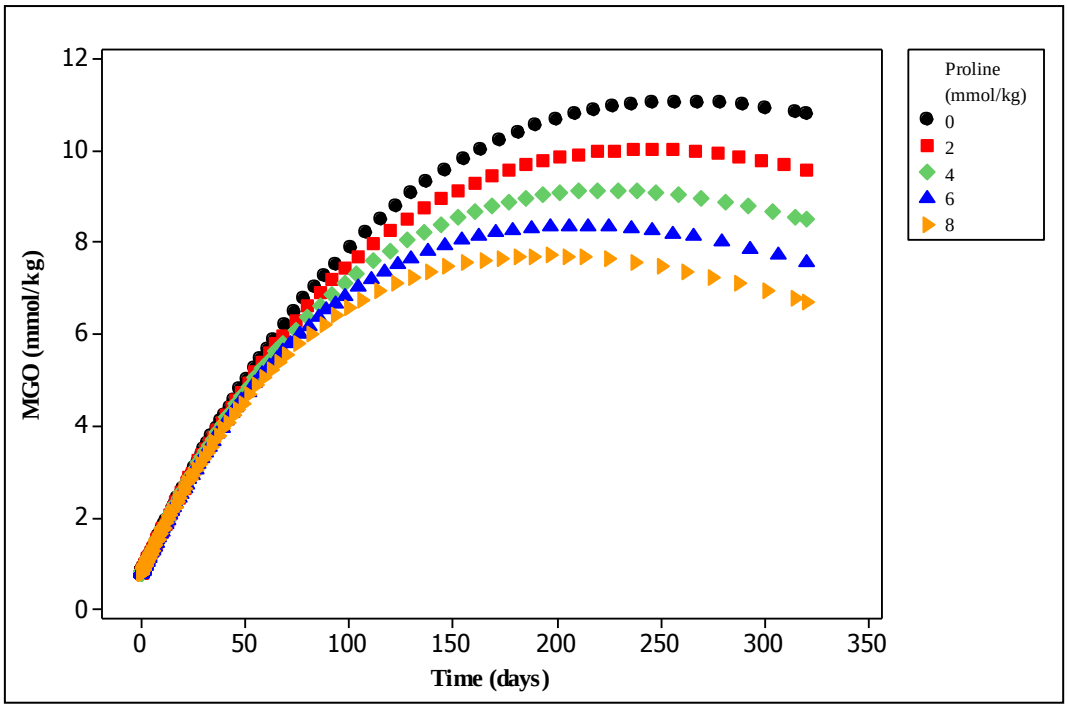


Figure 8.35 Simulated gain of MGO with various starting concentrations of proline (0-920 mg/kg). The simulation included total phenolic acids and Z (6 and 5 mmol/kg respectively).

Total phenolic acids

Table 8.6 summarises the starting concentrations of each species. The concentration of total phenolic acids is important because it influences both DHA and MGO; as the total phenolic acid concentration was increased, more DHA was consumed (Figure 8.36) and more MGO formed (Figure 8.37), showing the important influence of phenolic acids on the conversion. At 200 days, a sample with 0 mmol/kg phenolic acids reached 4 mmol/kg MGO (NPA 10) compared to a sample with 5 mmol/kg phenolic acids which has reached 11 mmol/kg (NPA 20). This is a difference of approximately 181 mg/kg, which is larger than the upper limit of the standard deviation of the 2013 ILCP results, indicating that phenolic acids have a significant effect on the MGO concentration.

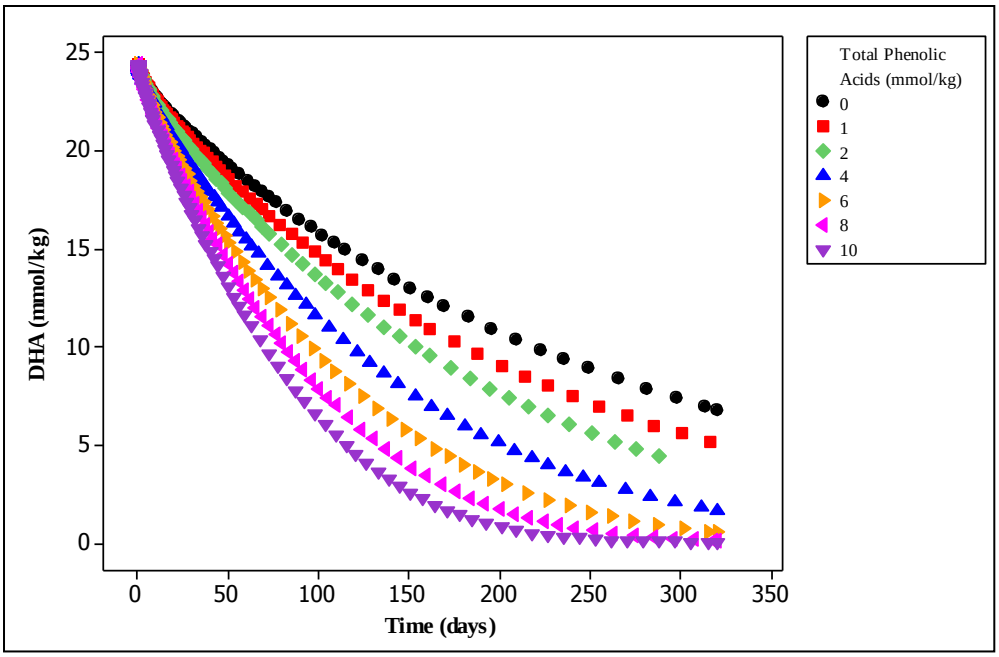


Figure 8.36 Simulated data for loss of DHA with various amounts of total phenolic acids. The higher the concentration of total phenolic acids, the greater the amount of DHA that was consumed.

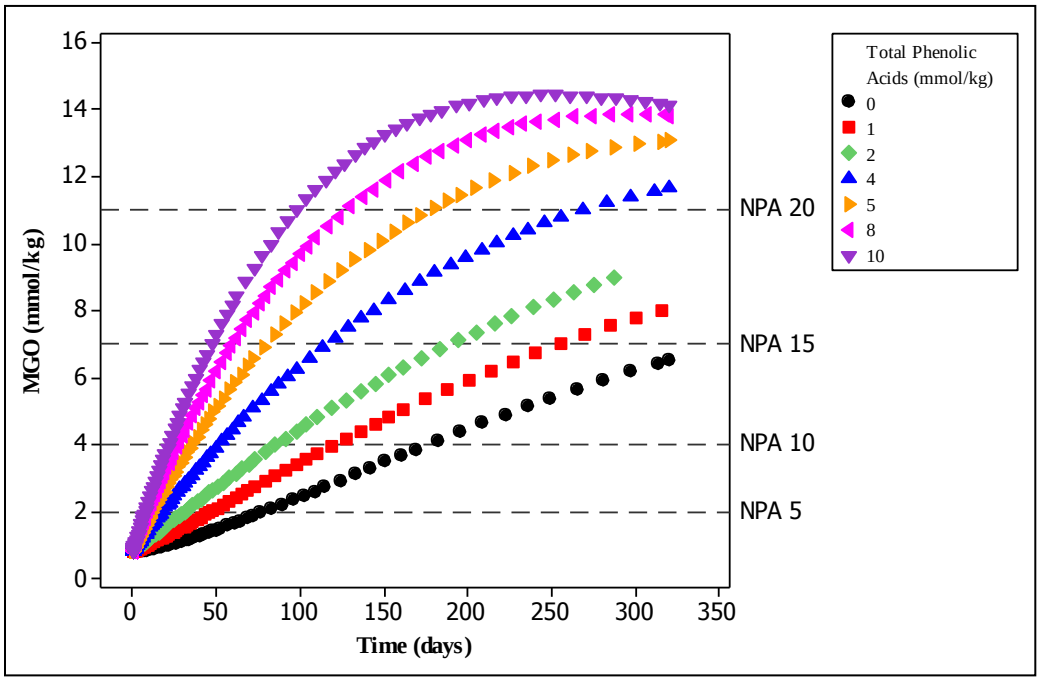


Figure 8.37 Simulated data for the gain of MGO with various amounts of total phenolic acids. The higher the concentration of total phenolic acids, the greater the amount of MGO that was formed, indicating that their concentration is important to the formation of MGO.

Compound Z

Table 8.6 summarises the starting concentrations of each species; total phenolics (6 mmol/kg) were held the same for all trials. There was no effect on the loss of DHA because Z only affects the removal of MGO. The chosen value of Z affected the shape of the MGO curve (Figure 8.38); when Z was set to 0, the MGO curve did not have a large deviation at later times and after 200 days the honey reached NPA 20 (11 mmol/kg MGO) compared to a samples with 4 mmol/kg Z, which only reached NPA 15 (8 mmol/kg MGO) and a sample with 10 mmol/kg Z, which was assigned as NPA 10 (6 mmol/kg MGO). These results show the importance of finding the compound/s that are responsible for Z. This will allow the simulation to be strengthened and to predict the maximum MGO that can be formed with more certainty.

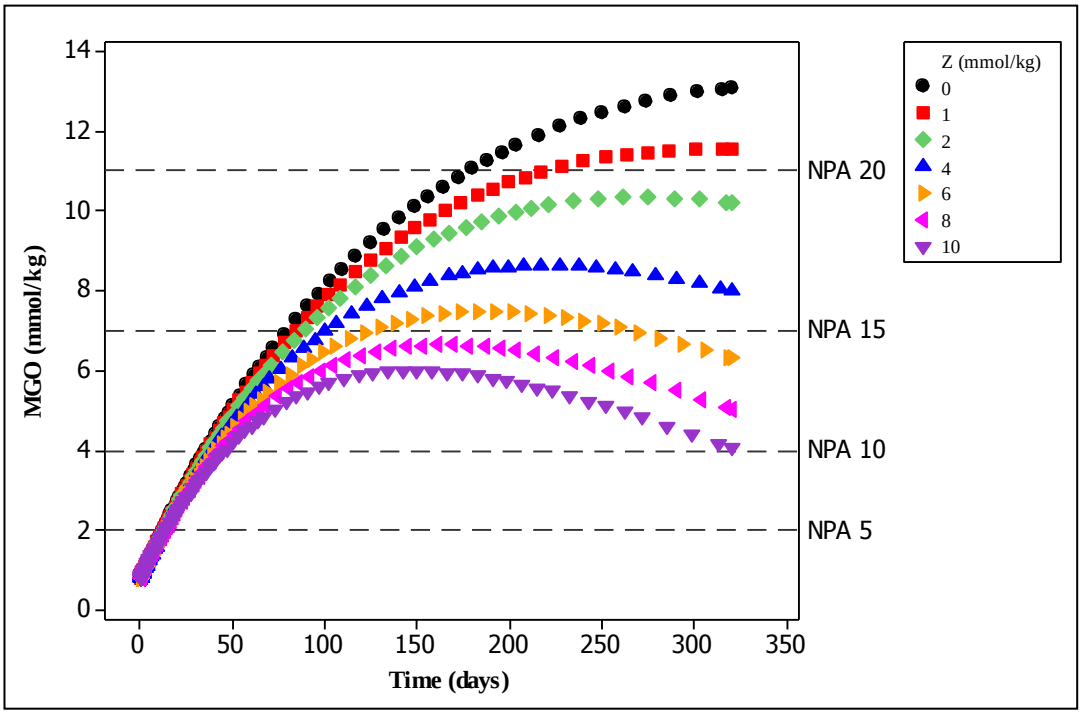


Figure 8.38 Simulated data for the gain of MGO with various amounts of Z. Z influences the shape of the curve; the higher the concentration of Z, the more MGO that was consumed, indicating that its concentration is important to the maximum formation of MGO.

8.5 Simulation of conversion of DHA to MGO in model systems at 27 °C

As discussed throughout this research, the conversion of DHA to MGO depends on the temperature. Samples stored at 27 °C convert DHA to MGO more slowly

than samples at 37 °C. Furthermore, side reactions appear to be lessened at lower temperatures.

For this simulation, the reactions that have been identified to occur in the honey matrix at 37 °C in Model 3 will be the same as those occurring at the lower temperature. However, the rate constants need to be adjusted in order to simulate the data at the lower temperature because the reactions will be slower. A similar approach that was used for the 37 °C model was used here – initially the artificial honey systems were used to suggest the correct rate constants. The simulation was fitted to the control sample experimental data, then to the DHA-alanine and DHA-proline systems before adding both perturbants together. The chosen rate constants for Model 3 for samples stored at 27 °C are summarised in Table 8.7. Plots of DHA vs. time are not shown due to the complication in reporting the DHA experimental data for this set of experiments as discussed in section 5.3.6.

Table 8.7 Summary of chemical equations and the estimated rate parameters for Model 3 for simulation of the conversion of DHA to MGO in artificial honey stored at 27 °C.

Chemical equations	Model	Rate constants for 27 °C (day^{-1})	Rate constants for 37 °C (day^{-1})	Factor reduced by
Dimer → 2 DHA	Control	0.0100	0.0500	5
2 DHA → Dimer	Control	1.6000	8.0000	5
DHA → Enediol	Control	0.0300	0.0550	1.8
Enediol →DHA	Control	0.0090	0.0090	1
Enediol → Enolic	Control	0.0400	0.0400	1
Enolic → MGO	Control	0.5000	0.5000	1
2 DHA → Aldol	Control	0.0900	0.4500	5
DHA + MGO → DHA-MGO product	Control	0.0012	0.0060	5
DHA-MGO product → DHA + MGO	Control	0.0012	0.0060	5
DHA-MGO product → Dead end DHA-MGO product	Control	0.0002	0.0010	5
Aldol + Alanine → 2 MGO + Alanine	Alanine	0.0200	0.0200	1
DHA + Alanine → Imine	Alanine	0.0200	0.0200	1
Imine → DHA + Alanine	Alanine	0.0300	0.0300	1
Imine → Maillard-like product	Alanine	0.0010	0.0010	1
Alanine + Compound A → Alanine-A product	Alanine	0.0020	0.0020	1
DHA + Proline → DHA-Proline product	Proline	0.0020	0.0100	5
DHA-Proline product → DHA + Proline	Proline	0.0020	0.0100	5
DHA + Proline → MGO + Proline	Proline	0.0006	0.0030	5
MGO + Proline → MGO-proline product	Proline	0.0001	0.0003	3
MGO-Proline product → MGO + Proline	Proline	0.0001	0.0003	3
MGO + Proline → MGO side product + Proline	Proline	0.0001	0.0003	3

Control

It is assumed that if the temperature is lowered, the dissociation of the dimer to DHA will be slower compared to 37 °C. As a starting point, all rate constants required for the control reaction were reduced by a factor of 5; however, the simulation was much too slow compared to the experimental data. It is proposed that the rate constants for conversion of DHA to MGO via the enediol and enolic form of MGO will not be altered because once the reaction starts this set of steps will readily occur. The rate constants for the conversion of dimer to monomer and the reverse reaction were reduced by a factor of 5 compared to the 37 °C rate constants. The rate constant for DHA to enediol was reduced from 0.055 to 0.03 (factor of 1.8) and the rate constants for the remaining steps to form MGO were not changed. This new set of rate constants mirrored the control system well for gain of MGO (Figure 8.39).

Perturbation by alanine

The new rate constants for the control system together with the rate constants for equations involving alanine at 37 °C were used to compare to the DHA-alanine system stored at 27 °C to investigate how different the simulated data was from the experimental data. The experimental data matched the gain of MGO well (Figure 8.40), indicating that reactions with alanine are not influenced by the change of temperature.

Perturbation by proline

At 27 °C, the system perturbed by proline did not show an anomaly in the experimental loss of DHA over time compared to that observed when stored at 37 °C. Initially the rate constants used for the 37 °C model for equations involving proline were used together with the rate constants for the control data at 27 °C to investigate how different the simulated data was from the experimental data. The loss of DHA and gain of MGO were both slightly too large. In addition, the simulated MGO concentration deviated more than the experimental data (Figure 8.41). This suggests that at a lower temperature the effect of proline on side reactions is not as large. This finding is in line with the observation that honey stored at a lower temperature has a better efficiency in the DHA to MGO conversion than honey which is stored at a higher temperature. At higher temperature, side reactions involving proline divert DHA from the formation of

MGO, but are reduced at lower temperature. Furthermore, as noted above, the rate constants for reactions with alanine were not changed, hence the conversion of DHA to MGO is greater than its conversion to side products.

The rate constants for proline used in the 37 °C simulation were reduced by a factor of 5; however, reduction by a factor of 3 gave the same result because the values are very small. Using these rate constants there was a good fit for the gain of MGO (Figure 8.42).

Perturbation by proline and alanine

The rate constants chosen for the control system and systems perturbed by alanine and proline were added together to compare to the DHA-proline-alanine system. The predicted values matched the experimental data well (Figure 8.43).

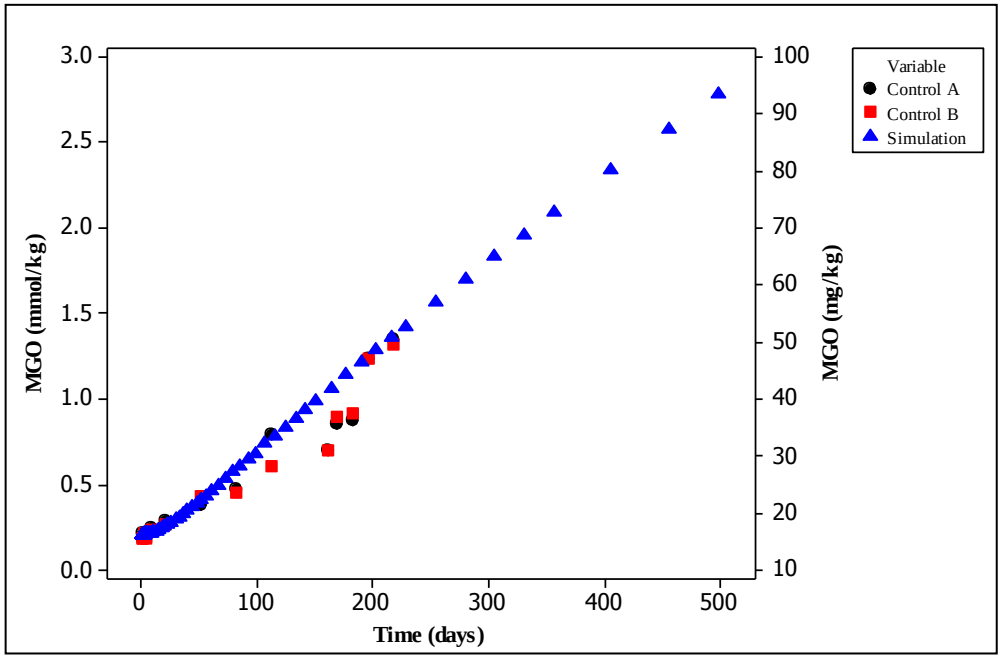


Figure 8.39 Experimental (circles and squares) and simulated (triangles) data for gain of MGO in the control experiment stored at 27 °C (2,000 mg/kg DHA added). The simulated data fits the experimental data well.

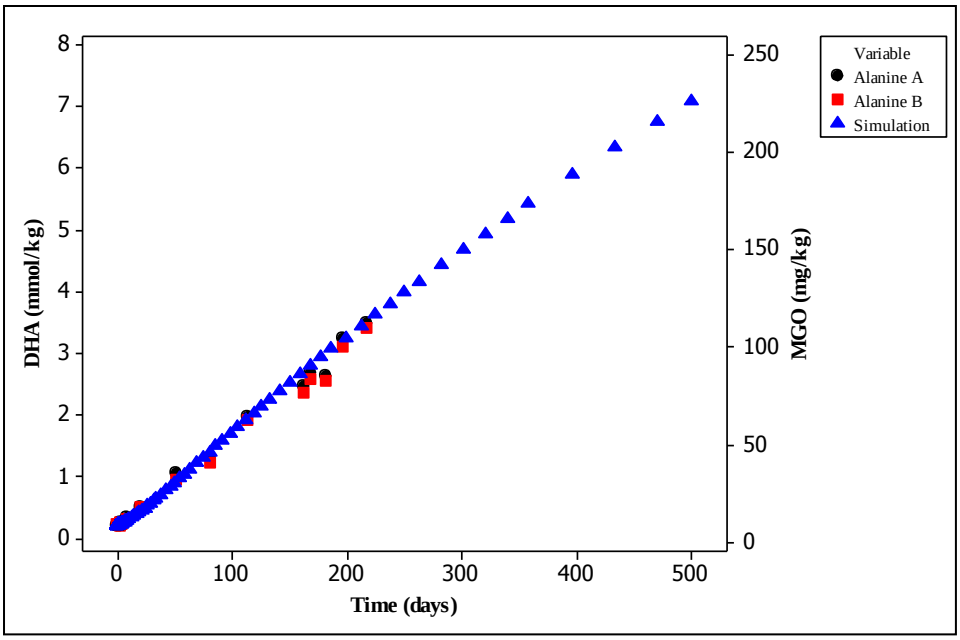


Figure 8.40 Experimental (circles and squares) and simulated (triangles) data for gain of MGO in the system perturbed by alanine and stored at 27 °C (2,000 mg/kg DHA added). The simulated data fits the experimental data well.

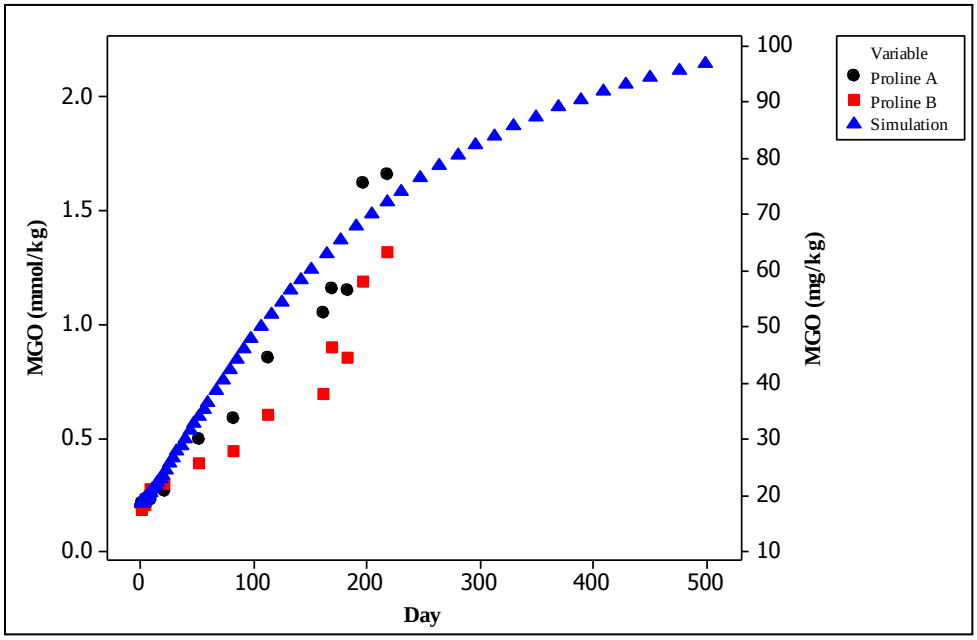


Figure 8.41 Experimental (circles and squares) and simulated (triangles) data for gain of MGO in the system perturbed by proline and stored at 27 °C (2,000 mg/kg DHA added). The simulation was run with rate constants chosen for the control system stored at 27 °C and rate constants for the proline equations for samples stored at 37 °C. Too much MGO was gained initially. Not enough experimental data was collected to observe if the MGO concentration deviated in the same manner as the simulated data.

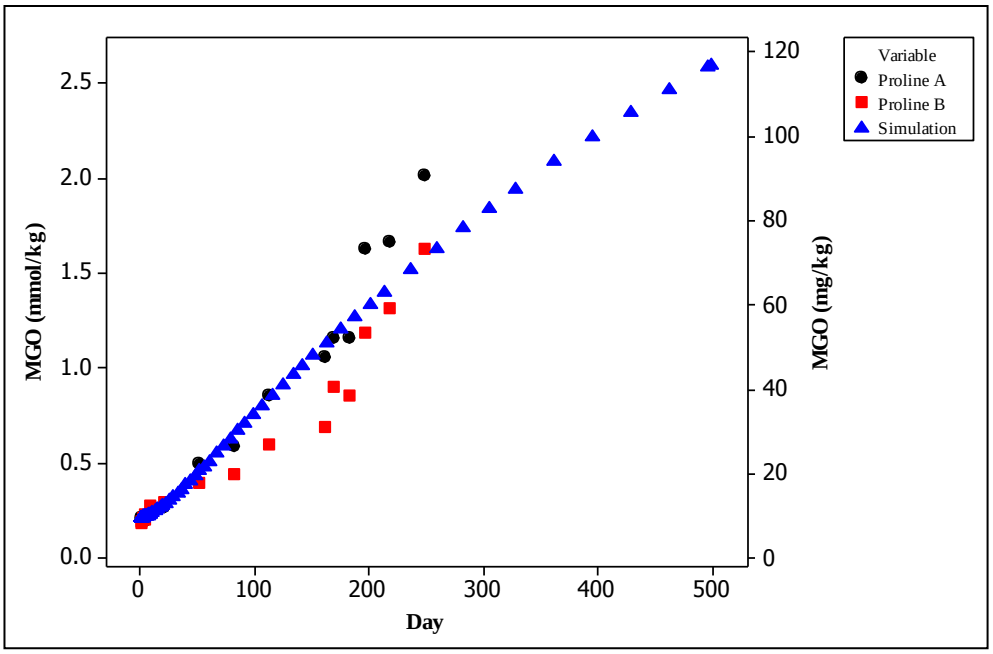


Figure 8.42 Experimental (circles and squares) and simulated (triangles) data for gain of MGO in the system perturbed by proline and stored at 27 °C (2,000 mg/kg DHA added). The simulation fits the experimental data well.

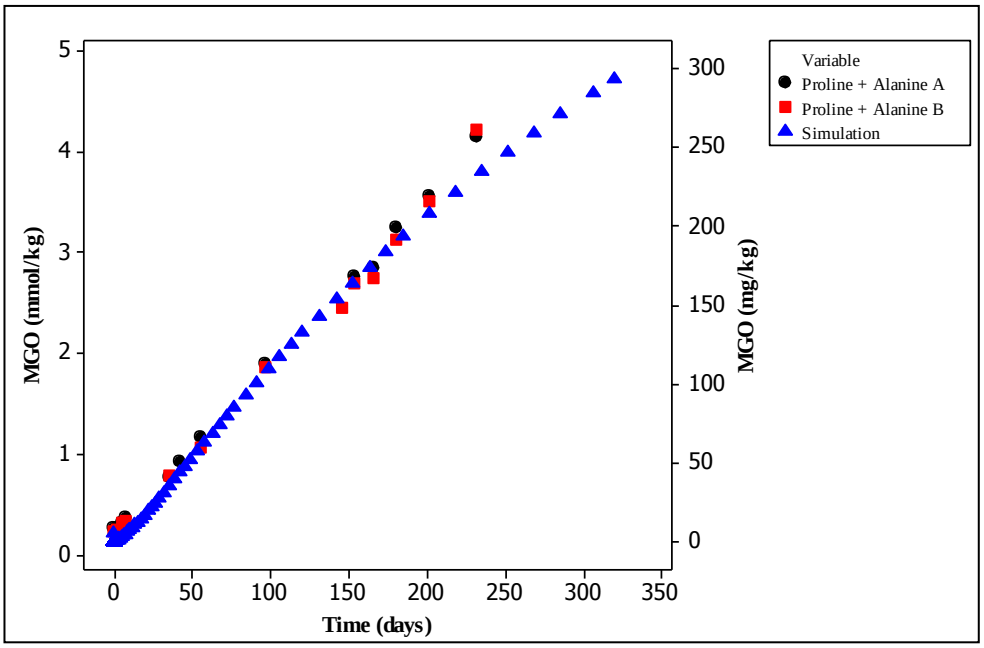


Figure 8.43 Experimental (circles and squares) and simulated (triangles) data for gain of MGO in the system perturbed by proline and alanine that was stored at 27 °C (2,000 mg/kg DHA added). The simulation fits the experimental data well.

8.6 Simulation of real honey at 27 °C

Rate constants used for the control sample and for equations involving alanine and proline were used as the start of the model for simulation of the conversion of DHA to MGO in real honey. As described in section 8.3 the concentration of total phenolic acids and also Z were required in the model to simulate the reaction in real honey. Rate constants for these equations were initially reduced by a factor of 5 as this was used for some rate constants in the model systems; however not enough MGO was formed. Therefore the rate equation for the reaction between DHA and the total phenolic acids was only reduced by a factor of three, which allowed the data to fit. The chosen rate constants are summarised in Table 8.8.

Table 8.8 Summary of rate constants that are required to simulate the conversion of DHA and MGO in real honey at 27 °C.

Chemical equation	Rate constant for 27 °C (day^{-1})	Rate constant for 37 °C (day^{-1})	Factor reduced by
DHA + total phenolic acids → MGO + total phenolic acids	0.0200	0.0600	3
Total phenolic acids → Y	0.0002	0.0010	5
MGO + Z → MGO-side product + Z	0.00014	0.0007	5
Z → W	0.00002	0.0001	5

Figure 8.44 shows the gain of MGO for sample 1404 stored at 27 °C (this sample was affected by the DHA measurement error). The simulated data fits the experimental data well. Sample 1404 was not analysed past the point where the MGO reaches a maximum and begins to decline, hence the rate constant chosen for Z may not reflect what will occur at later times. Four mānuka honeys were stored at 27 °C and analysed five times over 320 days. These samples were also stored at 37 °C and were discussed in section 8.3. The simulated DHA and MGO concentrations matched the experimental data well for all four samples, indicating that the chosen rate constants are appropriate. Plots for DHA and MGO for the simulation and experimental data for mānuka honeys can be seen in Figure 8.45.

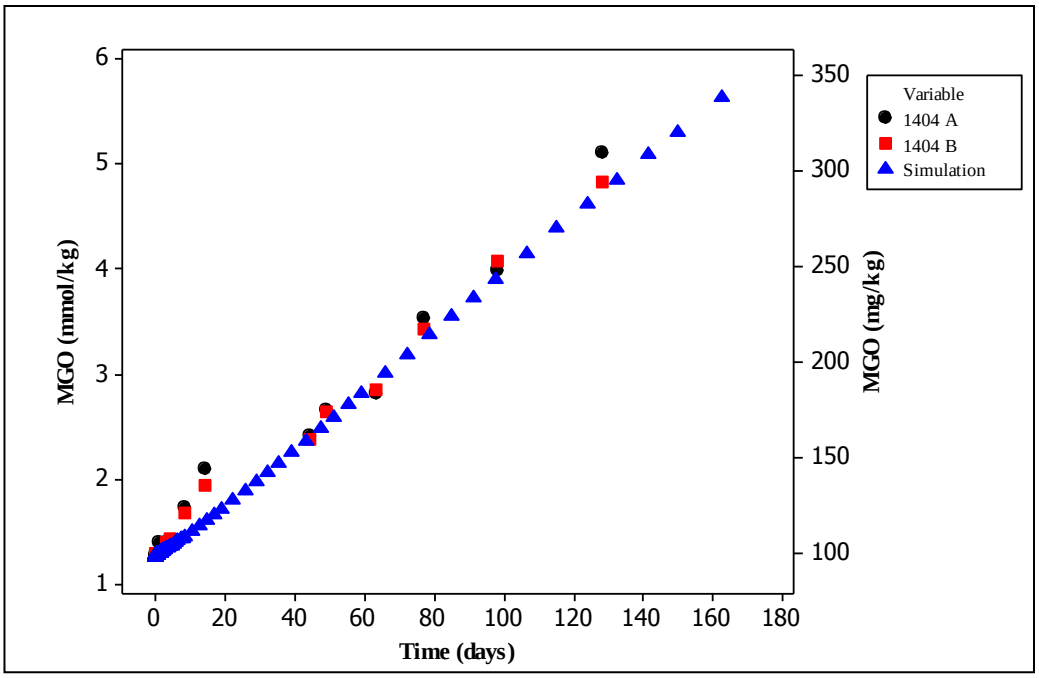


Figure 8.44 Experimental (circles and squares) and simulated (triangles) data for MGO gain in sample 1404 stored at 27 °C (2,000 mg/kg DHA added). The simulated data fits the experimental data well.

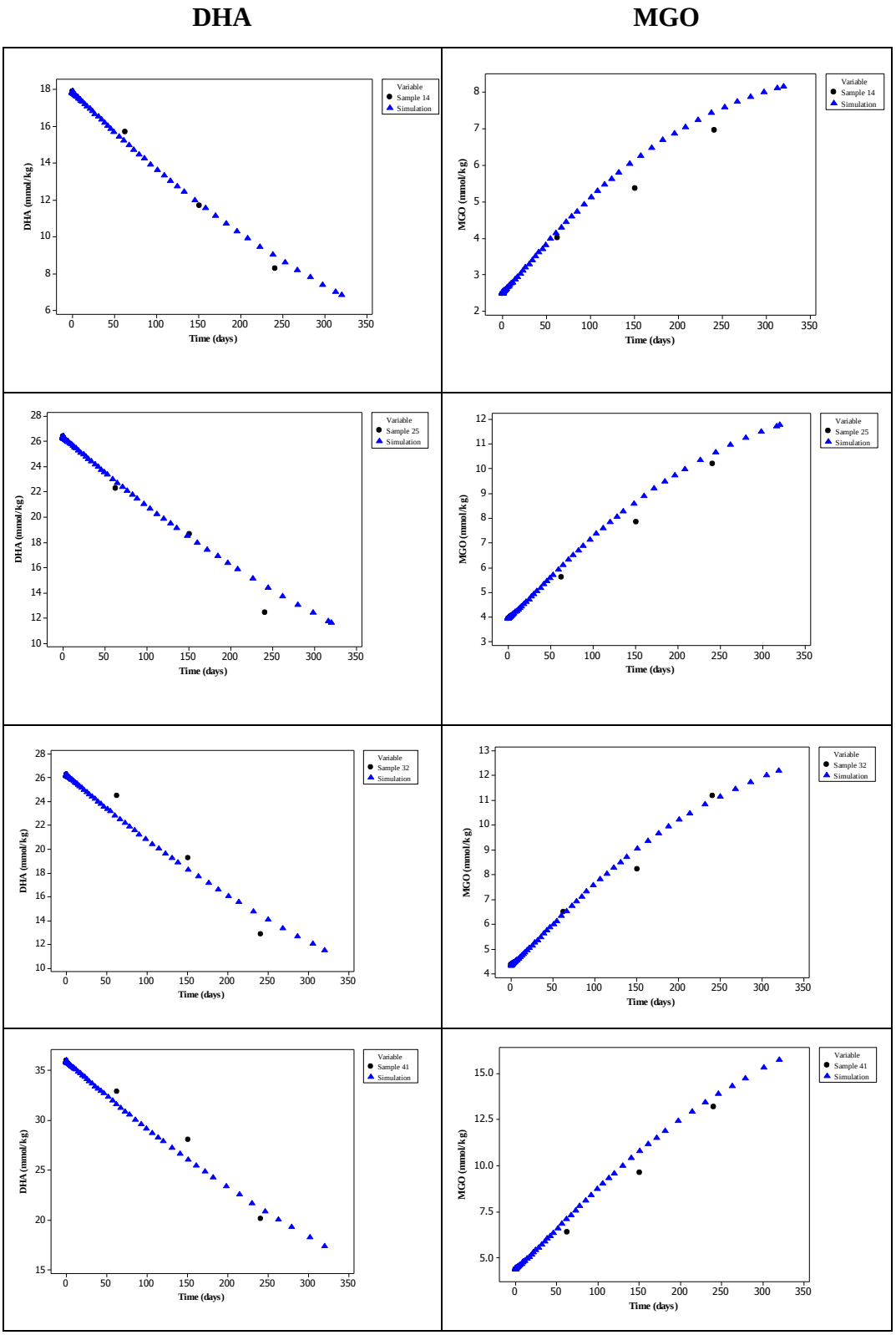


Figure 8.45 Simulated (triangles) and experimental (circles) data for mānuka honey samples 14, 25, 32 and 41 (top to bottom) stored at 27 °C. The simulation fits the experimental data well for both DHA and MGO.

8.7 Simulation of conversion of DHA to MGO in model systems and real honey at 20 °C

Artificial honeys systems were also stored at 20 °C, allowing the simulation to be tested at the lower temperature. Table 8.9 summarises the rate constants for each reaction. This set of samples were also affected by the measurement error in DHA, hence DHA vs. time plots are not shown.

Control

The control sample had the same rate constants for the dimer/monomer equations as the simulation at 27 °C, but the rate constants for the conversion of DHA to enediol and also the side reactions of DHA were reduced further so that the simulation fitted the experimental data. The simulated MGO fits well (Figure 8.46).

Proline

For the simulation of the sample perturbed by proline, the same rate constants used for 27 °C also worked for the 20 °C data. This was a factor of 2 reduction for DHA reaction with proline and a factor of 3 reduction for the reaction of MGO and proline compared to the 37 °C data.

Alanine

The system perturbed by alanine 27 °C was used to suggest the correct rate constants for the equations involving alanine. The rate equations for the reaction of alanine with the aldol and also the forward and reverse reactions of alanine with DHA were reduced by a factor of 5 compared to the 37 °C rate constants. It was essential that the reaction of alanine with the aldol was reduced by a factor of 5; however the other rate constants were so small that there was not a large difference in a 3-fold reduction. The MGO simulation fitted the experimental data well (Figure 8.48).

Proline + Alanine

The rate equations chosen for systems perturbed individually by alanine and proline were used in the model to simulation perturbation of the system by both compounds. This fitted the experimental data well for MGO (Figure 8.49).

Table 8.9 Summary of chemical equations and the estimated rate parameters for Model 3 for simulation of the conversion of DHA to MGO in artificial honey stored at 27 °C.

Chemical equations	Model	Rate constants for 20 °C (day^{-1})	Rate constants for 37 °C (day^{-1})	Factor reduced by
Dimer → 2 DHA	Control	0.0100	0.0500	5
2 DHA → Dimer	Control	1.6000	8.0000	5
DHA → Enediol	Control	0.0200	0.0550	2.75
Enediol →DHA	Control	0.0090	0.0090	1
Enediol → Enolic	Control	0.0400	0.0400	1
Enolic → MGO	Control	0.5000	0.5000	1
2 DHA → Aldol	Control	0.0900	0.4500	5
DHA + MGO → DHA-MGO product	Control	0.0010	0.0060	6
DHA-MGO product → DHA + MGO	Control	0.0010	0.0060	6
DHA-MGO product → Dead end DHA-MGO product	Control	0.0002	0.0010	5
Aldol + Alanine → 2 MGO + Alanine	Alanine	0.0040	0.0200	5
DHA + Alanine → Imine	Alanine	0.0050	0.0200	4
Imine → DHA + Alanine	Alanine	0.0150	0.0300	2
Imine → Maillard-like product	Alanine	0.0010	0.0010	1
Alanine + Compound A → Alanine-A product	Alanine	0.0010	0.0020	2
DHA + Proline → DHA-Proline product	Proline	0.0020	0.0100	5
DHA-Proline product → DHA + Proline	Proline	0.0020	0.0100	5
DHA + Proline → MGO + Proline	Proline	0.0006	0.0030	5
MGO + Proline → MGO-proline product	Proline	0.0001	0.0003	3
MGO-Proline product → MGO + Proline	Proline	0.0001	0.0003	3
MGO + Proline → MGO side product + Proline	Proline	0.0001	0.0003	3

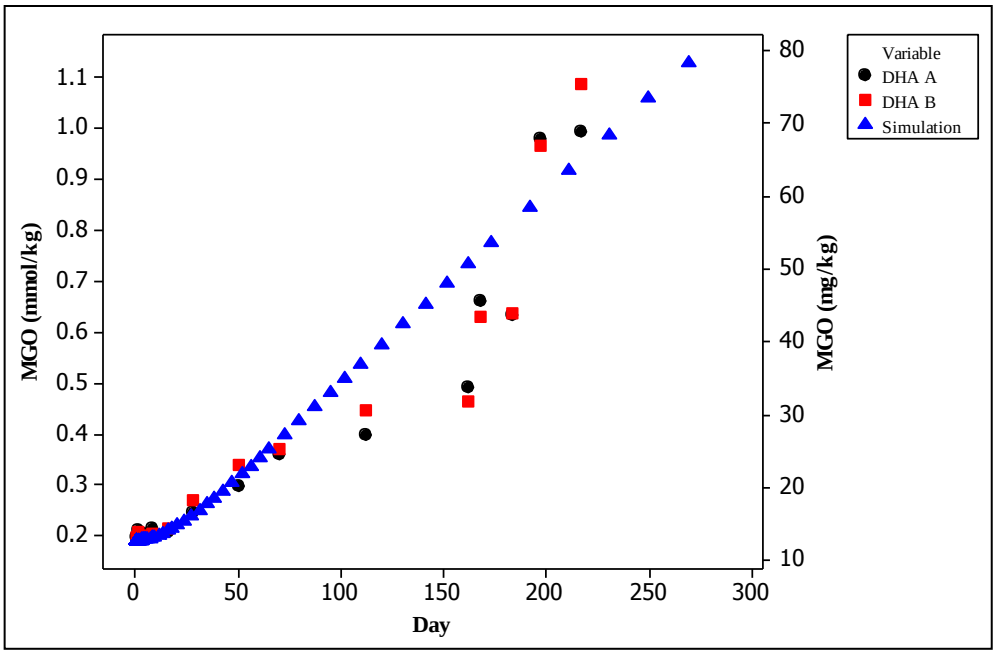


Figure 8.46 Experimental (circles and squares) and simulated (triangles) data for gain of MGO in the control experiment stored at 20 °C (2,000 mg/kg DHA added). The simulated data fits the experimental data well.

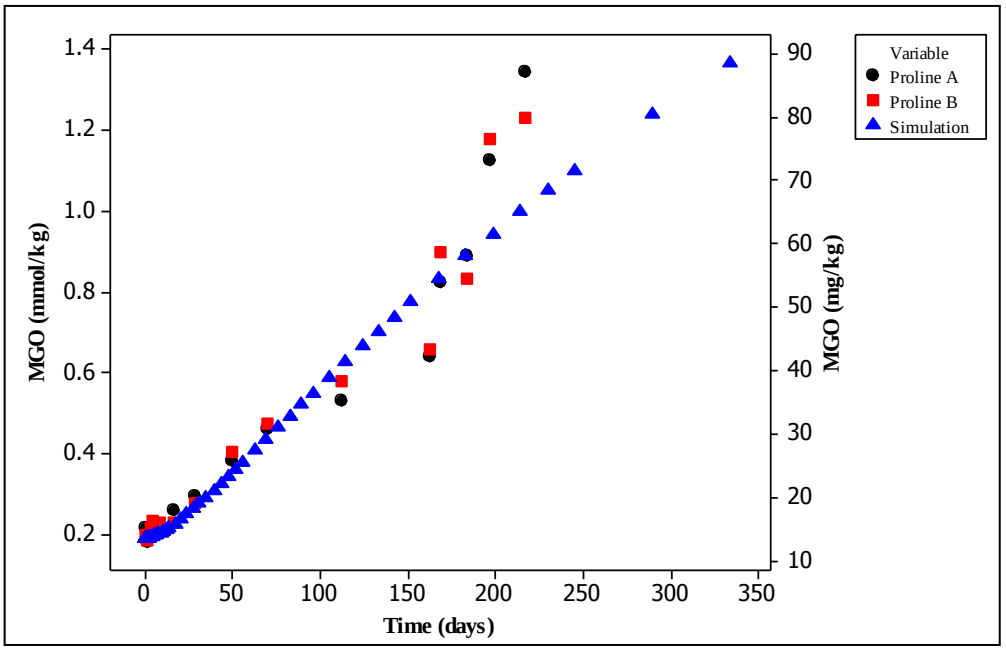


Figure 8.47 Experimental (circles and squares) and simulated (triangles) data for gain of MGO in the system perturbed by proline stored at 20 °C (approximately 2,000 mg/kg DHA and 800 mg/kg proline added). The simulated data fits the experimental data well using the same rate constants that were used at 27 °C.

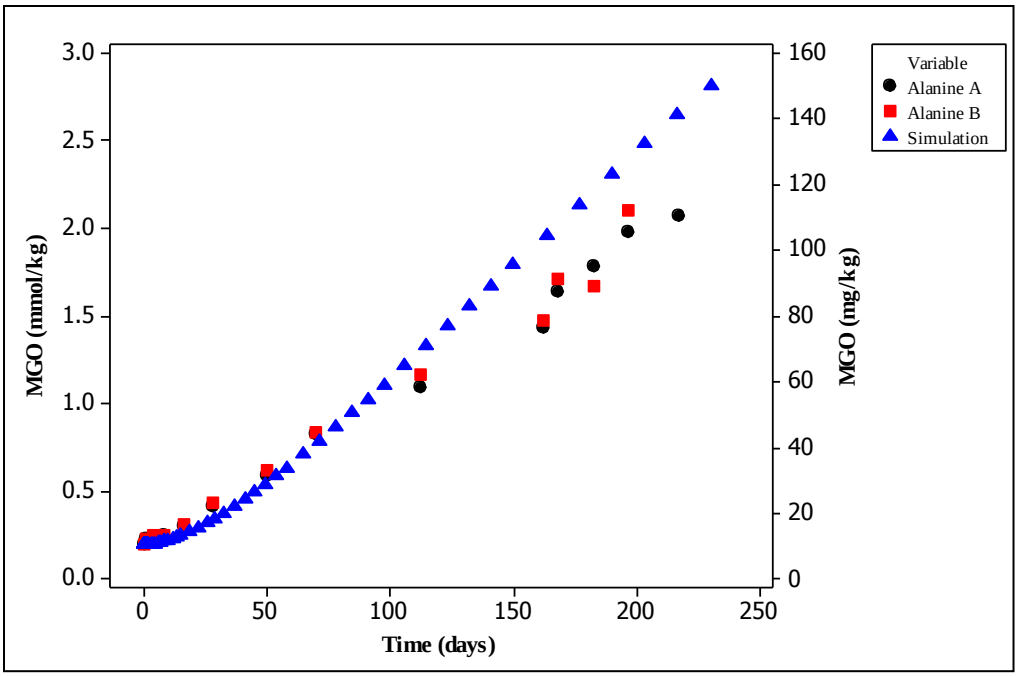


Figure 8.48 Experimental (circles and squares) and simulated (triangles) data for gain of MGO in the system perturbed by alanine that was stored at 20 °C (approximately 2,000 mg/kg DHA and 400 mg/kg alanine added). The simulated data fits the experimental data well when the rate constants were reduced by a factor of 5 compared to those used at 37 °C.

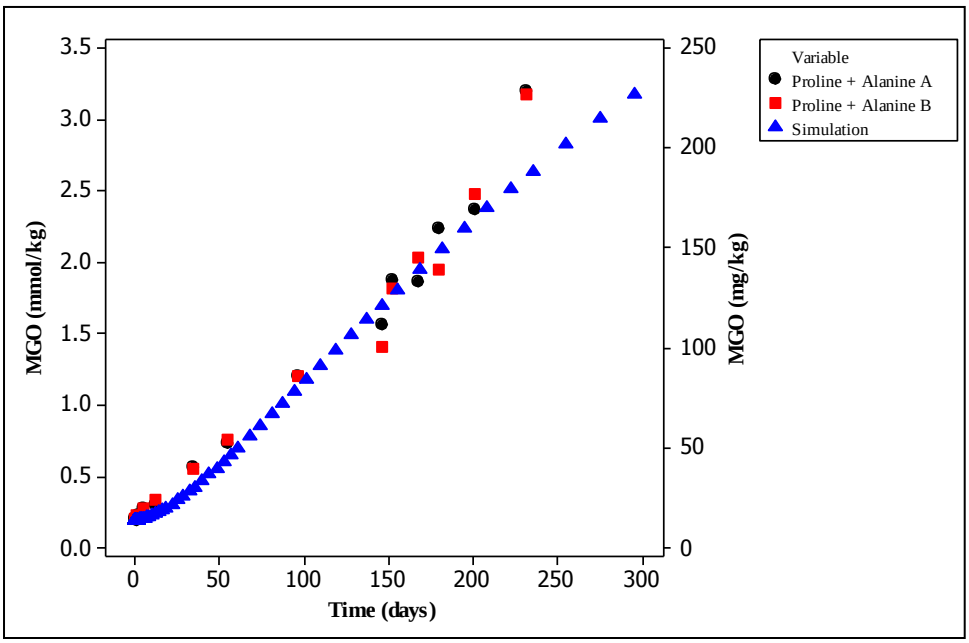


Figure 8.49 Experimental (circles and squares) and simulated (triangles) data for gain of MGO in the system perturbed by alanine and proline that was stored at 20 °C (approximately 2,000 mg/kg DHA, 400 mg/kg alanine and 800 mg/kg proline added). The simulated data fits the experimental data well.

Unfortunately only one mānuka honey was tested at 20 °C. The same rate constants used for the simulation at 27 °C for the extra reactions required for a real honey matrix were used and the simulation fitted the experimental data (Figure 8.50).

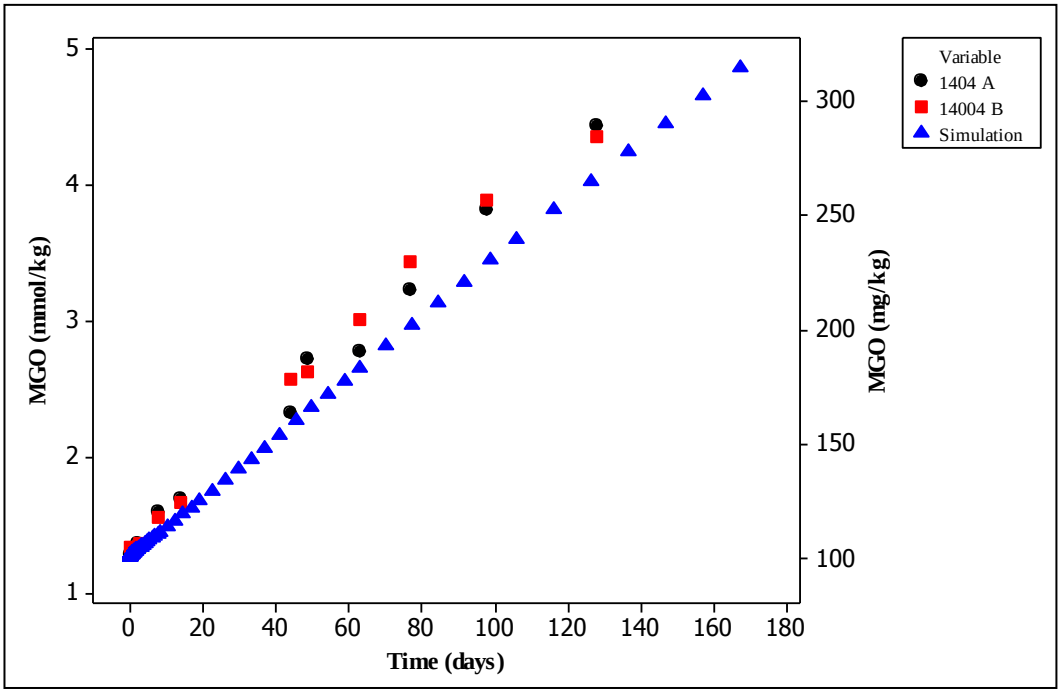


Figure 8.50 Experimental (circles and squares) and simulated (triangles) data for gain of MGO in sample 1404 that was stored at 20 °C. The simulated data fits the experimental data well.

8.8 Simulation of HMF formation at 37 °C

Knowledge of the kinetics of HMF formation in honey is useful because this would allow a compromise between low HMF and high MGO concentrations to be obtained. Capuano and Fogliano[172] reported that studies on HMF formation are limited and mainly based on empirical models describing HMF formation as zero-order kinetics or as following an exponential trend. Furthermore, most of these relate to food baked at high temperatures rather than storage of honey at ambient temperature.

A separate model was produced for the simulation of formation of HMF in honey to demonstrate that it can be achieved. Due to the iterative nature of this work and the time restraints of this research, the two models were not joined together and

refined. Concentrations of some species used in this model, such as amino acids, are influenced by the conversion of DHA to MGO and side reactions. Hence the rate constants will require adjusting when the two models are joined together. The amalgamation of the two models could be future work. Despite this, the simulation fits the experimental data well for HMF formation at 37 °C. The model was not assessed in detail as extra data from model systems are required (such as differences in pH) before a comprehensive model can be created. The model was also tested at lower temperatures 27 and 20 °C, but the induction period was noticeable in the simulation compared to the experimental data which was linear. This was addressed in chapter 7, where it was suggested that samples at lower temperatures remain in the induction period for a long time. Therefore further work is required to slow the induction period down in the model in order to fit the correct trend. A working model of the simulation was not fitted at 27 and 20 °C during this research.

The formation of HMF occurs faster from fructose than from glucose, therefore to simplify the simulation, only reactions involving fructose were considered; the rate constants chosen will reflect the formation of HMF from both fructose and glucose. The formation of HMF from fructose as reported by Antal *et al.*[380] was used as the basis of the model (Figure 8.51). It is assumed that the formation of the fructofuranosyl cation, intermediates 1 and 2 and formation of HMF will be the most important steps in the formation of HMF from fructose to create the model. Incorporation of the intermediate compounds allowed the initial lag phase to be reproduced and a curve to be plotted rather than a straight line in the simulation. Furthermore, this is important for slow formation of HMF due to the large excess of fructose. The addition of amino acids into an artificial honey matrix slowed down the rate of HMF formation (chapter 7), hence an equation for the formation of HMF by the Maillard reaction was not included in the simulation. It is possible that the amino acids were involved in reactions with DHA and MGO hence were not involved in the formation of HMF via the Maillard reaction. Further work is required to assess the effect of amino acids on the formation of HMF, including systems that do not contain DHA and MGO. Table 8.10 summarises the equations and rate constants used to simulate the formation of HMF in honey.

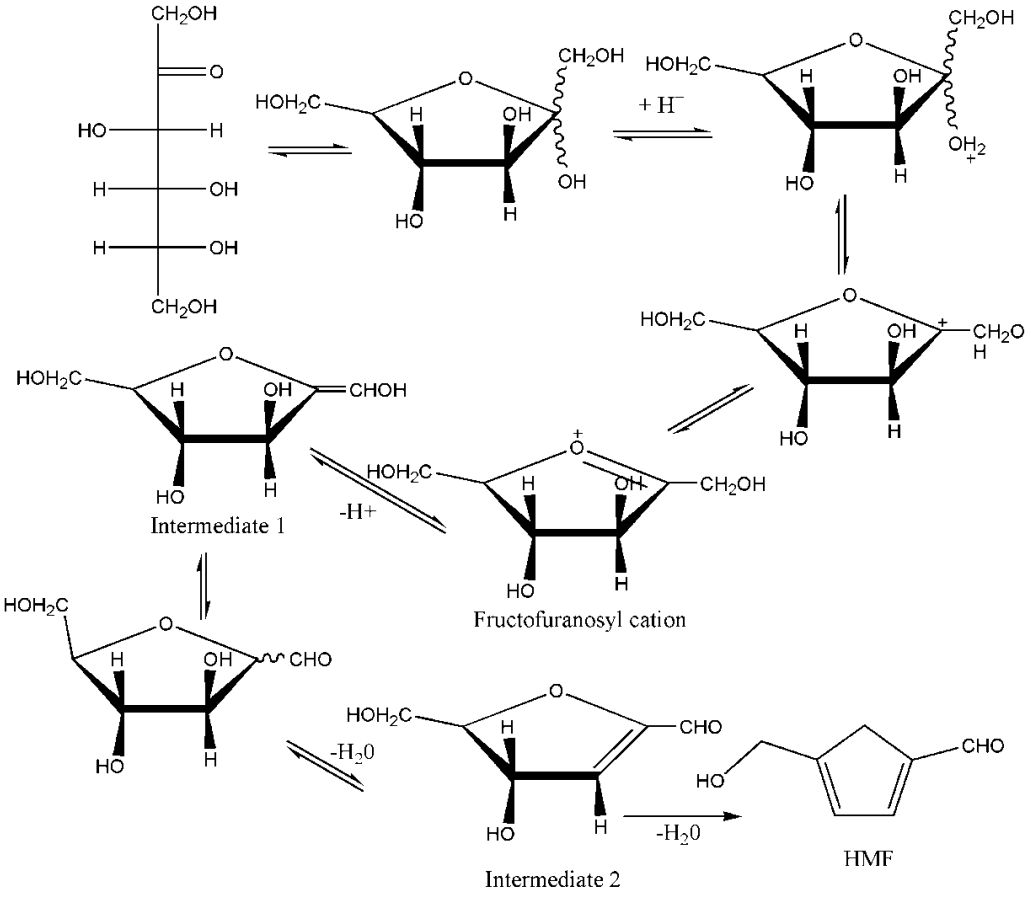


Figure 8.51 Proposed reaction scheme for the formation of HMF from fructose. Adapted from Antal *et al.*(1990)[380]

Table 8.10 Summary of rate equations and constants used to simulate the formation of HMF in honey.

Chemical equations	Model	Rate constant (day^{-1})
Fructose + H^+ → Fructofuranosyl cation	Control	0.01
Fructofuranosyl cation → Fructose + H^+	Control	0.01
Fructofuranosyl cation + H^+ → Intermediate 1	Control	0.005
Intermediate 1 → Intermediate 2 + H_2O	Control	0.003
Intermediate 2 → HMF + H_2O	Control	0.05
Primary amino acid + HMF →Primary amino acid-HMF	Alanine	0.002
DHA + Primary amino acid → DHA-Primary amino acid	Alanine	0.05
Proline + HMF → Proline-HMF	Proline	0.0025
DHA + Proline → DHA-Proline	Proline	0.05
Fructose + Unknown1 → Protonated Fructose + Unknown1	Real honey	0.001
HMF + Unknown2 → HMF-Unknown2	Real honey	0.001

Artificial honey matrices

The control sample (artificial honey with DHA) was used as a starting point to simulate the formation of HMF in honey. This is a simplified matrix with no added perturbants; an influence from DHA or MGO were not included in this model, but could be incorporated at a later date when more information is obtained.

The simulation fits the control experimental data well for most of the reaction, (Figure 8.52) but is slightly high at the end. As discussed in chapter 7, amino acids reduced the formation of HMF in the artificial honey matrix. Therefore reactions between alanine (representing all primary amino acids) and HMF were added to the simulation to reduce the amount of HMF formed. This gave a good fit for the system perturbed by alanine (Figure 8.53). An equation for the reaction between HMF and proline was also added to the model with good results compared to the experimental data for the system perturbed by proline (Figure 8.54). However, when equations for HMF with alanine and proline were added to the simulation and compared to the system perturbed by both alanine and proline not enough HMF was formed (Figure 8.55); this result was expected because there was no cumulative effect from alanine and proline to remove HMF (chapter 7). This may be due to DHA also reacting with amino acids in the system. Hence reactions between DHA with alanine and proline were added to the simulation, which improved the fit. The rate constants can be refined when this model is added to the main model for DHA conversion to MGO. Furthermore, in the simulation for the conversion of DHA to MGO there are equations for the removal of amino acids with unknown compounds; if the two models are added together, the rate constants could be adjusted so that the amino acids react with the HMF.

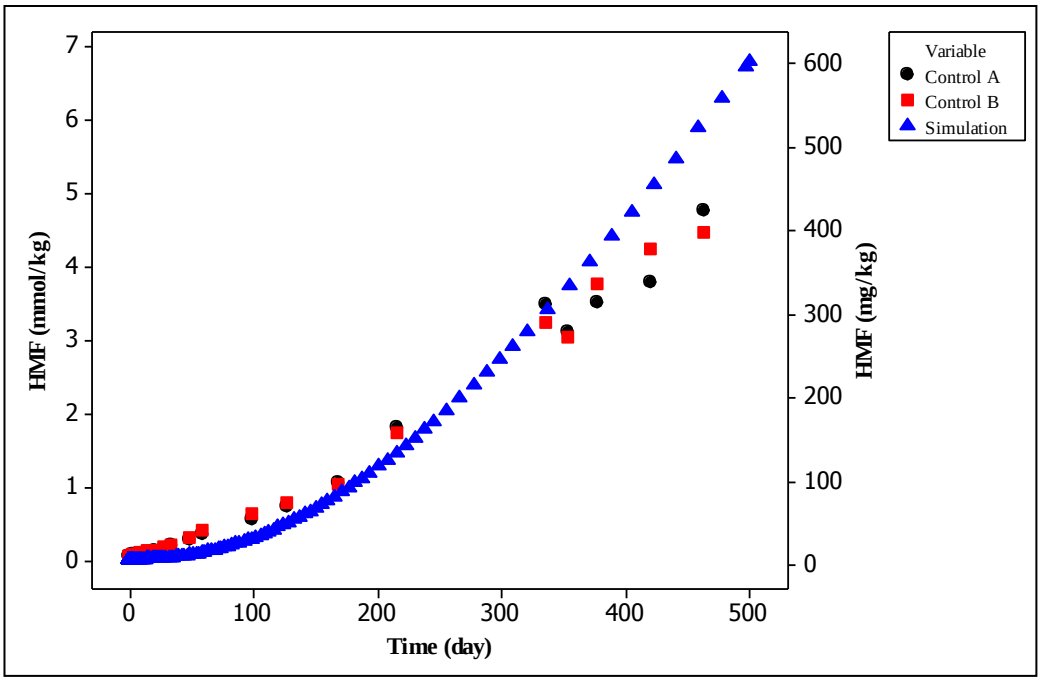


Figure 8.52 Formation of HMF in the control artificial honey matrix (circles and squares) and the simulation (triangles). The simulation models the experimental data well.

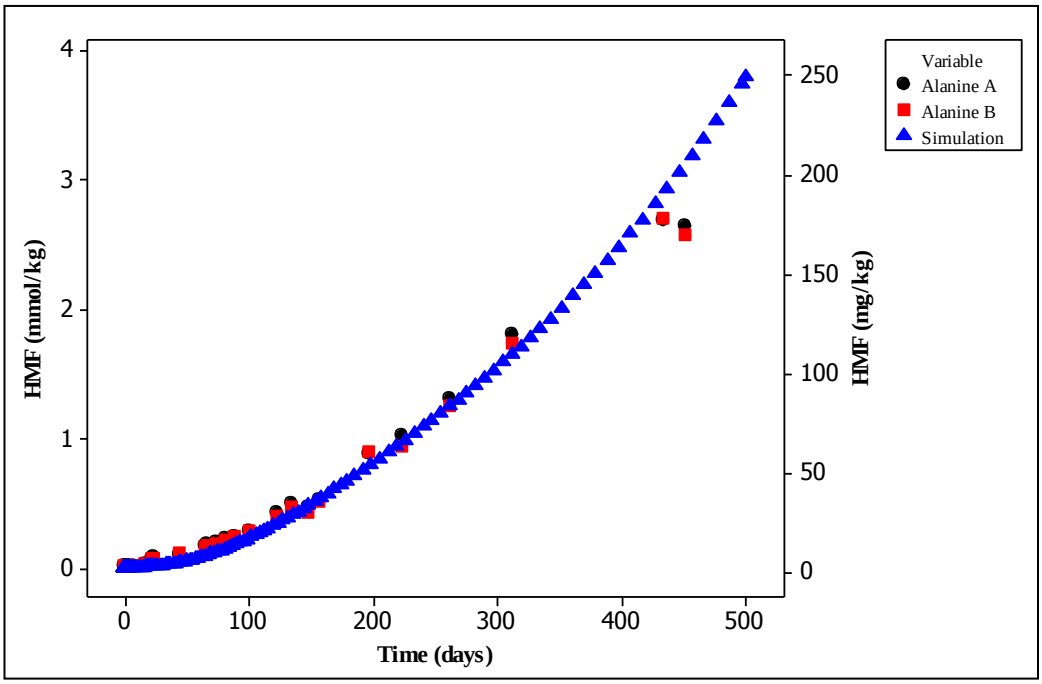


Figure 8.53 Formation of HMF for artificial honey perturbed by alanine (circles and squares) and the simulated data (triangles). The simulation fits the experimental data well.

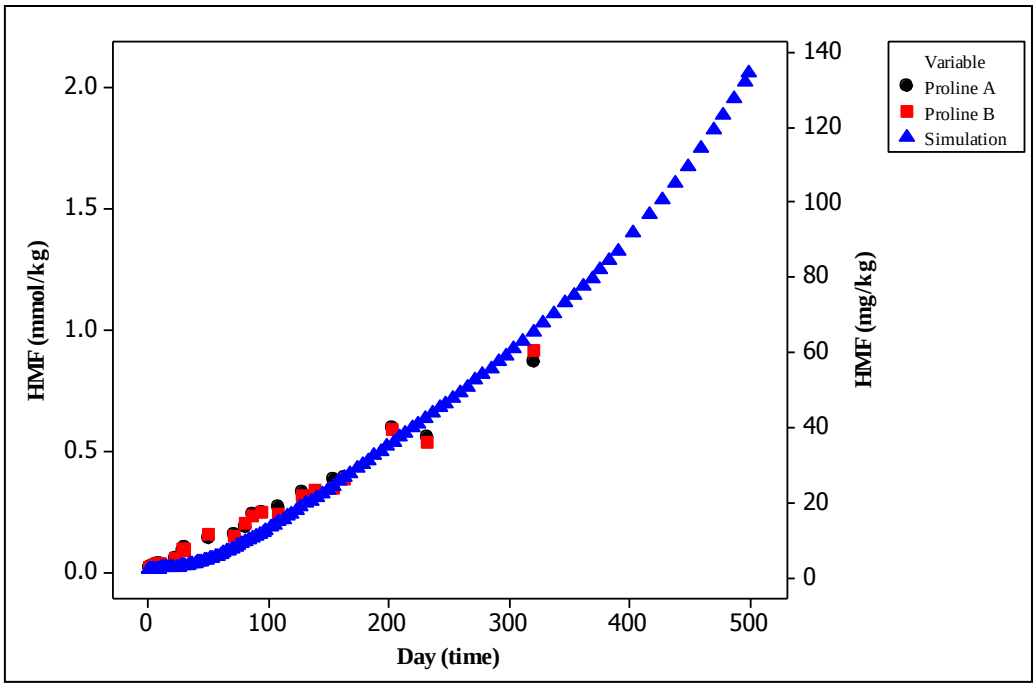


Figure 8.54 Formation of HMF for artificial honey perturbed by proline (circles and squares) and the simulated data (triangles). The simulation fits the experimental data well.

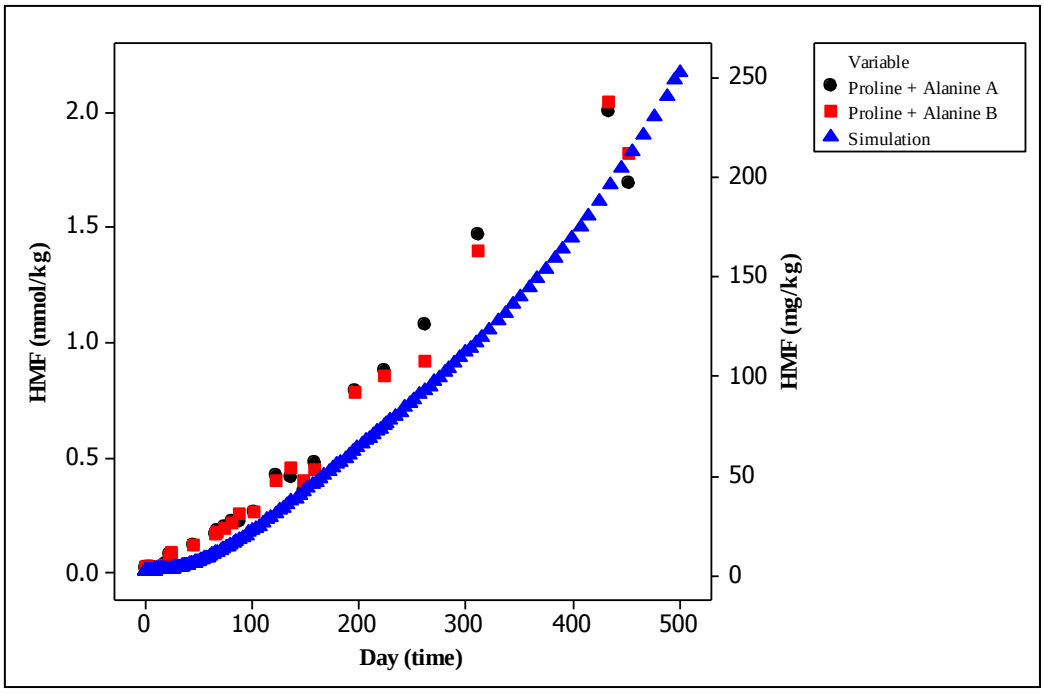


Figure 8.55 Formation of HMF for artificial honey perturbed by proline and alanine (circles and squares) and the simulated data (triangles). The simulated data too slow.

Real honey matrices

The simulation used for the formation of HMF in the artificial honey perturbed by proline and alanine was compared to real honey, but the simulation was slightly lower than the experimental data. Therefore an extra equation was added to account for protonation of fructose by unknown compound 1, similar to equations used to model the formation of DHA to MGO in real honey matrices. 5 mmol/kg unknown compound was added to the equation. A rate constant of 0.001 was originally chosen, but the plot does not change significantly when the rate constant is increased. It is possible that phenolic acids influence the protonation; however, large differences in the formation of HMF were not observed between mānuka and clover honeys. It is more likely that the formation is influenced by the pH or total acidity of the honey.

Addition of this equation gave a reasonable fit with the experimental data, but too much HMF is predicted at later times. This was refined by including an equation that removes HMF (5 mmol/kg initial concentration), which helped fit the simulated data with the experimental data at later times. An example of the fit is shown in Figure 8.56 for mānuka honey 953. Further examples are shown in Figure 8.57.

There was no observed change in the fit when the concentrations of alanine or proline were altered to match the amino acid concentration of each sample. Therefore further alterations may be required in the model if subtle differences in HMF formation are caused by amino acid concentration. Nevertheless the simulation fitted well for all samples examined.

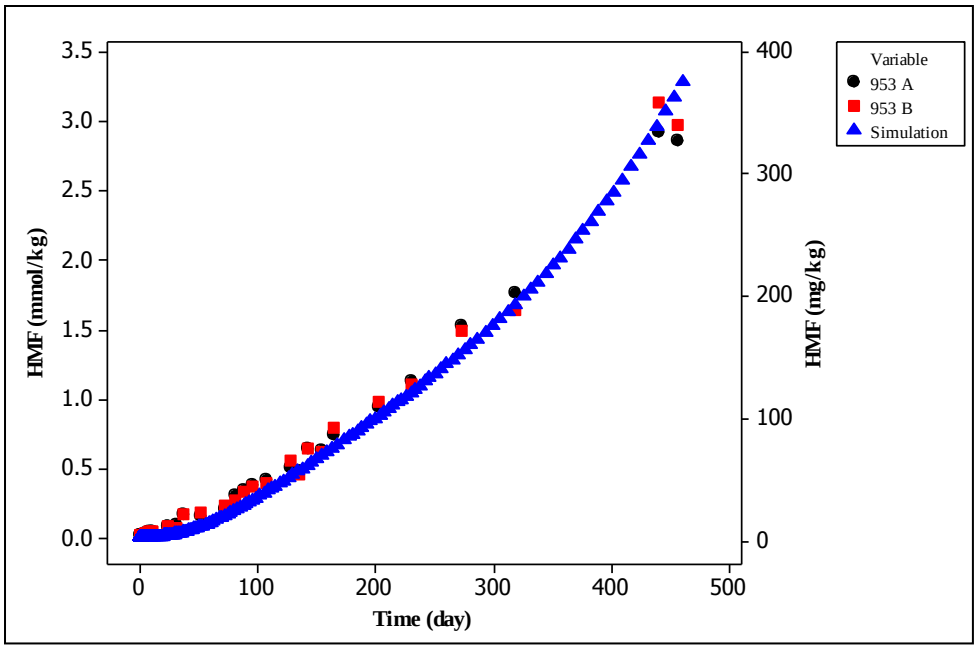


Figure 8.56 Formation of HMF in mānuka honey 953 (circles and squares) compared to the simulated data (triangles). There is good agreement of the simulated data with the experimental data.

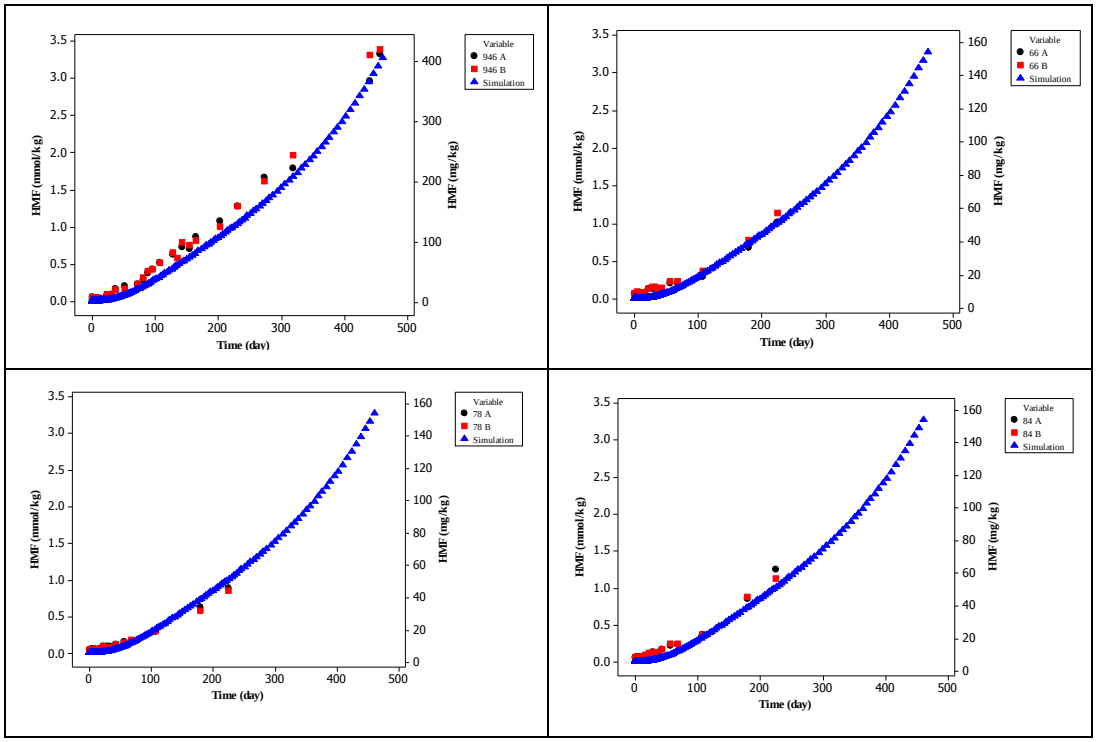


Figure 8.57 Experimental data (circles and squares) and simulated data (triangles) for the formation of HMF in manuka honey samples (946, 66, 78 and 84).

As previously mentioned, much of the literature investigates the formation of HMF at high temperatures. However, Escriche *et al.*[407] created a model for the prediction of HMF formation in honey at low temperatures (35-65 °C). The authors reported that only time, temperature and type of honey affected formation. The type of honey will have an effect due to various physical and chemical properties (for example, amino acids and pH). The model accounted for 75.48% of variation in the dataset and reported the standard error of estimation was 7.87. They modelled both storage and liquefaction, but reported that further improvement to the prediction would be to assess both separately.

This is the first version of the model; further refinements include addition of total acidity (which has a strong correlation with k_{HMF}) to the simulation and including this model into the main model for the simulation of DHA and MGO. However, because the main aim of the thesis was to investigate the conversion of DHA to MGO, refinement of the method will not be carried out in this work due to time constraints.

8.9 Conclusion

Model 3 is able to simulate the loss of DHA and gain of MGO with reasonable accuracy for most of the artificial honey systems studied. The model was constrained by the observations in the storage trials and also by the type of chemical reactions feasible in the system. When the model was compared to real honeys stored at 37 °C not enough MGO was produced, indicating that compounds other than amino acids play a role in the conversion of DHA to MGO and also the loss of MGO at later time periods. Phenolic acids are important for the conversion of DHA to MGO in mānuka honey; addition of equations for this gave a good fit for the loss of DHA and also for the gain of MGO at early times. An equation containing an as yet unknown compound was added to the model to simulate the loss of MGO at later times with reasonable accuracy compared to the experimental data.

Artificial honeys stored at 20 and 27 °C were used to test the simulation at lower temperatures. Rate constants for some reactions needed to be reduced, but other

reactions were unaffected. Experimental data was compared to the simulation with good accuracy.

As a first attempt, this model has been successful in simulating the conversion of DHA to MGO over time at 37, 27 and 20 °C. At present it is a valuable tool for investigating the influences of individual compounds on the conversion and also influences of multiple compounds in the complex system. However, further refinements are required if it is to be used as a predictive tool for the conversion of DHA to MGO in the mānuka honey industry. Analysis of a large number of fresh mānuka honeys from different geographical regions need to be compared with the simulation to ensure that it is robust; this includes storage at various temperatures.

The HMF formation model fitted the experimental data well at 37 °C. However, further refinements to the simulation are required; this includes addition of total acidity. Furthermore, the HMF model could be added to the main simulation for the conversion of DHA to MGO so that both MGO and HMF can be predicted depending on storage time and temperature. The simulation also needs to be fitted to the formation of HMF at lower temperatures.

9 Final conclusions and recommendations for future research

9.1 Conclusion

Mānuka honey is a multi-million dollar export industry in New Zealand. Mānuka honey that has a high level of MGO sells for a high price and is highly sought after due to its perceived health benefits. Prediction of the final MGO concentration from an immature mānuka honey is valuable for the entire supply chain. The intention of this research was to examine the conversion of DHA to MGO in honey and ultimately build a tool to predict the final concentration of MGO from an immature honey.

This research first compared four HPLC methods for the analysis of DHA, MGO and HMF in honey (chapter 3). Derivatisation with PFBHA and UV detection was deemed the best method for this research because all three compounds of interest could be quantified in a single 30 minute analysis. This method has low detection levels and a wide linear range of detection for all three compounds.

A database of mānuka honeys were analysed for various chemical and physical properties which may influence the conversion of DHA to MGO (chapter 4). Four clover honeys were also analysed for comparison. As expected, major compounds such as sugars did not vary much between samples. Phenolic acids were of interest due to the high levels found in mānuka honey compared to clover honey. Proline varied between samples and was at lower concentrations in older samples.

The work carried out in chapters 5, 6 and 7 on model honey systems and real honeys has gained many insights into the behaviour of DHA, MGO and HMF. It has been determined that not all honeys have the same rate constant for conversion of DHA to MGO, showing the requirement for a greater understanding of the influence of individual compounds in the honey in order to predict correctly the conversion of DHA to MGO. The conversion of DHA to MGO was determined to be a first-order reaction. However, at longer storage times the MGO first-order plot deviates from linearity due to side reactions; this was found to differ among honeys. Honey stored at lower temperature (20 and 27 °C) gained

more MGO over a longer period of time compared to samples stored at a higher temperature (37 °C) due to a larger loss of MGO at the higher temperature. Furthermore, it is proposed that DHA predominately exists as the dimer and only a small proportion is available for reaction (as the monomer), which influences the rate of the conversion of DHA to MGO and side reactions. Compounds that are readily able to donate a proton possibly catalyse the reaction by altering the equilibrium between the DHA dimer and monomer.

This research identified some factors that affect the conversion of DHA to MGO during storage at various temperatures. The concentration of proline, primary amino acids and phenolic acids were identified as important influences. Proline catalysed side reactions of DHA and MGO, hence the efficiency of the conversion of DHA to MGO was lowered. Primary amino acids have varying effects on the conversion; alanine increased the rate of MGO formation compared to the control sample. It was suggested that alanine either recovers DHA from a side product or prevents it from entering a side reaction, hence in high concentrations it allows more DHA to convert to MGO. In comparison, lysine and serine did not have a large influence on the conversion. High levels of iron accelerated the conversion of DHA to MGO. However, all mānuka honeys analysed contained very low levels of iron, therefore adulteration of mānuka honey with iron could be detected. In real honey matrices proline, primary amino acids and phenolic acids had a strong correlation with the first-order rate constant for the disappearance of DHA. However, the correlation between the concentration of amino acids or phenolic acids and the rate constant for MGO appearance was not high, even though they enhance the reaction, presumably due to the complex matrix.

Formation of HMF is predominately influenced by the length of time honey is stored and the temperature at which it is stored. However, the results show that physical and chemical properties of the honey can have a minor influence at the temperatures studied, which concurs with the literature. The difficulty in assigning a rate order to the formation of HMF was addressed. Samples stored at a lower temperature appear to be in an induction period for a long time, compared to samples at higher temperatures (37 °C) which pass through the induction period in about 40 days. The induction period follows first-order kinetics compared to the post-induction region which obeys zero-order kinetics. The induction period

strongly influenced the reported rate constant when only a few data points were taken at long time intervals. Amino acids reduced the rate constant for HMF formation at 37 °C, suggesting that they are not involved in forming HMF from the sugars via the Maillard reaction. This needs to be investigated further.

A strong positive correlation between k_{HMF} and the total acidity of the sample was observed, suggesting that donation of a proton may help form the fructofuranosyl cation, which is the first step in the formation of HMF from fructose.

A simulation to predict the level of DHA and MGO over time at various temperatures was created (chapter 8) using the information gained from the storage trials of artificial and real honeys. Influences from proline, total amino acids and phenolic acids were incorporated in the simulation in order to fit the simulated data with the experimental data. Initial concentrations of these compounds were entered into the model, along with DHA and MGO concentrations. The model had good accuracy for the samples analysed at 20, 27 and 37 °C. In most cases the error in prediction was no larger than the standard deviation (119 mg/kg) reported in the 2013 inter-laboratory comparison for quantification of MGO in honey. One or more compounds responsible for the loss of MGO at later times are yet to be identified. Not all rate constants were reduced for prediction at lower temperatures, suggesting that certain reactions are rate limiting. For example, the rate constant for the conversion of the DHA dimer to monomer and DHA to enediol were reduced but the subsequent steps to form MGO did not need to be altered. Furthermore, the rate constants for all alanine equations remained the same for the 37 and 27 °C models, but were reduced for the 20 °C model.

A simulation to predict the formation of HMF was also created (chapter 8); this had good accuracy for samples stored at 37 °C, but further work is required for samples at the lower temperatures. A compromise between reaching a high level of MGO and exceeding the recommended guideline for HMF needs to be considered.

9.2 Recommendations for future research

Chapter three compared four HPLC methods. From this research the following recommendations for analysis of DHA, MGO and HMF are suggested:

- MGO may be bound to sugars or other compounds in the honey matrix. Investigation of how various sample preparations influence the amount of MGO detected could be examined. This may include mildly warming/heating samples for different periods of time.
- Addition of DHA and HMF into the inter-laboratory comparison program to assess the variation in reported concentrations between laboratories.
- Standardise a method for the detection of all three analytes.

Analysis of mānuka honey samples in chapter 4 highlighted some areas of research that could be investigated. These include:

- Analysing a large set of mānuka honeys in which a wide number of mānuka varieties and geographical regions are represented. Other New Zealand mono-floral honeys should also be analysed for comparison. A large number of physical and chemical properties should be examined. Analysis of phenolic compounds would provide insight into compounds that are unique to mānuka honey. At the time of submission, initial stages of this work were being carried out by MPI.
- Analysis of phenolic compounds in the nectar of mānuka and kānuka flowers could be examined. Concentrations of compounds and relationships between syringic acid, methyl syringate and leptosperin as well as between phenyllactic acid and 4-methoxyphenyllactic acid would be of interest. This may elucidate the origin of leptosperin.

Storage of honey model systems, clover and mānuka honeys over extended time at regulated temperatures allowed the conversion of DHA to MGO and formation of HMF to be closely monitored (chapters 5, 6 and 7). Insights into these reactions were gained; however, there is still scope for more research to be carried out.

Further work could include:

- Continuing storage trials of honey model systems to isolate the effect of individual compounds on the conversion of DHA to MGO and side

reactions. Compounds to investigate include individual phenolic acids, with emphasis on phenyllactic acid and 4-methoxyphenyllactic acid, organic acids and amino acids not yet examined. Systems with varied total acidity could also be examined. Furthermore, systems with two or more of these compounds could also be considered.

- Storage trials of systems at lower temperatures (model honeys and real honey) should be carried out for longer periods of time than that used in this research to gain more information.
- Storage of control model samples and real honeys without DHA and MGO should be carried out to compare the HMF formation and influence of DHA and MGO on other compounds in the matrix.
- Systems should be stored at extra temperatures to strengthen the Arrhenius plot. Furthermore, Arrhenius plots from model systems with individual perturbants carried out at various temperatures will gain insight into the influence of temperature on the reactions.
- Sub-samples of each system could be analysed (possibly monthly) for certain compounds, such as phenolic acids and amino acids, to determine their concentration over time. This will help determine if perturbants are being consumed in side reactions with DHA or MGO. Depletion of perturbants in mānuka honey compared to other honeys may suggest a reaction with DHA or MGO.
- Mass spectrometry or NMR of the sub-samples could be carried out on each system to identify the side products of DHA and MGO.
- Identifying compounds that contribute to the loss of MGO at later times and determining rate constants for this loss by storing artificial honey systems with MGO as the starting compound and possible perturbants for extended periods of time at various temperatures.
- The effect of pH, acidity and water content of the conversion of DHA to MGO and formation of HMF could be investigated using model systems.
- Further investigation into the assignment of the HMF formation as a zero-order or first-order reaction could be carried out. In addition, the influence of individual perturbants on the formation and also the induction period could be investigated further.

Predictive software for the conversion of DHA to MGO and also the formation of HMF will be an important tool for the honey supply chain, including beekeepers and honey packers. As a first attempt, this research has created a good working model, but as with all research, a number of refinements and further work have been identified.

Further work to be carried out to refine and strengthen the predictive model includes:

- Testing a large number of mānuka honeys from various regions and beekeepers against the prediction software to ensure that it can correctly predict the MGO over time with a small margin of error.
- Incorporating temperature in the model as an initial input so that a wider range of samples can be predicted from one piece of software.
- Identifying compounds that contribute to the loss of MGO at later times (compound Z in the model).
- Investigating the effect of total acidity on the conversion of DHA to MGO. This may account for the 'baseline' conversion of DHA to MGO that was observed in clover honey and not artificial honey. If acidity has an effect the rate constant for the catalytic conversion of DHA to MGO by phenolic acid would need to be refined.
- Currently the model works with mānuka honey that has a known time zero. However, it is not likely that time zero will be known for all honey samples. Therefore an estimate of the age of the honey may need to be incorporated into the model. The ratio of DHA:MGO may be a good estimate of honey age.
- Testing a large number of mānuka honeys for their primary amino acid concentration and determine whether or not a median value can be used in the model so that primary amino acids do not have to be tested for every sample.
- The predictive models for DHA conversion to MGO and the formation of HMF could be joined into one model. This would require adjusting some rate parameters to account for the reactions between HMF and other perturbants.

In addition, further work that could be carried out either for interest or to strengthen the model includes:

- Examination of the equilibrium between monomeric and dimeric DHA in a honey model system. The influence of perturbants, temperature and moisture content on this equilibrium could also be investigated. An equilibrium constant could be obtained using NMR. A separate project is currently underway to determine the equilibrium constant in model systems.
- Other amino acids could be used in the storage trials to understand their influence on the reaction. Once their influence is known, the model can be adjusted. For example, amino acids with similar functional groups are likely to behave in the same way.
- Storage trials with different concentrations of the perturbing species could be investigated to see how they alter the model. While large differences may not be seen in nature, this may still be an interesting exercise to carry out. It may also give insight on the availability of monomeric DHA.
- The effect of amino acids on the formation of HMF in systems containing DHA and MGO needs to be investigated further. Formation of HMF can occur via the Maillard reaction in honey; however, in mānuka honey amino acids may not be readily available compared to honey of other floral types because of their reaction with DHA or MGO.

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Appendix A Conversion of NPA to MGO

Table A.1 Conversion of NPA to MGO (mg/kg and mmol/kg). Figures in bold are NPA points that are currently used to rate commercial products.

NPA	MGO (mg/kg)	MGO (mmol/kg)
0	91	1
1	92	1
2	98	1
3	106	1
4	118	2
5	**134**	**2**
6	153	2
7	175	2
8	201	3
9	230	3
10	**263**	**4**
11	299	4
12	339	5
13	382	5
14	428	6
15	**478**	**7**
16	531	7
17	588	8
18	648	9
19	712	10
20	**779**	**11**
21	850	12
22	924	13
23	1001	14
24	1082	15
25	**1167**	**16**
26	1254	17
27	1346	19
28	1440	20
29	1538	21
30	**1640**	**23**

Appendix B Inter-laboratory comparison programme for MGO using the OPD method

A collaborative study of quantification of MGO in honey was carried out in 2010. Following this study, inter-laboratory comparison tests were carried out in 2012 and 2013 for the antibacterial activity in mānuka honey. These tests were independently organised by Global Proficiency.* The aims of this work were to compare results between laboratories and identify possible causes of discrepancies between laboratories. The University of Waikato participated in all three programmes; however to protect confidentiality, the author is not allowed to identify any of the laboratories in these results. A written agreement to publish the results was given.[410]

2010 inter-laboratory comparison program

In 2010, seven laboratories from around the world took part in this collaborative study; laboratories have been arbitrarily assigned a number from 101 to 107, which does not correspond to the original number assigned during the comparison test in order to protect identity. All laboratories used OPD as the derivatising agent. Eight honey samples (H1-H8) were analysed. The results are shown in Table B.1. Samples 2 and 7 and samples 4 and 6 were blind duplicates. The average results for these samples are very close; there was only 0.12% and 1.34% difference between samples 2 and 7 and 4 and 6 respectively.

Samples ranged from 244 to 862 mg/kg, which represents a natural spread of MGO concentrations in honeys. However, a honey with a MGO concentration around 100 mg/kg would have been a good addition to the test.

* Global Proficiency is a specialist provider of proficiency testing, reference materials and related services.[409]

Table B.1 Summary of results for the 2010 collaborative study of MGO in honey.*

Lab	H1		H2		H3		H4		H5		H6		H7		H8	
	MGO (mg/kg)	Z-1	MGO (mg/kg)	Z-2	MGO (mg/kg)	Z-3	MGO (mg/kg)	Z-4	MGO (mg/kg)	Z-5	MGO (mg/kg)	Z-6	MGO (mg/kg)	Z-7	MGO (mg/kg)	Z-8
101	553	–0.33	585	–0.34	812	–0.14	427	–0.25	832	–0.23	429	–0.32	595	–0.25	225	–0.31
102	506	–0.74	544	–0.70	745	–0.68	389	–0.63	762	–0.77	387	–0.79	517	–0.91	191	–0.84
103	641	0.45	676	0.45	902	0.58	495	0.44	956	0.72	495	0.42	665	0.34	264	0.32
104	459	–1.16	499	–1.10	691	–1.11	352	–1.01	731	–1.00	366	–1.03	516	–0.91	182	–0.99
105	785	1.72	812	1.65	983	1.22	629	1.81	1016	1.18	616	1.77	852	1.91	352	1.73
106	668	0.69	716	0.81	978	1.18	503	0.53	1011	1.15	515	0.64	673	0.41	293	0.79
107	520	–0.62	535	–0.78	700	–1.04	365	–0.88	725	–1.05	395	–0.70	555	–0.59	200	–0.70
Average	590		624		830		451		862		457		625		244	
SD	113		114		125		98		130		89		119		62	
Median	553		585		812		427		832		429		595		225	

*All laboratories used OPD for derivatisation.

The reported standard deviation for each sample is very high (62-130 mg/kg). H8 had the lowest standard deviation, and also the lowest reported concentration of MGO (243 mg/kg). H5 had the highest reported reading of MGO (862 mg/kg) and the highest standard deviation (130 mg/kg).

Samples were standardised by calculating the Z-score; this calculates how far from the mean the reported value was in units of standard deviation and is calculated by subtracting the reported value by the mean and dividing this by the standard deviation. The average Z-score was below 2 for each laboratory which is acceptable.

2012 inter-laboratory comparison program

The inter-laboratory test was repeated in 2012 when five honey samples and an unknown solution of MGO were tested by eight laboratories. Laboratories were arbitrarily assigned a number from 201 to 208. Six laboratories used OPD derivatisation, one laboratory used PFBHA derivatisation and the final laboratories did not specify a derivatising agent.

Table B.2 summarises the reported concentration of honey samples for the eight laboratories. The standard deviation for the results dropped significantly since the 2010 test; it ranged from 14-70 mg/kg. The sample with the lowest reported MGO concentration (149 mg/kg) had the lowest standard deviation and the sample with the highest reported MGO concentration (785 mg/kg) had the highest standard deviation; this trend was also seen for the 2010 samples.

Table B.2 Summary of results for the 2012 inter-laboratory comparison program results for MGO concentration.*

Lab	H121		H122		H123		H124		H125		
	MGO (mg/kg)	Z-1	MGO (mg/kg)	Z-2	MGO (mg/kg)	Z-3	MGO (mg/kg)	Z-4	MGO (mg/kg)	Z-5	Average Z-Score
201	157	0.63	376	1.87	470	0.71	788	0.04	609	0.40	0.73
202	172	1.76	351	0.97	473	0.81	874	1.35	610	0.42	1.06
203	141	–0.60	312	–0.42	462	0.44	784.54	–0.01	602.63	0.23	–0.07
204	146	–0.20	313	–0.38	440	–0.25	787.33	0.03	594.33	0.02	–0.16
205	148	–0.08	307	–0.61	438	–0.30	751.15	–0.52	587.33	–0.16	–0.33
206	160	0.86	330	0.22	490	1.35	890.00	1.60	660.00	1.71	1.15
207	140	–0.66	325	0.04	425	–0.73	725.00	–0.92	573.00	–0.53	–0.56
208	126	–1.71	277	–1.68	384	–2.04	681.00	–1.59	512.00	–2.09	–1.82
Average	149		324		448		785		594		
SD	13		28		31		66		39		
Median	147		319		451		786		598		

* Lab 201 used PFBHA as the derivatising agent; lab 208 did not specify a derivatising agent; all other labs used OPD as the derivatising agent.

Analysis of the MGO standard solution showed a range of results, with all laboratories reporting concentrations lower than the expected value (155 mg/kg). The results are shown in Table B.3. Four laboratories reported less than 15% difference from the reported value. However, two laboratories had very large differences in reported values; one of these laboratories also had large reported differences for the honey – except that the reported concentrations for the honeys were high and the reported concentration for the MGO solution was low. It is unknown if this were due to a calculation error from the laboratory.

Table B.3 Inter-laboratory comparison results for MGO standard solution.

Lab	MGO (mg/kg)	Percentage difference	Z-score	Derivatising agent
201	109	–34.8	–1.66	PFBHA
202	149	–3.9	–0.22	OPD
203	151	–2.8	–0.16	OPD
204	141	–9.5	–0.51	OPD
205	137	–12.6	–0.66	OPD
206	80	–63.8	–2.71	OPD
207	86	–57.3	–2.49	OPD
208	117	–27.9	–1.37	Unknown
Mean	121.2			
Standard deviation	27.7			
Median	126.8			
Expected Value	155			

2013 inter-laboratory comparison

A third inter-laboratory comparison was set up in 2013; sixteen laboratories from around the world participated; these were arbitrarily assigned a number from 301 to 316. This included laboratories from New Zealand, Australia, Singapore, China and the USA. Five laboratories used PFBHA derivatisation, eight laboratories used OPD derivatisation and three laboratories did not specify which derivatising agent was used. Six honeys were analysed. A summary of the results can be found in Table B.4.

The standard deviations were large for the samples (ranging from 27 to 164 mg/kg) due to outliers in the sample set. Laboratories 305 and 316 had samples that had Z-scores larger than 2. When these samples were removed the standard deviation for each sample was lower, but some were still quite high (24 to 119 mg/kg). Honeys with low reported MGO had low standard deviations and honeys with

high reported MGO concentration had high standard deviation; this was also seen for the other two years. The increase in standard deviation compared to 2012 is most likely a reflection of double the number of participants. Furthermore this set of samples had more honeys with higher MGO concentrations than seen in the previous two studies, which will also affect the standard deviation.

Samples 3 and 6 were blind duplicates. There was only 3.7% difference in the reported average for these two samples, indicating that collectively the repeatability among the laboratories is high. However, three of the laboratories did not report the concentrations as similar, with the percentage differences between 13.37-38.05%. All other laboratories had less than 10% difference between the two samples.

A methylgloxal standard was also included for testing. The report from the comparison program compiled by Global Proficiency[411] stated that the reported results for the MGO standard were variable. The results were not given to participants, hence cannot be summarised here. They reported that the manufacturer stated that the MGO standard was not sufficiently stable. The 2012 results showed a small standard deviation; however, the samples were not transported to as many laboratories overseas, which may have had an impact on the stability of the MGO solution.

The results show that there are still some complications to be addressed when comparing MGO results between laboratories.

Table B.4 Summary of results for the 2013 inter-laboratory comparison program for MGO.

Lab	H1		H2		H3		H4		H5		H6		Method
	MGO (mg/kg)	Z-score	MGO (mg/kg)	Z-score	MGO (mg/kg)	Z-score	MGO (mg/kg)	Z-score	MGO (mg/kg)	Z-score	MGO (mg/kg)	Z-score	
301	447	1.02	225	1.44	860	0.32	448	0.94	726	0.74	826	0.24	PFBHA
302	374	–0.19	182	–0.13	744	–0.52	384	–0.09	621	–0.19	751	–0.21	OPD
303	306.4	–1.30	149.1	–1.33	648.2	–1.21	309.9	–1.28	524.3	–1.05	441	–2.10	OPD
304	352.7	–0.54	179.5	–0.22	719.3	–0.70	345	–0.72	572.8	–0.62	720.1	–0.40	PFBHA
305	503	1.94	242	2.06	1063	1.79	505	1.86	823	1.60	1090	1.85	OPD
306	354.6	–0.51	178.9	–0.24	697.5	–0.85	359.8	–0.48	574.8	–0.60	759.3	–0.16	OPD
307	330	–0.91	150	–1.30	740	–0.55	335	–0.88	530	–1.00	735	–0.31	Unspecified
308	314	–1.18	168	–0.64	642	–1.25	315	–1.20	499	–1.28	654	–0.80	OPD
309	396.38	0.18	185.64	0.00	889.07	0.53	409.55	0.32	655.92	0.12	870.73	0.52	Unspecified
310	357	–0.47	169	–0.61	767	–0.35	360	–0.47	581	–0.55	758	–0.17	PFBHA
311	402	0.28	192	0.23	818	0.02	404	0.23	830	1.67	630	–0.95	PFBHA
312	375.9	–0.16	179.6	–0.22	933.1	0.85	400.5	0.18	649.5	0.06	816.2	0.18	OPD
313	354.85	–0.50	165.59	–0.73	784.65	–0.22	361.75	–0.45	598.22	–0.40	784.31	–0.01	OPD
314	449	1.05	206	0.75	938	0.89	448	0.94	739	0.86	942	0.95	PFBHA
315	348.5	–0.61	167	–0.68	702.5	–0.82	335.5	–0.87	539	–0.92	696.5	–0.54	OPD
316	500	1.89	230	1.62	1100	2.06	510	1.94	820	1.58	1100	1.92	Unspecified
Average	385.33		185.58		815.40		389.44		642.72		785.88		
SD	60.52		27.35		138.22		62.05		112.35		164.01		
%RSD	15.71		14.74		16.95		15.93		17.48		20.87		
Median	365.50		179.55		775.83		372.88		609.61		758.65		

Appendix C Assignment of rate order

First- and second-order reactions are difficult to distinguish using the integrated formulae unless the reaction has been carried to at least 80% conversion.

The rate equation of a first-order reaction is

$$\frac{dx}{dt} = +k_1(a_0 - x) \tag{C-1}$$

and the integrated form is

$$k_{first}t = \ln\frac{x}{_0 - x} \tag{C-2}$$

The rate equation of a second-order reaction is

$$\frac{dx}{dt} = +k_2(a_0 - x) \tag{C-3}$$

and its integrated form is

$$k_{sec}ta_0 = \frac{x}{a_0 - x} \tag{C-4}$$

where

a_o = initial concentration

k_n = rate constant

t = time

x = amount reacted

Recasting the equation for $f = x/a_0$ (where f = the fraction reacted) yields

$$k_{first}t = ln\frac{1}{1 - f} \tag{C-5}$$

and

$$k_{sec}ta_0 = \frac{1}{1 - f} \tag{C-6}$$

for first- and second- order reactions, respectively.

For first-order data, a plot of ln (1/1−f) vs. t is linear with slope k and does not depend on the starting concentration. For second-order data, a plot of (1/1−f) vs. t is linear with a slope of ka_0 and does depend on the starting concentration, a_0. Therefore comparison of

experiments with different initial concentrations of DHA are helpful in distinguishing between first- and second-order data.

For a first-order reaction, where f is the fraction reacted and the expansion ln (1/1-f) is used, one obtains

$$k_{first}t = f + \frac{f^2}{2} + \frac{f^3}{3} + \ldots \frac{f^n}{n} \quad \text{(C-7)}$$

For a second-order reaction, where f is the fraction reacted and the binomial expansion of $1/1-f$ is used, one obtains

$$k_{sec}ta_0 = f + f^2 + f^3 + \ldots f^n \quad \text{(C-8)}$$

Ignoring terms beyond f^2 (due to their diminishing size and hence influence), the difference between the first- and second- order expansions is $\frac{f^2}{2}$ .Therefore if 25% of a first-order reaction has been completed then,

$$\frac{f^2}{2} = \frac{(0.25)^2}{2} = 0.031 \quad \text{(C-9)}$$

For a second-order reaction $f^2 = 0.25^2 = 0.063$. This means that when 25% of the reaction has occurred there is only 3% difference between the first- and second-order plots; hence they will both still be linear as this difference is within experimental error. Therefore the order cannot be accurately determined. If 80% of the reaction has been completed there will be 32% difference in the plots allowing enough difference to find the order of the reaction.

Appendix D Summary of initial concentrations of DHA and perturbants in artificial honeys used in storage trials

Appendix D contains the initial concentrations of DHA, perturbants and moisture content in artificial honey systems used throughout this research. This includes:

- 10,000 mg/kg DHA stored at 37 °C with model compounds
- 10,000 mg/kg DHA stored at 37 °C with naturally occurring perturbants
- 2,000 mg/kg DHA stored at 37 °C with naturally occurring perturbants
- 2,000 mg/kg DHA stored at 27 °C with naturally occurring perturbants
- 2,000 mg/kg DHA stored at 20 °C with naturally occurring perturbants

Table D.1 Summary of initial concentrations of DHA (~10,000 mg/kg) and model compounds in artificial honey stored at 37 °C.

Sample	Initial DHA (mg/kg)	Initial DHA (mmol/kg)	Perturbant	Perturbant (mg/kg)	Perturbant (mmol/kg)	Temperature (°C)	% Moisture
DHA Control	10694	119				37	26.8
Primary amine A	10817	120	Propylamine	868	15	37	23.8
Primary amine B	10938	121	Propylamine	863	15	37	23.2
Secondary amine A	10950	122	Diethylamine	1055	14	37	20.7
Secondary amine B	11035	123	Diethylamine	996	14	37	20.9
Amide A	10951	122	*N*-methylacetamide	1064	15	37	24.7
Amide B	11017	122	*N*-methylacetamide	1058	14	37	24.6

Table D.2 Summary of initial concentrations of DHA (~10,000 mg/kg) and naturally occurring perturbants in artificial honey stored at 37 °C.

Sample	Initial DHA (mg/kg)	Initial DHA (mmol/kg)	Perturbant	Perturbant (mg/kg)	Perturbant (mmol/kg)	Perturbant	Perturbant (mg/kg)	Perturbant (mmol/kg)	% Moisture
Control	10694	119							26.8
Proline A	10924	121	Proline	858	7				22.4
Proline B	10760	119	Proline	837	7				23.9
Proline A	10807	120	Proline	414	4				26.2
Proline B	10825	120	Proline	406	4				26.4
Iron A	10965	122	Iron Sulphate	814	3				21.9
Iron B	11108	123	Iron Sulphate	408	1				21.1
Proline + alanine A	10755	119	Proline	461	4	Alanine	456	3	N/D*
Proline + alanine B	10969	122	Proline	444	4	Alanine	438	3	N/D*
Proline + iron A	10823	120	Proline	834	7	Iron Sulphate	828	3	24.6
Proline + iron B	10774	120	Proline	823	7	Iron Sulphate	816	3	23.8

*N/D = Not determined

Table D.3 Summary of initial concentrations of DHA (~2,000 mg/kg) and naturally occurring perturbants in artificial honey stored at 37 °C.

Sample	Initial DHA (mg/kg)	Initial DHA (mmol/kg)	Perturbant	Perturbant (mg/kg)	Perturbant (mmol/kg)	Perturbant	Perturbant (mg/kg)	Perturbant (mmol/kg)	% Moisture
DHA A	2066	23							18.3
DHA B	2060	23							17.8
Proline A	2083	23	Proline	864	8				19.0
Proline B	2094	23	Proline	862	8				18.9
Alanine A	2054	23				Alanine	434	5	20.3
Alanine B	2055	23				Alanine	432	5	20.2
Iron A	2071	23				Iron Sulphate	869	3	19.3
Iron B	2074	23				Iron Sulphate	869	3	19.2
Lysine A	2062	23	Lysine	693	5				17.6
Lysine B	2034	23	Lysine	682	5				17.5
Serine A	2034	23	Serine	504	5				17.7
Serine B	2047	23	Serine	504	5				17.6
Proline + alanine A	2093	23	Proline	881	8	Alanine	434	5	20.3
Proline + alanine B	2065	23	Proline	887	8	Alanine	438	5	20.3
Proline + iron A	2067	23	Proline	885	8	Iron Sulphate	896	3	19.1
Proline + iron B	2072	23	Proline	885	8	Iron Sulphate	897	3	19.1
Alanine + potassium phosphate A	2075	23	Alanine	400	4	Potassium Phosphate	220	2	18.2
Alanine + potassium phosphate B	2081	23	Alanine	417	5	Potassium Phosphate	229	2	18.6
Alanine + iron A	2103	23	Alanine	443	5	Iron Sulphate	881	3	18.5
Alanine + iron B	2099	23	Alanine	409	5	Iron Sulphate	813	3	17.3
Proline + alanine + iron A	2106	23	Proline	852	7	Alanine / Fe	413.27/ 848.35	4.64 / 3.05	20.4
Proline+alanine+iron B	2056	23	Proline	841	7	Alanine / Fe	408.15/ 837.84	4.58 / 3.01	20.1

Table D.4 Summary of initial concentrations of DHA (~2,000 mg/kg) and naturally occurring perturbants in artificial honey stored at 27 °C.

Sample	Initial DHA (mg/kg)	Initial DHA (mmol/kg)	Perturbant	Perturbant (mg/kg)	Perturbant (mmol/kg)	Perturbant	Perturbant (mg/kg)	Perturbant (mmol/kg)	% Moisture
DHA A	1950	22							19.3
DHA B	1957	22							20.2
Proline A	1965	22	Proline	838	7				20.1
Proline B	1969	22	Proline	838	7				18.6
Alanine A	1967	22	Alanine	434	5				20.2
Alanine B	1945	22	Alanine	430	5				19.7
Lysine A	1937	22		717	5				19.3
Lysine B	1936	21	Lysine	708	5				19.8
Serine A	1992	22	Serine	490	5				20.1
Serine B	1974	22	Serine	488	5				19.9
Proline + alanine A	2095	23	Proline	834	7	Alanine	429	5	20.6
Proline + alanine B	2083	23	Proline	858	7	Alanine	441	5	20.0

Table D.5 Summary of initial concentrations of DHA (~2,000 mg/kg) and naturally occurring perturbants in artificial honey stored at 20 °C.

Sample	Initial DHA (mg/kg)	Initial DHA (mmol/kg)	Perturbant	Perturbant (mg/kg)	Perturbant (mmol/kg)	Perturbant	Perturbant (mg/kg)	Perturbant (mmol/kg)	% Moisture
DHA A	2323	26							21.0
DHA B	1688	19							20.9
Proline A	1969	22	Proline	846	7				20.3
Proline B	1967	22	Proline	836	7				21.2
Alanine A	1939	22	Alanine	431	5				20.9
Alanine B	1981	22	Alanine	430	5				20.3
Lysine A	1961	22	Lysine	708	5				20.6
Lysine B	2002	22	Lysine	709	5				20.8
Serine A	1944	22	Serine	508	5				20.3
Serine B	1948	22	Serine	485	5				20.6
Proline + alanine A	2058	23	Proline	863	8	Alanine	444	5	21.0
Proline + alanine B	2065	23	Proline	852	7	Alanine	439	5	20.9

Appendix E Summary of initial concentrations of MGO and perturbants in artificial honeys used in storage trials

Appendix E contains the initial concentrations of MGO, perturbants and % moisture in artificial honey systems used throughout this research. This includes:

- 2,000 mg/kg MGO stored at 37 °C with model compounds
- 2,000 mg/kg MGO stored at 37 °C with naturally occurring perturbants

Table E.1 Summary of initial concentrations of MGO (~2,000 mg/kg) and model compounds in artificial honey stored at 37 °C.

Sample	Initial MGO (mg/kg)	Initial MGO (mmol/kg)	Perturbant	Perturbant (mg/kg)	Perturbant (mmol/kg)	Temperature (°C)	% Moisture
MGO control A	2113	29	-	-	-	37	25.8
MGO control B	2084	29	-	-	-	37	26.6
Primary amine A	2073	29	Propylamine	936	16	37	18.9
Primary amine B	2075	29	Propylamine	933	16	37	18.9
Secondary amine A	2082	29	Diethylamine	951	13	37	19.2
Secondary amine B	2072	29	Diethylamine	963	13	37	18.9
Amide A	2089	29	*N*-methylacetamide	1216	17	37	19.5
Amide B	2092	29	*N*-methylacetamide	1218	17	37	19.1

Table E.2 Summary of initial concentrations of MGO (~2,000 mg/kg) and naturally occurring perturbants in artificial honey stored at 20, 27 or 37 °C.

Sample	Initial DHA (mg/kg)	Initial DHA (mmol/kg)	Perturbant	Perturbant (mg/kg)	Perturbant (mmol/kg)	Temperature (°C)	% Moisture
MGO A	2113	29				37	26.6
MGO B	2084	29				37	25.8
Proline A	2072	29	Proline	846	7	37	25.8
Proline B	2090	29	Proline	855	7	37	25.4
Alanine A	2094	29	Alanine	423	5	37	26.2
Alanine B	2082	29	Alanine	424	5	37	26.4
MGO A	2077	29				27	19.9
MGO B	2065	29				27	19.4
MGO A	2131	30				20	20.3
MGO B	2117	29				20	19.7

Appendix F Investigation on why mānuka honey fails the AOAC 998.12 C-14 sugar method

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Rogers, K. M.; Grainger, M. N. C.; Manley-Harris, M., The unique mānuka effect: Why New Zealand mānuka honey fails the AOAC 998.12 C-4 sugar method. *Journal of Agricultural and Food Chemistry* 2014, *62*, 2615-2622.

Nature of contribution by PhD candidate	I helped design this piece of research. I prepared the honeys samples and analysed them by HPLC. I collated the HPLC data.
Extent of contribution by PhD candidate (%)	33%

CO-AUTHORS

Name	Nature of Contribution
Merilyn Manley-Harris	Concept discussion and paper revision
Karyne Rogers	Developed research question. Analysis of honey samples for C-4 sugar determination. Collation and analysis of C-4 sugar data.

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

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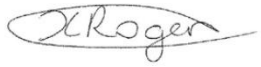
Karyne Rogers		09/10/14

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The Unique Manuka Effect: Why New Zealand Manuka Honey Fails the AOAC 998.12 C-4 Sugar Method

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ABSTRACT: Conversion of dihydroxyacteone (DHA) to methylglyoxal (MGO) has been shown to be the key mechanism for the growth in "apparent" C-4 sugar content in nonperoxide activity (NPA) manuka honey. This reaction is enhanced by heating and storage time and is demonstrated for the first time in clover honey adulterated with DHA purchased from a chemical supplier and in manuka honey containing naturally occurring DHA and MGO. After heating at 37 °C for 83 days, pure clover honey with no added DHA has the same apparent C-4 sugar content as at t = 0 days. The same clover honey adulterated with synthetic DHA added at t = 0 days and heated at 37 °C over the same time scale shows a change in apparent C-4 sugars from 2.8 to 5.0%. Four NPA manuka honeys heated over longer periods show an increase in apparent C-4 sugars of up to 280% after 241 days. This study strongly suggests that a protein fractionation effect occurs in the conversion of DHA to MGO in higher NPA manuka honey, rendering the remaining δ^{13}C protein value more negative and falsely indicating C-4 sugar addition when using the AOAC 998.12 method.

KEYWORDS: *New Zealand, manuka, Leptospermum scoparium, methylglyoxal, apparent C-4 sugars, 5-hydroxymethylfurfural, dihydroxyacetone, AOAC 998.12, adulteration, carbon isotope, honey*

INTRODUCTION

New Zealand manuka honey commands a premium price worldwide because of its nonperoxide antibacterial activity (NPA).[1] The NPA is principally due to the presence of unusually high levels of methylglyoxal (MGO).[2,3] MGO has been shown to derive from dihydroxyacetone (DHA) in the nectar of the manuka flower.[4] Immature honeys contain large amounts of DHA and little MGO; during the course of maturation and storage, DHA is chemically converted to MGO, although this conversion is not stochiometric.[4] The price for which manuka honey may be sold is directly proportional to the level of NPA and therefore proportional to the MGO content. To maximize MGO content in honey, beekeepers may resort to storage for protracted periods and/or warming (although it is generally accepted that warming of honey is not a good manufacturing practice and may affect honey quality parameters regulated in the Codex Alimentarius Standard).[5] When temperature-controlled storage is not available, stored honeys may be subject to fluctuations in temperature due to climatic variation.

During storage of honey and subsequent shelf life 5-(hydroxymethyl)-2-furaldehyde (hydroxymethylfurfural or HMF) also forms chemically from sugars, principally fructose, and the formation may be accelerated by heat.[6] HMF is regulated and, with certain specified exceptions, should not exceed 40 mg kg^{-1}.[5]

Recently, sugar adulteration concerns have arisen because the AOAC 998.12 C-4 sugar method (a test for illicit addition of C-4 cane sugar)[7] has shown the frequent occurrence of apparent C-4 sugars in manuka honey. It has been shown that 70% of manuka honeys (n = 220 samples) with MGO > 263 mg kg^{-1} (NPA > 10+) tested in a survey failed the AOAC 998.12 C-4 sugar method[8] and contained apparent C-4 sugars up to 15%. In contrast, manuka honeys (n = 156 samples) with MGO < 250 mg kg^{-1} (NPA < 10+) gave only 11% failure. These fails were proposed to actually be false-positive results, the result of some unknown mechanism within the honey.[8,9] False-positive results occurred when genuine honey exceeded the 7% threshold for apparent C-4 sugars because of a negative shift in the δ^{13}C protein rather than a positive shift in the δ^{13}C honey; the latter is usually attributed to C-4 sugar adulteration.[10−12] Previously false-positive results reported in manuka honey have been attributed to pollen contamination of the extracted protein;[13] however, even after filtration and centrifugation to remove pollen were applied, genuine manuka honey samples were still shown to exceed 7% C-4 sugars.[9]

This study reports for the first time the effects of storage temperature and storage time (aging and maturation) on apparent C-4 sugar, DHA, MGO, and HMF contents of clover honey with prior addition of DHA and of bioactive manuka honey to investigate why higher NPA honey has high apparent C-4 sugars.

MATERIALS AND METHODS

Chemicals and Solvents. MGO (40% w/w), DHA (97%), and HMF (99%) were supplied by Sigma-Aldrich. *o*-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA, 99+%) was supplied by Alfa Aesar. Hydroxyacetone (HA, ≥ 90%) was from Aldrich. Type I water (distilled and deionized) was obtained using a Barnstead E-pure system (18.0 MΩ·cm). HPLC grade acetonitrile (ACN) was obtained from Honeywell, Burdick and Jackson or Merck. HPLC grade methanol was supplied by Scharlau.

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Table 1. Honey and Protein Carbon Isotopes, Apparent C-4 Sugar Content, DHA Remaining, MGO and HMF Contents, and DHA/MGO Ratio of Honey Inoculated with Various Levels of DHA Incubated at 37 °C for 83 Days[a]

DHA dosage (mg kg^{-1})	δ^{13}C honey (‰)	δ^{13}C protein (‰)	apparent C-4 sugars (%)	DHA (mg kg^{-1})	MGO (mg kg^{-1})	HMF (mg kg^{-1})	DHA/MGO
0 (t = 0)	−26.3	−26.8	2.8	0.0	35.9	64.0	0
0 (t = 83)	−26.3	−26.8	2.8	0.0	39.1	86.0	0
250	−26.4	−26.9	2.8	105.9	87.4	94.6	1.2
500	−26.3	−26.9	3.0	290.4	140.9	86.7	2.1
1000	−26.4	−27.0	3.3	544.0	249.2	95.1	2.2
2000	−26.4	−27.1	3.6	1119.4	477.6	92.9	2.3
4000	−26.4	−27.2	3.9	2196.5	893.4	98.5	2.5
8000	−26.3	−27.3	5.0	4127.3	1649.3	102.2	2.5

[a]Values are reported as an average of three replicates. SDs are within 0.1‰ for isotopes and percentage relative SD of DHA, MGO, and HMF is <5%.

Clover Trial Samples. A 48000 mg kg^{-1} stock solution of DHA, supplied by Sigma-Aldrich, in type I water was prepared and diluted to give final concentrations of 8000, 4000, 2000, 1000, 500, 250, and 0 mg kg^{-1} DHA in honey. HappyBee clover blend honey (60 g) was weighed out, 1 mL of the corresponding DHA stock solution was added, and samples were thoroughly mixed. The control sample (0 mg kg^{-1} DHA) had 1 mL of type 1 water added. Replicate samples were incubated at 37 °C and tested after 83 days.

Manuka Trial Samples. Pure manuka honey samples were selected from four different regions in the southern North Island of New Zealand to see if common patterns existed during the aging of manuka honey. Samples were harvested from December 2011 to January 2012. Samples were held at 20 °C until the study began in October 2012. Replicate splits of each sample were taken in October 2012, with two sample splits (control) being frozen immediately to arrest any chemical transformations and the other paired splits being incubated at 27, 32, and 37 °C; samples withdrawn periodically (t = 21, 63, 151, and 241 days) and immediately frozen. Samples were analyzed for methylglyoxal, dihydroxyacetone, and hydroxymethylfurfural contents and carbon isotope ratios (δ^{13}C) of the honey and extracted protein.[14]

Analysis of DHA, MGO, and HMF. HA (3.34 mg mL^{-1}) was used as the internal standard solution. The PFBHA derivatizing solution (20 mg mL^{-1}) was made up in citrate buffer (0.1M) and adjusted to pH 4 with NaOH (4 M).

A six-point calibration curve was made up for each compound (DHA, MGO, and HMF) using a multicompound standard solution. Standards were made up in an artificial honey matrix (0.20 ± 0.02 g). The artificial honey comprised fructose (41%), glucose (37.5%), sucrose (2.9%), and water (18.6%), and the pH ranged from 3.13 to 3.76, reflecting the pH of honey[12] and so did not need to be adjusted. HA (internal standard, 250 μL), DHA stock (100 μL), and MGO + HMF stock (40 μL) were added, mixed, and left for 1 h. PFBHA derivatization solution (1200 μL) was added and mixed. Samples were left for 1 h to complete derivatization. Acetonitrile (6 mL) was added and the sample mixed until all crystals dissolved; water (2 mL) was added, and the standards were mixed before being transferred to autosampler vials and analyzed. A linear calibration curve was constructed using the HPLC peak area ratios of DHA/HA, MGO peak 1 + MGO peak 2/HA, and HMF peak 1/HA against the mass of the compounds.

Variability of the PFBHA method was determined by analyzing triplicate samples of each manuka honey over 4 days. This introduced real-world variability (such as sample preparation and instrumentation). The percentage relative standard deviation is <5% for all three compounds, and values are reported as averages.

Sample Preparation. Honey samples (0.20 ± 0.02 g) were weighed into test tubes, HA (internal standard, 250 μL) was added, and the sample was mixed and left for 1 h, at which time PFBHA derivatizing solution (1,200 μL) was added, mixed, and left for a further hour. ACN (6 mL) was added and mixed until all crystals were dissolved; water (2 mL) was added and mixed before analysis.

HPLC Method. HPLC to determine DHA, MGO and HMF was performed at the University of Waikato on an HPLC system fitted with two Waters 515 pumps, a 996 photodiode array (PDA) detector (240−400 nm), an autosampler (set at 20 °C), and an Alltech Elite degassing system. A Symmetry shield RP18 column (5 μm, 3.0 × 250 cm) was used for separation of compounds; a 20 μL injection was used. The system was controlled using Waters Empower 2 chromatography software. The system was operated with gradient elution using 30:70 (v/v) ACN/H_2O as mobile phase A and 100% ACN as mobile phase B. The gradient elution was run at a constant combined flow rate of 0.8 mL min^{-1}. Gradient steps were (%B) 0 (10), 2.5 (10), 10.5 (50), 18 (100), 25 (10), and 35 (10). DHA and HA were quantified at 260 nm; HMF and MGO were quantified at 279 and 244 nm, respectively.

C-4 Sugar Determination. δ^{13}C values of honey and corresponding extracted protein were measured using stable isotope analyses according to the internationally used AOAC 998.12 method.[7] Honey and extracted protein (∼0.5 mg) were transferred into 6 × 4 mm tin capsules. Samples were analyzed at the Stable Isotope Laboratory at GNS Science, using a Eurovector elemental analyzer coupled to an Isoprime mass spectrometer, for isotopic abundance in continuous flow mode (EA-IRMS). International and working reference standards (IAEA-CH_6, leucine, EDTA, and cane and beet sugars), two honey standards (honey and protein), and blanks were included during each run for calibration. Isotopic ratios (^{13}C/^{12}C) are expressed as isotopic deviations δ defined as

$$\delta\ (\%) = \frac{R_s - R_{ref}}{R_{ref}} \times 1000$$

where R_s is the isotopic ratio measured for the sample and R_{ref} that of the international standards. The δ^{13}C value is relative to the international Vienna Pee Dee Belemnite (VPDB) standard. Results are expressed in δ (‰) versus the specific reference, and analytical precision is within ±0.2‰ for carbon. Apparent C-4 sugar content is calculated by

$$\text{apparent C-4 sugars (\%)} = [\delta^{13}\text{C(protein)} - \delta^{13}\text{C(honey)}]/[\delta^{13}\text{C(protein)} - (-9.7)] \times 100$$

where AOAC 998.12 method interprets pure honey as generally yielding a value of C-4 sugars of ≤7%. Some unusual varieties may slightly exceed the range, but will have δ^{13}C values for honey that are in the normal range (more negative than −24.0‰).[7] It is noted that apparent C-4 sugar is not a direct measurement of actual C-4 sugar levels but an estimation based on the positive δ^{13}C shift that occurs when C-4 sugars (which range from δ^{13}C ∼9.7 to 11‰) are added to honey (typically ranging from δ^{13}C ∼−24 to −27‰ in New Zealand).[8]

RESULTS AND DISCUSSION

Addition of Synthetic DHA to Clover Honey. Clover honey (which neither displays nonperoxide antibacterial activity nor contains measurable DHA) was used as matrix to investigate the effect of higher levels of DHA and MGO on the apparent C-4 sugar assay. Prior to any addition of DHA, the baseline DHA and MGO contents of the clover honey were tested and were 0 and 35.9 mg kg^{-1}, respectively (Table 1); the MGO result for clover honey is higher than would be expected but may be due to some analytical background interference. Clover honey samples were inoculated with DHA at various concentrations from 0 to 8000 mg kg^{-1} and incubated (37 °C, 83 days) with testing for DHA, MGO, and HMF at 0, 35, and 83 days and for apparent C-4 sugars at 0 and 83 days. At 35 and 83 days clover honeys to which DHA had been added were assayed for MGO content (Figure 1). Results showed

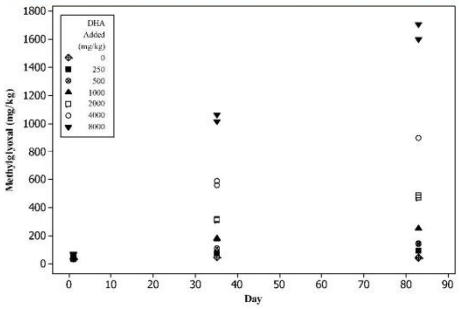

Figure 1. MGO levels (mg kg^{-1}) in replicate clover honey samples after 0, 35, and 83 days of incubation at 37 °C with different additions of DHA (mg kg^{-1}) at 0 days. (Some replicates have excellent repeatability and lie on top of the first point.)

decreased levels of DHA, increased levels of MGO (Table 1), as has been observed previously,[4] and increased levels of HMF. The highest levels of MGO and HMF observed in this trial were 1649 and 102.2 mg kg^{-1}, respectively.

At 0 and 83 days, apparent C-4 sugars were assayed (Table 1; Figure 2). At 0 days, apparent C-4 sugars were 2.8% and were comparable with an average clover honey.[8] After 83 days of incubation, the control sample still measured 2.8% apparent C-4 sugars, but the apparent C-4 sugar content of clover honey with the highest DHA addition (8000 mg kg^{-1}) was 5.0% (a 78.6% increase), the DHA content had decreased (4127 mg kg^{-1}), and the MGO content had increased (1649 mg kg^{-1}) (Figure 2). The ratio of DHA/MGO observed after 83 days was 2.0, similar to that usually observed in mature manuka honey.[15,16]

The HMF level of the clover honey before the trial (at t = 0 days) was 64 mg kg^{-1}, which exceeded recommended Codex limits at the time of purchase. After maturation at 37 °C for 83 days, the clover honey had darkened from a light brown color (at t = 0 days) to a rich caramel color and attained a maximum HMF level (in the DHA, 8000 mg kg^{-1} sample) of 102 mg kg^{-1}. The color and HMF changes are expected given the high temperature and length of incubation. A similar conversion of DHA to MGO might be achieved by using a lower temperature and a longer storage time without the formation of the high

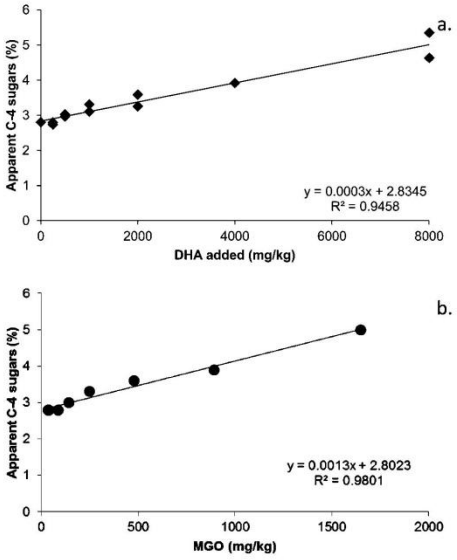

Figure 2. (a) Evolution of apparent C-4 sugars in clover honey after 83 days of incubation at 37 °C after different additions of DHA (mg kg^{-1}) at 0 days; (b) apparent C-4 sugars and corresponding MGO (mg kg^{-1}) evolved in clover honey after 83 days.

levels of HMF because the two reactions, although occurring simultaneously, are not chemically related.

All DHA inoculated clover samples and the clover honey control sample had the same $\delta^{13}C$ honey value of −26.4 ± 0.1‰, and these were not found to vary during the trial (Table 1). In contrast, the $\delta^{13}C$ protein values, which were unchanged in the control sample between 0 and 83 days, showed an increasing negative trend with increasing initial DHA content; this caused a progressive increase in $\Delta(\delta^{13}C$ honey-protein) and apparent C-4 sugars (Table 1). This suggests that freshly harvested honey with a higher initial DHA content would be more likely to fail the AOAC 998.12 C-4 test once maturation had occurred because the chemical reaction converting DHA to MGO was in some way affecting the protein in the honey, causing a false-positive fail. Adams et al.[4] demonstrated that conversion of DHA to MGO was not stoichiometric; that is, not all DHA lost had been converted to MGO, suggesting that DHA and possibly MGO were reacting with other honey components. Other possible reactions that DHA or MGO might undergo include Maillard-type reactions with proteins and/or amino acids. It has been reported that DHA was more reactive with amino acids than glucose; more specifically, basic amino acids (proline, lysine, etc.) were the most reactive with DHA.[17] High concentrations of sugar relative to amino acid and low water activity, both being conditions that pertain in honey, might accelerate the reaction.

MGO is well-known for its capacity to react with and cross-link protein chains.[18] The effect of these Maillard-type reactions would be to reduce the apparent content of amino acids; further cross-linking may also change the solubility and cause

Table 2. Incubation Time, Incubation Temperature, Average DHA, HMF, and MG Contents, and Apparent C-4 Sugar Content of Four Manuka Honeys (Samples 1–4)[a]

sample	time (days)	temp (°C)	DHA (mg kg^{-1})	DHA remaining (%)	HMF (mg kg^{-1})	MGO (mg kg^{-1})	δ^{13}C honey (‰)	δ^{13}C protein (‰)	C-4 sugars (%)	DHA/ MGO
1	0	27	1612.4	100	7.3	178.5	−25.9	−26.6	4.2	9.0
1	63	27	1414.4	87.8	9.8	289.1	−25.9	−26.7	4.9	4.9
1	151	27	1051.6	65.2	15.5	386.1	−25.9	−26.8	5.4	2.7
1	241	27	746.4	46.3	48.7	500.9	−26.0	−27.0	6.1	1.5
1	0	32	1612.4	100	7.3	178.5	−25.9	−26.6	4.2	9.0
1	63	32	1242.0	77.0	13.3	337.7	−25.9	−26.8	5.6	3.7
1	151	32	816.0	50.6	27.0	419.5	−26.0	−27.0	6.6	1.9
1	241	32	557.9	34.6	68.7	459.7	−25.9	−27.2	8.0	1.2
1	0	37	1612.4	100	7.3	178.5	−25.9	−26.6	4.2	9.0
1	21	37	1350.6	83.8	12.7	282.9	−25.9	−27.0	5.0	4.8
1	63	37	950.1	58.9	28.3	369.6	−25.9	−26.9	6.3	2.6
1	151	37	468.4	29.0	75.9	393.3	−25.9	−27.2	7.9	1.2
1	241	37	294.4	18.3	213.1	364.6	−25.9	−27.7	11.1	0.8
2	0	27	2376.6	100	7.9	283.6	−25.3	−26.6	8.3	8.4
2	63	27	2005.9	84.4	10.0	404.0	−25.3	−26.7	9.0	5.0
2	151	27	1677.5	70.6	15.6	565.5	−25.3	−26.9	10.3	3.0
2	241	27	1116.3	47.0	46.9	734.3	−25.3	−27.0	10.9	1.5
2	0	32	2376.6	100	7.9	283.6	−25.3	−26.6	8.3	8.4
2	63	32	1705.8	71.8	12.8	485.5	−25.3	−26.8	9.8	3.5
2	151	32	1302.1	54.8	27.6	625.7	−25.3	−27.1	11.5	2.1
2	241	32	782.7	32.9	71.0	706.4	−25.2	−27.2	12.9	1.1
2	0	37	2376.6	100	7.9	283.6	−25.3	−26.6	8.3	8.4
2	21	37	1916.0	80.6	11.0	389.4	−25.2	−26.6	8.9	4.9
2	63	37	1332.1	56.1	25.3	559.2	−25.3	−27.1	11.9	2.4
2	151	37	780.4	32.8	83.3	627.8	−25.3	−27.2	12.4	1.2
2	241	37	444.4	18.7	200.7	641.5	−25.6	−27.6	12.6	0.7
3	0	27	2367.0	100	6.7	313.8	−25.2	−26.0	4.8	7.5
3	63	27	2204.5	93.1	11.5	468.7	−25.2	−26.1	5.7	4.7
3	151	27	1733.3	73.2	14.3	592.9	−25.2	−26.2	6.4	2.9
3	241	27	1154.3	48.8	51.5	805.4	−25.2	−26.6	9.0	1.4
3	0	32	2367.0	100	6.7	313.8	−25.2	−26.0	4.8	7.5
3	63	32	1964.6	83.0	15.5	538.6	−25.2	−26.2	6.4	3.6
3	151	32	1367.4	57.8	25.6	658.1	−25.2	−26.4	7.1	2.1
3	241	32	824.7	34.8	73.9	755.4	−25.2	−26.7	9.7	1.1
3	0	37	2367.0	100	6.7	313.8	−25.2	−26.0	4.8	7.5
3	21	37	2104.7	88.9	12.5	385.0	−25.2	−26.0	5.4	5.5
3	63	37	1525.2	64.4	29.6	467.4	−25.2	−26.4	7.7	3.3
3	151	37	814.3	34.4	73.7	652.1	−25.2	−26.7	9.5	1.2
3	241	37	460.5	19.4	199.7	669.8	−25.2	−26.9	11.0	0.7
4	0	27	3242.6	100	9.2	315.7	−26.1	−26.7	3.9	10.3
4	63	27	2964.6	91.4	11.9	459.2	−26.0	−27.1	6.7	6.5
4	151	27	2526.7	77.9	15.2	692.1	−26.0	−27.3	7.7	3.7
4	241	27	1811.0	55.9	33.1	950.0	−26.0	−27.5	9.2	1.9
4	0	32	3242.6	100	9.2	315.7	−26.1	−26.7	3.9	10.3
4	63	32	2579.2	79.5	14.7	538.1	−26.0	−27.1	6.7	4.8
4	151	32	2035.7	62.8	26.6	753.9	−26.0	−27.2	9.0	2.7
4	241	32	1375.5	42.4	47.3	964.0	−26.0	−27.7	10.4	1.4
4	0	37	3242.6	100	9.2	315.7	−26.1	−26.7	3.9	10.3
4	21	37	2849.0	87.9	14.7	473.3	−26.0	−27.0	5.9	6.0
4	63	37	2149.9	66.3	26.5	665.3	−26.0	−27.2	7.3	3.2
4	151	37	1378.7	42.5	73.2	883.2	−26.0	−27.5	9.3	1.6
4	241	37	832.5	25.7	164.8	972.1	−26.0	−28.1	12.9	0.9

[a]Values are reported as an average of three replicates. SDs are within 0.1‰ for isotopes, and percentage relative SD of DHA, MGO, and HMF is <5%.

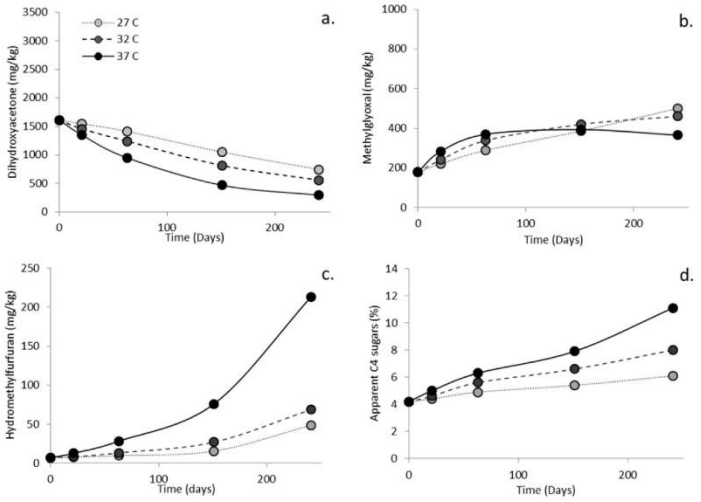

Figure 3. (a) DHA, (b) MGO, (c) HMF (mg kg^{-1}), and (d) apparent C-4 sugar contents (%) of manuka honey sample 1.

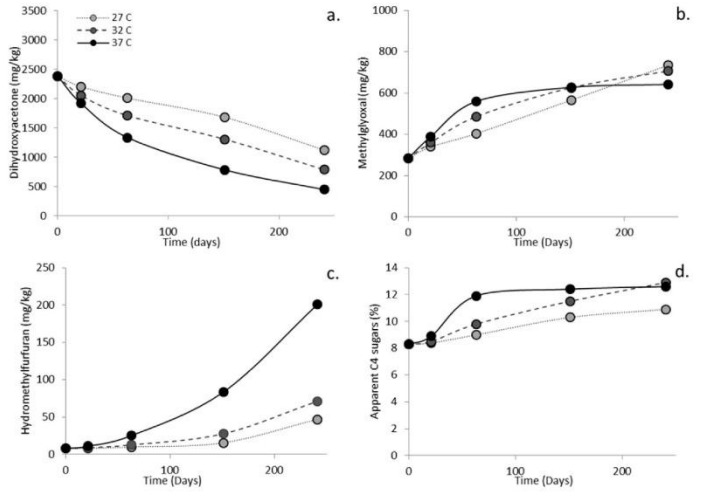

Figure 4. (a) DHA, (b) MGO, (c) HMF (mg kg^{-1}), and (d) apparent C-4 sugar contents (%) of manuka honey sample 2.

aggregation of protein. Such phenomena have been well reported in the production of milk powder.[19−21]

Isotopic enrichment of amino acids in plant seeds and leaves has been studied using LC-IRMS[22] over a range of plant types; the mean $\Delta^{13}C$ values (difference from mean $\delta^{13}C$ for all amino acids) for lysine for leaf and seed were 1.9 and 1.3, respectively. The difference between leaf and seed for lysine was significant, and it might be expected that nectar and hence honey may differ somewhat from these figures. However, it is clear that the loss of an amino acid that makes a positive contribution to the mean would render the mean more negative, similar to the more negative shift seen in clover honey $\delta^{13}C$ protein values (Table 1). A similar positive contribution by lysine was observed in plants in an earlier study.[23] Furthermore, following the demonstrated transfer of essential amino acids including lysine from pollen to eggs of the butterfly *Heliconius charitonia*,[24] it can be assumed that lysine originating from the plant might be transferred to larval bees and hence that contributions from the bee to the honey would have similar

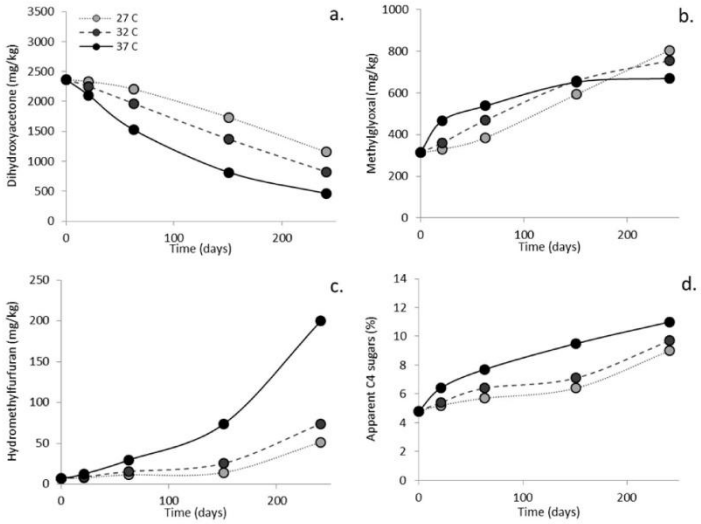

Figure 5. (a) DHA, (b) MGO, (c) HMF (mg kg^{-1}), and (d) apparent C-4 sugar contents (%) of manuka honey sample 3.

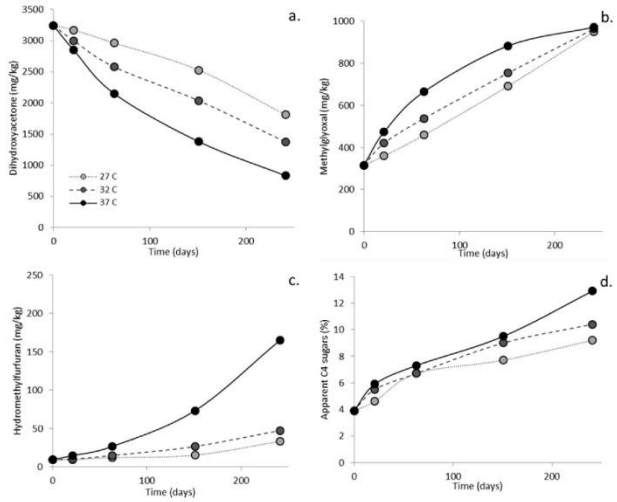

Figure 6. (a) DHA, (b) MGO, (c) HMF (mg kg^{-1}), and (d) apparent C-4 sugar contents (%) of manuka honey sample 4.

stable isotope values as the plants from which the bee derives pollen.

Manuka Honey. Four manuka honeys with different DHA and MGO contents were selected for storage trials to assess apparent C-4 sugar evolution as DHA, MGO, and HMF evolve at 27, 32, and 37 °C over 241 days (Table 2; Figures 3–6). Initial DHA levels ranged from 1612.4 to 3242.6 mg kg^{-1}. At t = 0, all samples had some MGO content ranging from 178.5 to 315.7 mg kg^{-1} (NPA 7.9+ to 11.2+) as they had been harvested and stored at 20 °C for 10 months prior to the trial.

During the course of incubation, DHA content decreased and MGO content increased (Table 2), as previously demonstrated by Adams et al.[4] The highest MGO contents attained in the study were 459.7 mg kg^{-1} in sample 1, 734.3 mg kg^{-1} in sample 2, 805.4 mg kg^{-1} in sample 3, and 972.1 mg kg^{-1} in sample 4 (which equates to NPA values between 14+ and

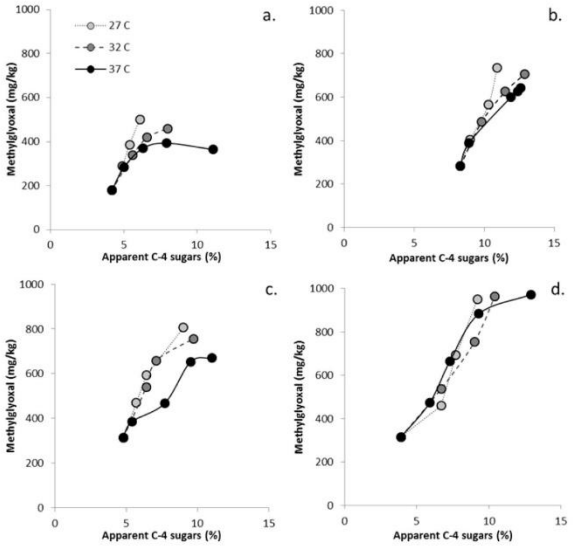

Figure 7. MGO content (mg kg^{-1}) of the four manuka honeys [(a) sample 1; (b) sample 2; (c) sample 3; (d) sample 4] and their corresponding apparent C-4 sugars (%) at 27, 32, and 37 °C.

22+). The highest MGO growth did not always occur in those samples that lost the most DHA (i.e., samples stored at 37 °C for 241 days). After heating at 37 °C, the MGO usually attained a maximum value around 150 days and began to decrease, apart from sample 4, which had a higher initial DHA content than the other samples, so its MGO growth was just attaining its maximum at t = 241 days. At 32 °C, it appeared that all samples except sample 4 were beginning to plateau at a maximum at t = 241 days, whereas sample 4 still had increasing MGO content. At 27 °C, MGO content was still increasing in all samples at t = 241 days, which suggested that at lower storage temperature, MGO content is slower to develop; however, given sufficient time, the MGO content will grow to higher levels than if stored at higher temperatures to accelerate the reaction.

The MGO growth was also accompanied by a similar increasing growth in HMF and apparent C-4 sugars. All four manuka honey samples had initial HMF levels <10 mg kg^{-1}, and by day 241 all but one replicate had exceeded 40 mg kg^{-1} (the Codex recommended threshold), with some in excess of 200 mg kg^{-1}. HMF continued to grow at a much faster rate when the honey was incubated at 37 °C than at 27 or 32 °C.

Apparent C-4 sugars increased with both increasing storage temperature and increasing time. In samples 1, 3, and 4, the apparent C-4 sugars were 4.2, 4.8, and 3.9%, respectively, before incubation, which were well below the permissible AOAC 998.12 C-4 sugar threshold of 7%. Sample 2 had apparent C-4 sugars of 8.3% at 0 days, suggesting a possible apparent C-4 sugar contamination prior to incubation. However, none of these samples were from hives that had undergone sugar feeding prior to nectar collection by bees. Apart from sample 2 at 37 °C, in which apparent C-4 sugars had rapid growth and then remained fairly constant from 63 days onward, all other samples showed steady growth of apparent C-4 sugars over the entire incubation period, and by 151 days, the average apparent C-4 sugars exceeded 7% in all cases regardless of the temperature, suggesting that storage temperatures considerably lower than 27 °C may be a critical factor in retarding the growth of apparent C-4 sugars. A plot of MGO levels and corresponding apparent C-4 sugars of the four manuka honey samples in this study showed that even though MGO growth began to peak and stabilize after t = 150 days, C-4 sugars continued to grow. This phenomenon would explain higher C-4 sugar contents found in older, low NPA or MGO honey (Figure 7). Because DHA and MGO continue to evolve in the jar, even after packaging of honey, the evolution of apparent C-4 sugars would also continue (similar to HMF content) and would be exacerbated when honey is stored at higher ambient temperatures, even for as little as 3 weeks. It is likely that temperatures exceeding 37 °C would increase apparent C-4 sugars at a faster rate.

In this study, the average apparent C-4 sugars of the four manuka honeys after 241 days of storage were 11.1, 12.9, 11.0, and 12.9%, respectively. This research strongly supports the conclusions drawn by Rogers et al.[8] that excess C-4 sugar feeding to bees is not the main cause for C-4 sugars exceeding 7% in New Zealand manuka honey. The key cause is a protein effect, which is activated in the presence of higher levels of DHA and/or MGO found in bioactive manuka honey, which react and cause isotopic fractionation and may also be responsible for other chemical changes.[25] This reaction most likely consumes acidic (^{13}C enriched) amino acids, causing the δ^{13}C protein value to become more negative and thereby generating a bigger Δ (δ^{13}C honey-protein), which artificially enhances the apparent C-4 sugar value as seen after the aging and heating of NPA honey in this study.

When honey is analyzed using the AOAC 998.12 method, unadulterated New Zealand manuka honeys with higher NPA levels (>MGO 260 mg kg^{-1} or > NPA 10+) may have apparent C-4 sugars up to 12.9% (highest C-4 sugar content found in this study), and further increases will occur if the honey is stored for a longer duration at ambient temperature.

We therefore recommend that an exception to the AOAC method 998.12 be considered for all New Zealand manuka honey that has demonstrated DHA and/or MGO levels with the potential to grow beyond MGO > 250 mg kg^{-1}; on the basis of this study and past research,[8] allowable C-4 sugar levels should be at least 13% in manuka honey.

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Notes

The authors declare no competing financial interest.

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Appendix G Summary of initial DHA and water content of real honey matrices used in storage trials

Appendix G contains the initial concentrations of DHA and water content in real honey matrices (mānuka and clover) used throughout this research. This includes:

- Clover honey doped with 10,000 mg/kg DHA and stored at 37 °C
- Mānuka honey doped with 10,000 mg/kg DHA and stored at 37 °C
- Clover honey doped with 2,000 mg/kg DHA and stored at various temperatures (27, 20 and 4 °C)
- Mānuka honey (some doped with extra DHA) and stored at various temperatures (37, 27 and 20 °C)

Table G.1 Summary of DHA-doped clover honeys (~10,000 mg/kg) stored at 37 °C.

Sample	Initial DHA (mg/kg)	Initial DHA (mmol/kg)	Initial Water Content (% Moisture)
Happy Bee A	10253	114	19.9
Happy Bee B	10337	115	20.0
Airborne A	11670	130	18.2
Airborne B	11235	125	18.3
Holland A	10940	121	18.3
Holland B	11124	124	18.6
Katikati A	11063	123	18.1
Katikati	11168	124	18.1

Table G.2 Summary of DHA-doped (~10,000 mg/kg) mānuka honeys used in storage trials at 37 °C.

Sample	Initial DHA (mg/kg)	Initial DHA (mmol/kg)	Proline (mg/kg)	Proline (mmol/kg)	Initial Water Content (% Moisture)
946 A	11123	123			21.9
946 B	11007	122			21.7
953 A	12915	143			21.3
953 B	13395	149			21.4
1394 A	11410	127			19.3
1394 B	11738	130			19.5
1394 + proline A	10221	113	779	7	22.6
1394 + proline B	10875	121	784	7	22.4

Table G.3 Summary of DHA-doped clover honeys (~2,000 mg/kg) used in storage trials at various temperatures.

Sample	Initial DHA (mg/kg)	Initial DHA (mmol/kg)	Temperature (°C)	Initial Water Content (% Moisture)
Happy Bee A	1994	22	27	20.5
Happy Bee B	1985	22	27	21.0
Airborne A	1987	22	27	22.9
Airborne B	1997	22	27	22.6
Holland A	1962	22	27	21.3
Holland B	1955	22	27	22.2
Happy Bee A	1968	22	20	20.7
Happy Bee B	2019	22	20	20.8
Airborne A	1981	22	20	23.0
Airborne B	1953	22	20	22.5
Holland A	1958	22	20	22.0
Holland B	1964	22	20	22.4
Happy Bee A	1979	22	4	22.1
Happy Bee B	1958	22	4	21.2
Airborne A	1979	22	4	22.6
Airborne B	1980	22	4	22.8
Holland A	1981	22	4	21.9
Holland B	1977	22	4	21.7

Table G.4 Summary of DHA-doped mānuka honeys stored at various temperatures.

Sample	Initial DHA (mg/kg)	Initial DHA (mmol/kg)	Temperature (°C)	Initial Water Content (% Moisture)
946 A	2542	28	37	20.7
946 B	2548	28	37	20.8
953 A	4909	55	37	20.0
953 B	4937	55	37	20.2
66 A	3548	39	37	22.6
66 B	3523	39	37	21.7
78 A	3007	33	37	20.2
78 B	3017	33	37	20.7
84 A	3961	44	37	22.8
84 B	3896	43	37	22.9
1404 A	3547	39	37	19.2
1404 B	3550	39	37	19.2
1404 A	3551	39	27	19.1
1404 B	3557	39	27	19.1
1404 A	3546	39	20	19.1
1404 B	3565	40	20	19.1

Appendix H High pressure processing

Table H.1 Concentrations of DHA, MGO and HMF for each honey replicate sample after each HPP treatment regime measured by the PFBHA method.

Sample	Run	Time (min)	Pressure (MPa)	DHA (mg kg)	HMF (mg/kg)	MGO (mg/kg)
1218 A	0	0	0	1175	7	164
1218 A	1	15	100	1165	7	165
1218 A	2	45	100	1169	8	161
1218 A	3	90	100	1178	8	163
1218 A	4	15	400	1176	8	163
1218 A	5	45	400	1201	8	180
1218 A	6	90	400	1197	7	163
1218 A	7	15	600	1178	8	159
1218 A	8	45	600	1174	8	161
1218 A	9	90	600	1186	7	161
1218 B	0	0	0	1170	8	162
1218 B	1	15	100	1182	7	168
1218 B	2	45	100	1154	7	159
1218 B	3	90	100	1170	7	157
1218 B	4	15	400	1181	8	164
1218 B	5	45	400	1184	8	166
1218 B	6	90	400	1170	7	160
1218 B	7	15	600	1183	6	159
1218 B	8	45	600	1187	8	163
1218 B	9	90	600	1155	8	166
976 A	0	0	0	1770	15	626
976 A	1	15	100	1747	13	627
976 A	2	45	100	1777	16	621
976 A	3	90	100	1730	11	608
976 A	4	15	400	1739	16	628
976 A	5	45	400	1749	16	624
976 A	6	90	400	1752	14	622
976 A	7	15	600	1727	16	575
976 A	8	45	600	1767	16	625
976 A	9	90	600	1792	15	623
976 B	0	0	0	1766	16	636
976 B	1	15	100	1723	15	616
976 B	2	45	100	1780	17	620
976 B	3	90	100	1682	14	596
976 B	4	15	400	1759	16	627
976 B	5	45	400	1748	16	612
976 B	6	90	400	1748	15	615
976 B	7	15	600	1744	15	591
976 B	8	45	600	1753	16	621
976 B	9	90	600	1737	14	617
Arataki Clover A	0	0	0	1254	11	27
Arataki Clover A	1	15	100	1296	12	28
Arataki Clover A	2	45	100	1283	13	30
Arataki Clover A	3	90	100	1300	13	30
Arataki Clover A	4	15	400	1288	13	29
Arataki Clover A	5	45	400	1310	13	28
Arataki Clover A	6	90	400	1284	14	29

Sample	Run	Time (min)	Pressure (MPa)	DHA (mg kg)	HMF (mg/kg)	MGO (mg/kg)
Arataki Clover A	7	15	600	1200	13	33
Arataki Clover A	8	45	600	1280	12	28
Arataki Clover A	9	90	600	1295	12	28
Arataki Clover B	0	0	0	1243	11	27
Arataki Clover B	1	15	100	1290	13	30
Arataki Clover B	2	45	100	1274	13	28
Arataki Clover B	3	90	100	1281	12	29
Arataki Clover B	4	15	400	1311	13	31
Arataki Clover B	5	45	400	1299	13	29
Arataki Clover B	6	90	400	1289	14	29
Arataki Clover B	7	15	600	1230	12	27
Arataki Clover B	8	45	600	1290	14	30
Arataki Clover B	9	90	600	1255	12	26
Arataki Mānuka A	0	0	0	624	65	358
Arataki Mānuka A	1	15	100	635	69	372
Arataki Mānuka A	2	45	100	645	73	365
Arataki Mānuka A	3	90	100	639	65	366
Arataki Mānuka A	4	15	400	625	73	365
Arataki Mānuka A	5	45	400	622	72	358
Arataki Mānuka A	6	90	400	628	65	355
Arataki Mānuka A	7	15	600	622	50	358
Arataki Mānuka A	8	45	600	610	68	350
Arataki Mānuka A	9	90	600	623	62	367
Arataki Mānuka B	0	0	0	640	65	366
Arataki Mānuka B	1	15	100	642	66	368
Arataki Mānuka B	2	45	100	645	71	361
Arataki Mānuka B	3	90	100	616	63	363
Arataki Mānuka B	4	15	400	627	71	365
Arataki Mānuka B	5	45	400	647	72	365
Arataki Mānuka B	6	90	400	638	65	361
Arataki Mānuka B	7	15	600	633	73	355
Arataki Mānuka B	8	45	600	630	68	359
Arataki Mānuka B	9	90	600	621	60	357
Artificial Honey A	0	0	0	2044	8	19
Artificial Honey A	1	15	100	1871	11	19
Artificial Honey A	2	45	100	2011	7	19
Artificial Honey A	3	90	100	1978	8	21
Artificial Honey A	4	15	400	1982	9	20
Artificial Honey A	5	45	400	1855	8	20
Artificial Honey A	6	90	400	1988	10	19
Artificial Honey A	7	15	600	2038	11	21
Artificial Honey A	8	45	600	1988	9	20
Artificial Honey A	9	90	600	1966	8	18
Artificial Honey B	0	0	0	2033	9	20
Artificial Honey B	1	15	100	1947	8	18
Artificial Honey B	2	45	100	2045	7	19
Artificial Honey B	3	90	100	1983	8	18
Artificial Honey B	4	15	400	2074	10	21
Artificial Honey B	5	45	400	1865	8	18
Artificial Honey B	6	90	400	1943	8	18
Artificial Honey B	7	15	600	2101	11	20
Artificial Honey B	8	45	600	1970	8	18
Artificial Honey B	9	90	600	1983	7	19
Hollands Clover A	0	0	0	1212	17	23
Hollands Clover A	1	15	100	1234	16	22

Sample	Run	Time (min)	Pressure (MPa)	DHA (mg kg)	HMF (mg/kg)	MGO (mg/kg)
Hollands Clover A	2	45	100	1241	18	22
Hollands Clover A	3	90	100	1262	18	25
Hollands Clover A	4	15	400	1278	17	22
Hollands Clover A	5	45	400	1256	18	23
Hollands Clover A	6	90	400	1269	18	23
Hollands Clover A	7	15	600	1218	17	23
Hollands Clover A	8	45	600	1260	18	24
Hollands Clover A	9	90	600	1273	17	24
Hollands Clover B	0	0	0	1259	17	25
Hollands Clover B	1	15	100	1244	16	22
Hollands Clover B	2	45	100	1245	18	22
Hollands Clover B	3	90	100	1263	18	26
Hollands Clover B	4	15	400	1376	19	24
Hollands Clover B	5	45	400	1240	18	24
Hollands Clover B	6	90	400	1251	19	24
Hollands Clover B	7	15	600	1258	18	23
Hollands Clover B	8	45	600	1240	17	25
Hollands Clover B	9	90	600	1270	17	23

Table H.2 Percentage change of DHA, MGO and HMF, analysed by the PFBHA method, for each HPP treatment compared to the untreated control for all honey matrices.

		Treatment number								
Sample[a]	**Assay by PFBHA**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
976 A	**DHA**	–1.28	0.42	–2.27	–1.77	–1.21	–1.01	–2.44	–0.15	1.27
976 B	**DHA**	–2.49	0.77	–4.91	–0.39	–1.01	–1.05	–1.26	–0.77	–1.65
1218 A	**DHA**	–0.87	–0.48	0.29	0.13	2.23	1.83	0.31	–0.09	0.91
1218 B	**DHA**	0.99	–1.37	0.02	0.98	1.20	0.02	1.09	1.42	–1.26
AM A	**DHA**	1.76	3.33	2.38	0.25	–0.28	0.73	–0.29	–2.15	–0.04
AM B	**DHA**	0.39	0.85	–3.87	–2.02	1.19	–0.24	–1.14	–1.63	–2.97
AC A	**DHA**	3.31	2.30	3.59	2.70	4.36	2.36	–4.44	2.03	3.24
AC B	**DHA**	3.68	2.44	2.98	5.28	4.41	3.62	–1.09	3.71	0.96
HC A	**DHA**	1.72	2.36	3.99	5.26	3.56	4.56	0.45	3.83	4.88
HC B	**DHA**	–1.22	–1.14	0.29	8.82	–1.55	–0.66	–0.10	–1.51	0.82
AH A	**DHA**	–8.84	–1.63	–3.26	–3.07	–9.69	–2.76	–0.29	–2.78	–3.88
AH B	**DHA**	–4.33	0.60	–2.49	1.99	–8.64	–4.55	3.28	–3.15	–2.48
976 A	**MGO**	0.06	–0.77	–2.95	0.23	–0.36	–0.69	–8.57	–0.20	–0.48
976 B	**MGO**	–3.34	–2.64	–6.54	–1.47	–3.95	–3.38	–7.38	–2.51	–3.10
1218 A	**MGO**	0.94	–1.60	–0.64	–0.52	9.32	–0.72	–3.03	–1.82	–2.03
1218 B	**MGO**	4.03	–1.63	–2.95	1.32	2.54	–1.19	–1.78	0.97	2.46
AM A	**MGO**	3.83	2.08	2.38	2.05	0.06	–0.62	–0.03	–2.14	2.54
AM B	**MGO**	0.49	–1.38	–0.79	–0.46	–0.26	–1.40	–3.11	–1.91	–2.71
AC A	**MGO**	4.21	10.80	10.84	6.72	2.62	5.00	19.90	1.62	2.93

Sample[a]	Assay by PFBHA	Treatment number 1	2	3	4	5	6	7	8	9
AC B	**MGO**	11.82	5.44	7.20	13.24	8.68	8.11	–1.73	10.89	–2.12
HC A	**MGO**	–3.75	–5.23	9.94	–2.13	0.76	2.35	1.50	5.69	5.31
HC B	**MGO**	–10.20	–9.11	4.81	–2.29	–3.13	–2.87	–7.15	0.85	–7.44
AH A	**MGO**	0.92	–2.38	9.09	3.39	2.70	–0.18	8.93	5.84	–2.68
AH B	**MGO**	–11.48	–7.85	–8.79	2.01	–9.17	–11.16	–1.42	–8.59	–7.82
976 A	**HMF**	–13.50	5.67	–28.13	7.46	6.26	–5.27	5.20	6.29	0.98
976 B	**HMF**	–10.38	3.99	–11.79	–1.38	–1.99	–4.91	–4.37	–0.87	–11.55
1218 A	**HMF**	1.50	9.07	3.48	13.04	10.79	–0.97	8.11	7.89	–1.30
1218 B	**HMF**	–2.56	–3.31	–3.70	6.03	7.65	–6.25	–27.79	6.91	–1.27
AM A	**HMF**	6.53	12.02	0.84	11.78	10.37	0.64	–26.33	4.41	–3.74
AM B	**HMF**	2.49	9.69	–2.56	10.15	10.89	0.04	12.47	4.62	–6.69
AC A	**HMF**	3.94	15.96	9.80	10.59	13.53	17.05	9.64	6.85	2.29
AC B	**HMF**	11.66	14.80	7.08	–9.96	14.11	20.26	3.99	20.87	0.93
HC A	**HMF**	–4.14	5.76	4.24	2.68	4.42	6.78	–0.10	4.09	2.24
HC B	**HMF**	–3.87	4.98	3.68	8.35	3.79	10.20	3.23	–1.72	–1.61
AH A	**HMF**	30.51	–7.85	2.62	12.94	0.33	22.67	33.66	5.85	–4.17
AH B	**HMF**	–12.00	–27.04	–11.78	7.61	–10.30	–9.78	18.97	–12.59	–18.27

[a] AM = Arataki Mānuka, AC = Arataki Clover, HC = Hollands Clover, AH = Artificial Honey

Published article on HPP

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Chapter 6, section 6.5 contains work carried out that has resulted in the following publication.

Grainger, M. N. C.; Manley-Harris, M.; Fauzi, M.; Farid, M. M., Effect of high pressure processing on the conversion of dihydroxyacetone to methylglyoxal in New Zealand mānuka (*Leptospermum scoparium*) honey and models thereof. *Food Chemistry* 2014, *153*, 134-139.

This is attached as appendix H in the thesis

Nature of contribution by PhD candidate	For this research I designed the experiment with Merilyn Manley-Harris, prepared samples for analysis (doped honeys and helped prepare them for HPP), compiled and analysed the data and wrote the manuscript.
Extent of contribution by PhD candidate (%)	60%

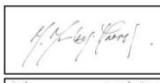
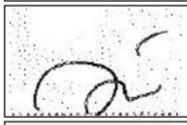
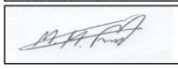
CO-AUTHORS

Name	Nature of Contribution
Merilyn Manley-Harris	Concept discussion and paper revision
Noor Fauzi	Preparation of samples for HPP and analysis of honey samples by HPP
Mohammed Farid	Concept discussion , HPP experimental planning and paper revision

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

Name	Signature	Date
Merilyn Manley-Harris		29/09/2014
Noor Fauzi		08/10/2014
Mohammed Farid		29/09/2014

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Effect of high pressure processing on the conversion of dihydroxy acetone to methylglyoxal in New Zealand mānuka (*Leptospermum scoparium*) honey and models thereof

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ABSTRACT

The effect of high pressure processing (HPP) on the conversion of dihydroxyacetone (DHA) to methylglyoxal (MGO) was examined in New Zealand mānuka honey and models thereof. The objective was to confirm that previously reported increases of MGO with HPP treatment originated from conversion of DHA. RP-HPLC was used to quantify DHA, MGO and hydroxymethylfurfural (HMF) after derivatisation with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) or (in the case of MGO) separately with *o*-phenylenediamine (OPD). Fresh and stored mānuka honey, clover honey with DHA added and artificial 26 honey with DHA added were subjected to nine different pressures and holding times and compared to untreated samples. There was no consistent trend of decrease in DHA or increase in MGO for any of the samples with any treatment. Samples showed random change generally within 5–10% of an untreated sample for MGO, DHA and HMF. HPP does not accelerate the conversion of DHA to MGO in honey.

1. Introduction

New Zealand mānuka (*Leptospermum scoparium*) honey commands a premium price world-wide because of its non-peroxide antibacterial (NPA) activity. NPA is largely attributable to the unusually high levels of methylglyoxal (MGO) found in mānuka honey (Adams et al., 2008; Atrott & Henle, 2009). The remuneration received by beekeepers and the retail price of the packaged mānuka honey is dependent upon the measured NPA and thus upon the content of MGO.

MGO forms during maturation of the honey by the chemical conversion of dihydroxyacetone (DHA), which is derived from the nectar of the mānuka tree (Adams, Manley-Harris, & Molan, 2009). In order to maximise commercial returns, beekeepers and honey producers may store honey for long periods of time and/or use warming to maximise the extent of the conversion; this process may take many months to achieve optimum MGO content. During the storage period, as in all honeys, 5-hydroxymethylfurfuraldehyde (HMF) also increases through an unrelated reaction, which is exacerbated by heat. Thus storage becomes a delicate balance between optimising the MGO content and minimising HMF content in order to produce an export-quality product. The Codex standard for HMF in honey is, with certain specified exceptions, 40 mg kg^{-1} (Codex Alinorm 01/25, 2001), and many importing agencies routinely measure HMF content.

There was thus considerable interest when Al-Habsi and Niranjan (2012) reported increases in MGO concentration (up to ~17%) in one sample of mānuka honey, which had an initial MGO content of 385.9 mg kg^{-1} (Unique Manuka Factor, UMF 10+), when it was subjected to various high pressure processing (HPP) regimes. There was no concomitant increase in HMF content or change in diastase number and the increase in MGO content, as would be expected, correlated linearly to the percentage inhibition as measured by minimum inhibitory concentration.

HPP is a non-thermal treatment which, as an alternative to classical thermal processing, has been reported to have a positive effect on product quality with characteristics of freshness and improved functionality (Butz, 2010; Nguyen & Balasubramaniam, 2011, chap. 1; Patras, Brunton, Da Pieve, & Butler, 2009). Although there is extensive literature describing the effects of HPP on the quality of other foods such as juices, dairy products, meats, fruits and vegetables, the effects of HPP on honey are less documented; Akhmazillah, Farid, and Silva (2013) demonstrated that HPP increased total phenolic content in mānuka honey by 47%, which was associated with the production of higher antioxidant activity.

HPP is excellent for pasteurisation because it affects microbial cells and large molecules such as enzymes but is generally thought not to affect small molecules (Li et al., 2006; Ramaswamy, Balasubramaniam, & Kaletunç, 2013; Shook, Shellhammer, & Schwartz,

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2001). Although not exactly analagous, since the experiment was conducted at elevated temperature, it has been demonstrated in a model system that high pressure promotes the initial condensation of the Maillard reaction but inhibits further reaction of the Amadori compound (Guan et al., 2011); thus it may be that, where temperature is not limiting, pressure can affect small molecules.

Fermentation (spoilage) of honey by yeast occurs within a 12 month period if water content rises above 17.1% (Lochhead, 1934), thus a treatment system that could prevent spoilage and increase MGO content without concomitant increase in HMF has many attractions for honey producers.

Al-Habsi and Niranjan (2012) tested for MGO using *o*-phenylenediamine (OPD) derivatisation following the method of Adams et al. (2008) but did not directly assay DHA, thus although accelerated conversion of DHA to MGO was claimed, it was not proven since there remains the possibility that this change was due to a release of MGO bound to protein or amino acids in the early, reversible stages of a Maillard-type reaction (Klöpfer, Robert Spanneberg, & Glomb, 2011; Van Lancker, Adams, & De Kimpe, 2012). If this is the case, it is possible that the change in MGO content resulting from HPP might not be permanent. Al-Habsi and Niranjan (2012) did not comment upon the durability of the observed changes.

This paper reports an extended study of the effects of HPP upon the conversion of DHA to MGO in New Zealand mānuka honey in an attempt to confirm whether the increase in MGO observed by Al-Habsi and Niranjan (2012) originated from the conversion of DHA and to confirm the possibility that the technique might be of use to commercial honey producers.

2. Materials and methods

2.1. Samples and materials

Arataki mānuka honey with a stated unique mānuka factor (UMF)+10, Arataki clover blend honey (Havelock North, New Zealand) and Hollands premium white clover (New Zealand Honey Ltd.) were purchased locally; two fresh mānuka honeys (976, 1218) were kindly supplied by Steens Honey Ltd., Te Puke.

Methylglyoxal (MGO, 40% w/w), 1,3-dihydroxyacetone (DHA, 97%) and 5-hydroxymethyl-2-furaldehyde (HMF, 99%), D-(+)-glucose, sucrose, D-(−)-fructose and D-gluconic acid lactone (99–100%) were obtained from Sigma–Aldrich.

O-(2,3,4,5,6-Pentaflurobenzyl)hydroxylamine hydrochloride (PFBHA, 99+%) was obtained from Alfa Aesar. Hydroxyacetone, (HA, ⩾90%) and *o*-phenylendiamine (OPD, ⩾98.0%) were obtained from Aldrich.

HPLC grade water was prepared from a Barnstead E-Pure water system (18.1 MΩ cm). The use of the term water refers to HPLC grade water unless otherwise stated.

2.2. Sample preparation

Artificial honey was made by adding fructose (24.00 g), glucose (21.72 g) and sucrose (1.68 g) to water (10.5 g) which had DHA added to give a final concentration of (2000 mg kg^{-1}); the DHA solution was pH adjusted to 4.0 with gluconic acid before addition to the sugar. Clover honeys (60 g) were doped with DHA solution (1.5 ml) to a final concentration of ~1200 mg kg^{-1}.

Duplicate samples were subjected to HPP; each sample was divided into ten sub-samples; the tenth sub-sample was the untreated control. The other 9 sub-samples were packed in 5 × 5 cm transparent plastic film pouches (Cas-Pak plastic vacuum pouch, New Zealand) and thermosealed under vacuum (this plastic film is made of cast polypropylene for excellent transparency and heat sealing qualities and can withstand temperatures up to 125 °C). Each of the 9 sub-samples was subjected to a different HPP treatment.

Treated samples were stored at 0 °C until required. Samples were subsequently analysed for DHA, MGO and HMF; if the results of an analysis for the duplicates differed by more than 10% the analysis was repeated for each of the duplicates.

2.3. High pressure processing

High pressure processing was carried out in the Department of Chemical and Materials Engineering at The University of Auckland. A high pressure processing (HPP) unit Avure 2 L – 700 Laboratory Food Processing System (Avure Technologies, Columbus, OH, USA) was used. The equipment consisted of a 2 L cylindrical shaped pressure treatment chamber, water circulation, a pumping system and a control system operated through a computer with software supplied by the manufacturer. Distilled water was used as the medium in the chamber in which the honey samples were placed. The fluid pressure acted uniformly in all directions due to the almost instantaneous, isostatic pressure transmission (Abdul Ghani & Farid, 2007). The equipment operated at a maximum pressure of 600 MPa and a temperature up to 90 °C. The treatment time was the holding pressure time and did not include the pressure 'come-up' time and the decompression time. The temperature of the pressure medium (distilled water) was measured using a thermocouple. Duplicates of vacuum-packed honey samples (5 g) were subjected to different conditions of pressure (100, 400 and 600 MPa) at close to ambient temperature for 15, 45 and 90 min. The adiabatic heating of 100, 400 and 600 MPa gave an average processing temperature of 25.75 ± 0.95, 28.71 ± 0.90 and 30.18 ± 2.14 °C, respectively during the holding pressure phase. Pressure 'come-up' time was approximately 1.5 min while the decompression time was <20 s. After treatment, the samples were immediately cooled in ice water.

2.4. High performance liquid chromatography (HPLC) – PFBHA method

O-(2,3,4,5,6-Pentaflurobenzyl)hydroxylamine hydrochloride (PFBHA) was used as a derivatising agent for HPLC analysis (PFBHA method, Windsor, Pappalardo, Brooks, Williams, & Manley-Harris, 2012). This method was able to quantify MGO, DHA and HMF simultaneously. Hydroxyacetone (HA, 3.34 mg ml^{-1}) was used as the internal standard solution. The PFBHA derivatising solution (20 mg ml^{-1}) was made up in citrate buffer (0.1 M) and adjusted to pH 4 with NaOH (4 M). Honey samples (0.20 ± 0.02 g) were weighed into test tubes, HA solution (internal standard, 250 μl) was added, the sample was mixed and left to stand for 1 h, at which time PFBHA derivatising solution (1,200 μl) was added, mixed and left to stand for a further hour. ACN (6 ml) was added and mixed until all crystals were dissolved; water (2 ml) was added and mixed before analysis. Standards were made up in an artificial honey matrix (0.20 ± 0.02 g); HA (internal standard, 250 μl), DHA stock (100 μl) and MGO + HMF stock (40 μl) were added, and the standards prepared in the same manner as the samples. Standards ranged from 0–13,000 mg kg^{-1} for DHA, 0–1,400 mg kg^{-1} for MGO and 0–100 mg kg^{-1} for HMF. HPLC was performed on an HPLC system fitted with two Waters 515 pump, 996 photodiode array (PDA) detector (240–400 nm) and autosampler (set at 20 °C), and an Alltech Elite degassing system. A Symmetry Shield RP18 column (5 μm, 3.0 × 250 cm) was used for separation and a 20 μl injection was used. The system was controlled using Waters Empower™ 2 Chromatography software. The system was operated with gradient elution using 30:70 (v:v) ACN:H_2O as mobile phase A and 100% ACN as mobile phase B and a constant combined flow rate of 0.8 ml min^{-1}. Gradient steps

were [min (%B)]: 0 (10), 2.5 (10), 10.5 (50), 18 (100), 25 (10), 35 (10). A linear calibration curve was constructed using the HPLC peak area ratios of DHA: HA, (MGO peak 1 + MGO peak 2): HA and (HMF peak 1 + HMF peak 2): HA.

2.5. HPLC–OPD method

o-Phenylendiamine (OPD) was used as the derivatising agent for the second HPLC method (OPD method; Adams et al., 2008). This method was used to quantify MGO alone as an adjunct to the PFBHA method and for fairer comparison with the work reported by Al-Habsi and Niranjan (2012), in which this method was used. Honey (0.6 g) was dissolved in water (30% (w/v)). Honey solution (1.5 ml) was reacted with 2% (w/v) OPD in 0.5 M phosphate buffer (0.75 ml, pH 6.5) and left to react (16 h). Reactions were performed in the dark at room temperature. Samples were filtered using a 0.45 μm filter before analysis. MGO standards were made up in water, ranging from 0 to 0.2 mg ml^{-1}. Standard (1.5 ml) was added to 2% (w/v) OPD in 0.5 M phosphate buffer (0.75 ml, pH 6.5) and reacted (16 h) at room temperature with light excluded. HPLC was performed on the same HPLC system as used for the PFBHA method. The system was operated with gradient elution with 0.075% acetic acid in water as mobile phase A and 80:20 (v/v) MeOH: H2O as mobile phase B and a constant combined flow rate of 0.3 ml min^{-1}. Gradient steps were [min (%B)]: 0 (10), 4 (10), 5 (42), 30 (55), 31 (100), 34 (100), 35 (10), 40 (10). A linear calibration curve was created using MGO concentration versus peak area.

Table 1
HPP treatment regimes of honey samples.

Treatment number	Time (min)	Pressure (MPa)
1	15	100
2	45	100
3	90	100
4	15	400
5	45	400
6	90	400
7	15	600
8	45	600
9	90	600
Control	0	0

2.6. Statistical analysis

Statistical analysis was performed using either Microsoft Office Excel 2007® or Minitab 16® Statistical Software.

2.7. HPP treatment regimes

Three mānuka honeys (one store-bought and two freshly harvested), two clover honeys (doped with DHA) and an artificial honey (doped with DHA) were subjected to a range of pressures (100, 400 and 600 MPa) and times (15, 45 and 90 min). This resulted in 10 different duplicate treatments, including the untreated control, Table 1. All samples were analysed by the PFBHA method to quantify DHA, MGO and HMF simultaneously. In addition, the untreated control and samples treated for 90 min at 600 MPa (treatment 9) were analysed for MGO alone by the OPD method to permit direct comparison with the work of Al-Habsi and Niranjan (2012) in which this method was used to assay MGO.

3. Results and discussion

For the maximum treatment pressure and duration (run 9, 600 MPa, 90 min), there was less than 8% difference, randomly distributed, in MGO content between the treated samples and untreated control when analysed using the PFBHA method and for all but three samples the difference was less than 5% (Fig. 1). The three exceptions were observed in the clover and artificial honeys which had an initial MGO content which was an order of magnitude smaller than the mānuka honeys (Supplementary Table 1), making assay more difficult. A similar result was obtained for DHA but HMF showed slightly more variation. The results for all treatments of all samples are given in Table 2; greater variation of MGO content within and between replicates is again seen in the honeys with low initial concentrations of MGO.

These results were in sharp contrast to the observations of Al-Habsi and Niranjan (2012) who described a clear trend of time and pressure-dependent increase in MGO content for all pressures (100–800 MPa) and times (15–120 min) used; the percentage

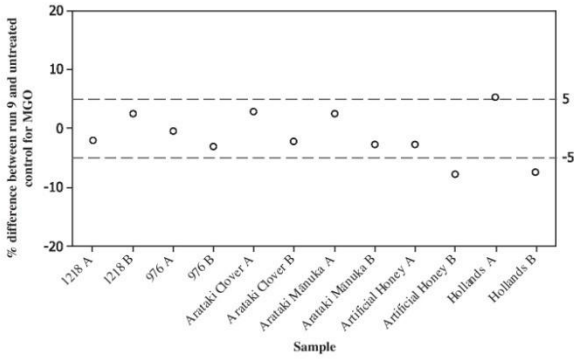

Fig. 1. Percentage difference in MGO content as a result of the maximum treatment pressure and duration (run 9, 600 MPa, 90 min) compared to untreated sample (mean of two samples) for each matrix tested. There was less than 5% difference between the treated samples and untreated control (with three exceptions) when analysed using the PFBHA method.

Table 2
Percentage change of DHA, MGO and HMF, analysed by the PFBHA method, for each HPP treatment compared to the untreated control for all honey matrices.

Sample[a]	Assay by PFBHA	Treatment number							
		1	2	3	4	5	6	7	8	9
976 A	DHA	−1.28	0.42	−2.27	−1.77	−1.21	−1.01	−2.44	−0.15	1.27
976 B	DHA	−2.49	0.77	−4.91	−0.39	−1.01	−1.05	−1.26	−0.77	−1.65
1218 A	DHA	−0.87	−0.48	0.29	0.13	2.23	1.83	0.31	−0.09	0.91
1218 B	DHA	0.99	−1.37	0.02	0.98	1.20	0.02	1.09	1.42	−1.26
AM A	DHA	1.76	3.33	2.38	0.25	−0.28	0.73	−0.29	−2.15	−0.04
AM B	DHA	0.39	0.85	−3.87	−2.02	1.19	−0.24	−1.14	−1.63	−2.97
AC A	DHA	3.31	2.30	3.59	2.70	4.36	2.36	−4.44	2.03	3.24
AC B	DHA	3.68	2.44	2.98	5.28	4.41	3.62	−1.09	3.71	0.96
HC A	DHA	1.72	2.36	3.99	5.26	3.56	4.56	0.45	3.83	4.88
HC B	DHA	−1.22	−1.14	0.29	8.82	−1.55	−0.66	−0.10	−1.51	0.82
AH A	DHA	−8.84	−1.63	−3.26	−3.07	−9.69	−2.76	−0.29	−2.78	−3.88
AH B	DHA	−4.33	0.60	−2.49	1.99	−8.64	−4.55	3.28	−3.15	−2.48
976 A	MGO	0.06	−0.77	−2.95	0.23	−0.36	−0.69	−8.57	−0.20	−0.48
976 B	MGO	−3.34	−2.64	−6.54	−1.47	−3.95	−3.38	−7.38	−2.51	−3.10
1218 A	MGO	0.94	−1.60	−0.64	−0.52	9.32	−0.72	−3.03	−1.82	−2.03
1218 B	MGO	4.03	−1.63	−2.95	1.32	2.54	−1.19	−1.78	0.97	2.46
AM A	MGO	3.83	2.08	2.38	2.05	0.06	−0.62	−0.03	−2.14	2.54
AM B	MGO	0.49	−1.38	−0.79	−0.46	−0.26	−1.40	−3.11	−1.91	−2.71
AC A	MGO	4.21	10.80	10.84	6.72	2.62	5.00	19.90	1.62	2.93
AC B	MGO	11.82	5.44	7.20	13.24	8.68	8.11	−1.73	10.89	−2.12
HC A	MGO	−3.75	−5.23	9.94	−2.13	0.76	2.35	1.50	5.69	5.31
HC B	MGO	−10.20	−9.11	4.81	−2.29	−3.13	−2.87	−7.15	0.85	−7.44
AH A	MGO	0.92	−2.38	9.09	3.39	2.70	−0.18	8.93	5.84	−2.68
AH B	MGO	−11.48	−7.85	−8.79	2.01	−9.17	−11.16	−1.42	−8.59	−7.82
976 A	HMF	−13.50	5.67	−28.13	7.46	6.26	−5.27	5.20	6.29	0.98
976 B	HMF	−10.38	3.99	−11.79	−1.38	−1.99	−4.91	−4.37	−0.87	−11.55
1218 A	HMF	1.50	9.07	3.48	13.04	10.79	−0.97	8.11	7.89	−1.30
1218 B	HMF	−2.56	−3.31	−3.70	6.03	7.65	−6.25	−27.79	6.91	−1.27
AM A	HMF	6.53	12.02	0.84	11.78	10.37	0.64	−26.33	4.41	−3.74
AM B	HMF	2.49	9.69	−2.56	10.15	10.89	0.04	12.47	4.62	−6.69
AC A	HMF	3.94	15.96	9.80	10.59	13.53	17.05	9.64	6.85	2.29
AC B	HMF	11.66	14.80	7.08	−9.96	14.11	20.26	3.99	20.87	0.93
HC A	HMF	−4.14	5.76	4.24	2.68	4.42	6.78	−0.10	4.09	2.24
HC B	HMF	−3.87	4.98	3.68	8.35	3.79	10.20	3.23	−1.72	−1.61
AH A	HMF	30.51	−7.85	2.62	12.94	0.33	22.67	33.66	5.85	−4.17
AH B	HMF	−12.00	−27.04	−11.78	7.61	−10.30	−9.78	18.97	−12.59	−18.27

[a] AM = Arataki Mānuka, AC = Arataki Clover, HC = Hollands Clover, AH = Artificial Honey

change in MGO was between ~0.5% and 17% from the mildest treatment to the most severe, respectively.

For a fairer comparison, the MGO content after the most severe HPP treatment (run 9, 600 MPa, 90 min) was also assayed using the OPD method (Adams et al., 2008) as used by Al-Habsi and Niranjan (2012). The OPD method showed a much larger variation in MGO content between samples than did the PFBHA method but the results were still randomly distributed (Table 3); once again the largest variations in change between replicates were observed in honeys with the lowest initial concentration of MGO. The very long development time in the presence of base for the OPD assay may be the source of the greater variation in this data. In this laboratory, a shorter development time for this assay had been introduced prior to this study to prevent inadvertent conversion of DHA to MGO catalysed by the derivatising agent. Notwithstanding this a 16 h development time as outlined by Adams et al. (2008) was used in the current study for fairer comparison with Al-Habsi and Niranjan (2012), who used the longer development time.

Table 3
Percentage change of MGO analysed by the OPD method, for the most severe HPP treatment (run 9, 600 MPa, 90 min) compared to the untreated control for all honey matrices.

Sample	Percentage change in MGO content after treatment 9 compared with the control
976 A	−6.37
967 B	−8.78
1218 A	−1.20
1218 B	10.78
Arataki Mānuka A	−9.79
Arataki Mānuka B	−14.60
Arataki Clover A	31.28
Arataki Clover B	5.66
Hollands A	−7.91
Hollands B	−12.48
Artificial Honey A	−20.60
Artificial Honey B	−12.98

Measurements of DHA and MGO by the PFBHA method for the Arataki mānuka honey generally fell within ±5% of the untreated sample and were randomly distributed for all pressures, and all times at any given pressure; similar behaviour was also observed for HMF but with slightly greater variation, (Fig. 2, Table 2). This result again contrasted with Al-Habsi and Niranjan (2012), who observed, for every pressure used, an initial, linear increase of MGO content with increasing time eventually levelling off at a higher concentration. The magnitude of this effect increased with pressure.

During the course of maturation of honey DHA converts to MGO, however, the conversion is not stoichiometric (Adams et al., 2009) as was predicted by kinetic studies in aqueous media (Federoňko & Konigstein, 1969) and both MGO and DHA are consumed presumably in Maillard-type side reactions with other honey components such as amino acids. Store-bought honeys are likely to have a DHA:MGO ratio of about 2:1 (Atrott, Haberlau, & Henle,

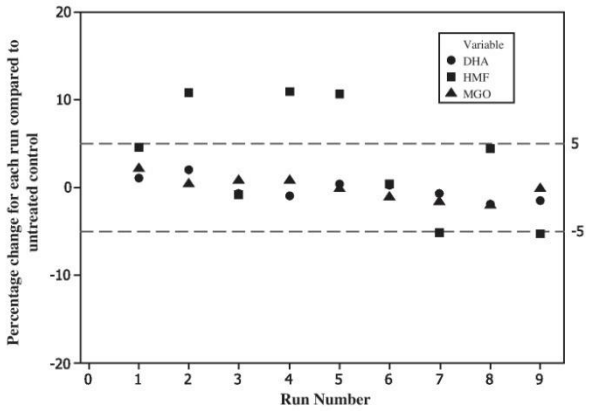

Fig. 2. Percentage difference of the DHA, MGO and HMF concentrations (analysed by the PFBHA method) in Arataki mānuka honey (store bought) for each HPP treatment, compared with the untreated sample (mean of two samples). None of the treatments show over 5% difference in MGO and DHA; all but three HMF samples also had 5% or less difference in concentration.

2012), but with prolonged storage this ratio changes (Windsor et al., 2012) as DHA either continues to convert to MGO or is consumed by side reactions. To eliminate the possibility that the store-bought honey contained insufficient DHA to cause a measurable change in MGO with HPP treatment, two measures were undertaken. Fresh mānuka honeys (976 and 1218) containing respectively ~1767 and 1172 mg kg^{-1} of DHA were used and clover and artificial honeys were doped with DHA at levels, which, based upon previous kinetic trials (Adams et al., 2009), were sufficient to show a clear increase in MGO content over ~3 months of storage at 37 °C. Under HPP treatment all of these honeys manifested the same behaviour as the store-bought Arataki mānuka honey – that is they showed random change within ±10% of the untreated sample for all DHA samples and most of the MGO samples (Table 2). Somewhat wider variation, but still random, was seen in HMF concentrations, which may have been a function of the assay or because the concentration of HMF was very much lower than that of DHA.

The difference between the results observed in this study and those of Al-Habsi and Niranjan (2012) may relate to the fact that HPP has been generally observed to affect bacterial cells and large molecules such as enzymes but not affect small molecules (Li et al., 2006; Ramaswamy et al., 2013; Shook et al., 2001); both DHA and MGO are three carbon molecules and it thus seems unlikely that the kinetics of the conversion would be affected by HPP. As indicated previously, if temperature is not rate limiting, pressure may well affect the reactions of small molecules (Guan et al., 2011); however in this instance temperature was regulated between 25 and 30 °C to retain parity with the conditions used by Al-Habsi and Niranjan (2012). Elevated temperatures are generally contraindicated in treatment of mānuka honey since they can accelerate HMF formation and also lead to reduced yields of MGO (Adams et al., 2008). It is nevertheless possible that careful study of the respective activation energies of the DHA to MGO conversion and the various condensation reactions that consume DHA or MGO, or give rise to HMF, might permit judicious use of high pressure at moderate treatment temperatures to optimise MGO formation. The latter is however outside the scope of the current study.

It is also possible, that denaturation of protein under HPP may somehow release reversibly bound MGO but this change might not be permanent since MGO, thus released could react again with amino acids or proteins. It was not possible to confirm this reversibility in the current study since no sufficiently large and reproducible change was observed under any HPP treatment.

4. Conclusion

We conclude that the observation of increasing MGO content with HPP treatment by Al-Habsi and Niranjan (2012) was not due to conversion of DHA to MGO and that high pressure processing at ambient temperatures cannot at present be recommended as a routine means to achieve rapid conversion of DHA to MGO in mānuka honey. Notwithstanding the foregoing, the possibility that HPP might be used to prevent spoilage of honey during maturation or to alter the phenolic content still remains.

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Appendix A. Supplementary data

Supplementary Data: Actual concentrations (mg kg^{-1}) of MGO, DHA and HMF in all samples for PFBHA assay can be found in Supplementary Table 1. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.12.017.

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Appendix I Initial concentrations of DHA/MGO and HMF in artificial honey systems

Table I.1 Initial concentrations of DHA/MGO and HMF in artificial honey stored at various temperatures.

Sample	Temp (°C)	Initial DHA/MGO (mg/kg)	Initial DHA/MGO (mmol/kg)	Initial HMF (mg/kg)	Initial HMF (mmol/kg)	Initial water content (%)
DHA control A	37	2066	23			18.3
DHA control B	37	2060	23			17.8
MGO control A	37	2113	29			26.6
MGO control B	37	2084	29			25.8
DHA-HMF A	37	2072	23	40	0	18.9
DHA-HMF B	37	2078	23	41	0	18.8
MGO-HMF A	37	2100	29	41	0	18.8
MGO-HMF B	37	2072	29	41	0	18.8
DHA control A	27	1950	22			19.3
DHA control B	27	1957	22			20.2
MGO control A	27	2077	29			19.9
MGO control B	27	2065	29			19.4
DHA-HMF A	27	2079	23	41	0	20
DHA-HMF B	27	2078	23	41	0	19.8
MGO-HMF A	27	2063	29	41	0	20
MGO-HMF B	27	2081	29	41	0	20.1
DHA control A	20	2323	26			21
DHA control B	20	1688	19			20.9
MGO control A	20	2131	30			20.3
MGO control B	20	2117	29			19.7
DHA-HMF A	20	2068	23	40	0	21.2
DHA-HMF B	20	2054	23	41	0	20.9
MGO-HMF A	20	2080	29	43	0	21.1
MGO-HMF B	20	2091	29	41	0	21.2

Appendix J The MATLAB Program

MATLAB (MATrixLABoratory) is a high-performance language for technical computing. It has a user friendly interface that provides useful tools for kinetic simulations of chemical data.[412] MATLAB language requires programmes to be written to solve ordinary differential equations. The rate equations can be estimated and the equations can be run to produce a model of the data. Experimental data can be compared to the model to check the fit.

In order to create a programme each reaction that is occurring in the honey matrix needs to be known, written out separately and assigned a rate constant. To create a programme each species is assigned a number. Differential equations can then be created to describe the situation. Each equation is assigned a rate constant that has been estimated from experimental data and is further refined via MATLAB simulation to get the best fit. Estimates of the starting concentration of each species is also entered into the programme. The programme attempts to encompass all the possible reactions that are occurring and assign each one a rate of reaction.

An artificial honey with DHA and iron added is used to illustrate the process used to create a programme file and to explain the workings of MATLAB. Note that the values and rate numbers used here are not reflective of the final programme. Also note that the non-catalysed reaction of DHA to MGO is not included in this illustration.

1. Each equation is written out and assigned a rate constant

DHA + Iron → DHA-Iron-Complex	k1
DHA-Iron-Complex → DHA + Iron	k2
DHA-Iron-Complex → MGO + Iron	k3

2. Differential equations are written for each species

d[DHA]/dt = –k1 [DHA] [Fe] + k2 [DHA-Iron-Complex]
d[DHA-Iron-Complex]/dt = + k1 [DHA] [Fe] – k2 [DHA-Iron-Complex] – k3 [DHA-Iron-Complex]
d[MGO]/dt = + k3 [DHA-Iron-Complex]

Note that as other reactions are added, they must be added into the correct differential equations or new equations must be formed.

3. Differential equations are converted to a suitable form for MATLAB

MATLAB must be able to recognise each component in the programme. Therefore each species must be assigned a number in a bracket, and an asterisk is used to indicate multiplication.

For the purpose of this illustration, the following assignments were made:
(1) = DHA
(2) = Fe
(3) = DHA-Iron-Complex
(4) = MGO

A semi-colon is used at the end of each line that you do not want MATLAB to print out in the command window after running the script. A percentage sign is used for lines MATLAB will ignore (e.g. text labels and comments). An array, y, with elements y(1), y(2) ... y(n) is assigned as the concentration of each dynamic species, (1) ... (n).

Therefore the equations become:
dy(1)/dt = –k1*y(1)*y(2) +k2*y(3);
dy(2)/dt = –k1*y(1)*y(2) +k2*y(3);
dy(3)/dt = +k1*y(1)*y(2) –k2*y(3);
dy(4)/dt = +k3*y(3);

Failure to have the correct format will prevent MATLAB from running the script.

4. Write a MATLAB script file

A MATLAB programme file (M-file) holds all of the information necessary to execute multiple mathematical operations sequentially. This file holds all of the information required, including all other files to use, starting concentrations, rate constants and plotting commands. A second file holds the differential equations.

Table J.1 shows an example programme file for solving the differential equations). Table J.2 shows an example of a programme file of equilibrium differential equations.

The rates and starting concentrations can be varied accordingly to help improve the fit between the modelled and experimental data.

The command 'xslwrite' was used to convert the output into an excel file. This data was transferred to Minitab and plotted against the experimental data.

Table J.1 Programme for solving the differential equations describing the chemical dynamics.

Line	Information in programme	Explanation
1	% Fe – DHA Simulation	Title of programme
2	Global k1 k2 k3	Tells MATLAB to use these values in all files used in this programme
3	k1 =	Estimates of the three rate constants
4	k2 =	
5	k3 =	
6	%	Spacer
7	DHA0 =	Starting concentrations of the four dynamic species
8	Iron0 =	
9	DHA-Iron-Complex0 =	
10	MGO0 =	
11	%	Spacer
12	t0 = 0;	Time scale of simulation
13	tfinal =	
14	tfinal = tfinal*(1+esp)	esp is the noise-level of the computer
15	y0 = [DHA0;Fe0;Fe-Iron-Complex0;MGO0]	Set initial concentrations of dynamic species
16	% Carry out the integration	

Line	Information in programme	Explanation
17	options = odeset('RelTol',1e–5,'abstol',[1e–5 1e–5 1e–5 1e–5]);	Lines 17 and 18 control the MATLAB ordinary differential equation solver function. Note: 'Equations' is the name of the programme file containing the differential equations to be solved.
18	[t,y] = ode15s('Equations',[t0 tfinal],y0,options,k1,k2,k3);	

19	for n = 1:100000000
	if (t(n) – tfinal) < 0
	else
	npoints = n
	break
	end
	end
		Control the length of integration from t = 0 to t = tfinal

20	% Export results to Excel	Note to explain what following lines are for
21	xlswrite('Simulations',t,1,A2)	Sends time to column A row 2 of excel
22	xlswrite('Simulations',y,1,B2)	Sends simulated data to excel started at column B row 2 of excel

Table J.2 Programme for the chemical dynamics differential equations

Line	Information in programme	Explanation
1	Function dy = Equations(t,y)	Designates these variables to be accessible to all functions
2	Global k1 k2 k3	Calls on the values from main programme
3	dy = zeros (4,1)	Establishes and initialises dy as an array (matrix) of dimension four rows and one column
4	dy(1) = –k1*y(1)*y(2) +k2*y(3)	Differential equations in MATLAB form
5	dy(2) = –k1*y(1)*y(2) +k2*y(3)	
6	dy(3) = +k1*y(1)*y(2) –k2*y(3)	
7	dy(4) = +k3*y(3)	