An investigation of the accelerated loss of diastase (α-amylase) activity in mānuka honey

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Abstract

New Zealand's multi-million dollar apiculture industry exports over 10,000 tonnes of honey annually. Each country has import guidelines to ensure honey quality. The diastase activity assay is used to determine whether honey has been exposed to harsh conditions (excess storage time or high temperatures will denature diastase) and reports the activity as diastase number (DN), where DN must be >8 for exports. Unfortunately, mānuka honey (NZ's high value honey with unique properties) fails the diastase assay more often than other NZ floral honeys. Bioactive compounds [methylglyoxal (MGO), dihydroxyacetone (DHA) and 3-phenyllactic acid (3PLA)] in mānuka honey were predicted to decrease diastase activity by forming modifications on diastase. Hence, the extent, diversity, location and functional impacts of these modifications on diastase were investigated.

Two fresh mānuka honeys, a clover control and clover spiked (MGO, DHA, 3PLA, 3PLA+MGO and 3PLA+DHA) honey samples were stored at 27 °C for ~200 days and periodically tested to observe chemical compositional changes in relation to the loss of diastase activity. The results corroborated previous data; DN decreased in honey over time with increased loss rates observed in mānuka. In the spiked clover samples both DHA and MGO increased rates of diastase activity loss and these rates were enhanced by 3PLA (a proton donor). Enzyme kinetics (enzyme-substrate binding and enzyme rate constants) suggest that denaturation is not the sole mechanism for reduced DN values in honey over time, rather, surface level modifications from bioactive compounds are affecting the affinity of diastase for the substrate.

Diastase was isolated from the honeys (days 0 and ~200) using SDS-PAGE gels and analysed with LC-MS/MS to identify surface modifications on diastase. Results showed evidence of diastase modification over the time trial for all honeys. This occurred in unique locations for both mānuka honeys and MGO and 3PLA+DHA spiked clover honeys. Some of these locations were in proximity to the active site of diastase.

The most abundant honey protein, MRJP1, was used to accurately identify trends in modification changes on proteins over time as a model for the effect occurring on diastase (since similar reactive amino acids are present on both MRJP1 and diastase). All honeys (except the clover control) had an increased number of modifications. The largest increases were observed for the MGO, DHA and 3PLA+MGO spiked honeys. Fewer modifications were detected for the 3PLA+DHA spiked honey despite this having the most pronounced changes in the enzyme kinetic parameters. This is likely because the modification library focused on MGO-induced modifications and did not account for DHA modifications.

In summary, MGO modifications on proteins in honey were observed. DHA modifications and the role of 3PLA as a proton donor were deduced from the enzyme kinetic parameters. While time and temperature denature diastase, surface modifications on diastase may be contributing to the accelerated loss of diastase activity observed for mānuka honey. This necessitates the reassessment of diastase activity as a test for honey quality, especially in the context of complex honey matrices (such as mānuka honey).

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Table of Contents

Abstract ii

Acknowledgements iv

Table of Contents vi

List of Figures ix

List of Tables xiv

List of Abbreviations xvi

Modification Abbreviations xviii

1 Introduction and Literature Review 19

- 1.1 Introduction 19
- 1.2 Regulations of NZ honey for domestic and international sale 20
- 1.3 Diastase (α-amylase) 23
  - 1.3.1 Diastase (α-amylase) structure 29
- 1.4 Honey composition 41
  - 1.4.1 Mānuka honey 42
  - 1.4.2 Sugars 44
  - 1.4.3 Water content 45
  - 1.4.4 Minerals 46
  - 1.4.5 Vitamins 47
  - 1.4.6 Proteins and Enzymes 47
  - 1.4.7 Free amino acids 49
  - 1.4.8 Pollen 50
  - 1.4.9 pH and acids 50
  - 1.4.10 Phenolic compounds 51
  - 1.4.11 Dihydroxyacetone (DHA) and Methylglyoxal (MGO) 56
- 1.5 Research aims 60

1.6 Thesis outline 61
2 Methods 62
2.1 Honey samples 62
2.2 Solvents, standards, reagents, consumables 62
2.2.1 Solvents 62
2.2.2 Standards and reagents 62
2.3 General methods 63
2.3.1 Apparatus temperature management 63
2.3.2 Glassware cleaning 63
2.3.3 Image generation, spectrophotometric and statistical analysis 64
2.3.4 Honey storage time trial 64
2.4 Quantification of DHA, MGO and HMF (3in1 method) 65
2.4.1 Matrices, reagents and Standards 65
2.4.2 Sample preparation 65
2.4.3 Instrument method 66
2.4.4 Data work-up 67
2.5 Diastase activity analysis 67
2.6 Enzyme kinetic analysis 67
2.6.1 Reagents and standards 67
2.6.2 Sample preparation 68
2.6.3 Data work-up 69
2.7 Mānuka marker analysis 70
2.8 Protein modification identification 70
2.8.1 Reagents 70
2.8.2 Sample preparation 71
2.8.3 Data work-up 72
3 Method Validation for Enzyme Kinetics 73
3.1 Method development for enzyme kinetics analysis 73

3.2 Validation of the enzyme kinetic method 75
4 Time Trial Results and Discussion 78
4.1 Time trial set-up... 79
4.2 Changes in HMF over time 82
4.3 Changes in DHA and MGO over time.. 84
4.4 Changes in mānuka markers and phenolic compounds over time 86
4.5 Changes in diastase activity over time... 90
5 Protein Modifications Occurring in Honey 99
5.1 Changes in size of diastase over time in honey.................................. 99
5.2 LC-MS/MS modification databank formation 107
5.3 LC-MS/MS analysis of modifications on honey proteins.................. 117
5.3.1 Modification changes on diastase in honey over time................. 118
5.3.2 MRJP1 modification changes in honey as a case study............... 126
6 Conclusion and Future work.. 129
6.1 Conclusion.. 129
6.2 Recommendations for future research 131
7 References 133
8 Appendix 162

List of Figures

Figure 1.1. Amino acid sequence of *A. mellifera* diastase (UniProt primary accession no. Q8N0N7), yellow-highlighted amino acids form the signal peptide and green-highlighted amino acids are the catalytic triad (full forms of each amino acid given in the Appendix in Table 8.1). 29

Figure 1.2. (a) *A. mellifera* diastase (AlphaFold model, UniProt ID: Q8N0N7) with domains highlighted (cyan = domain A, magenta = domain B and brown = domain C), catalytic triad amino acids highlighted red and disulfide bonds highlighted yellow. (b) Surface charge distribution of diastase. (c) *A. mellifera* diastase (UniProt ID: Q8N0N7) aligned to *D. melanogaster* diastase (PDB ID: 8ORP). (d) *A. mellifera* diastase with substrate (orange) positioned in the active site based on overlay with *D. melanogaster* diastase (c; PDB ID: 8ORP). 30

Figure 1.3. The general mechanism for the double displacement of starch by diastase modified from Lim and Oslan (2021).[4, 77] 31

Figure 1.4. *A. mellifera* diastase (UniProt ID: Q8N0N7) AlphaFold structure depicting the level of confidence of the model by the colour scheme; blue highlighted amino acids have a pLDDT >90 (very high confidence), cyan highlighted amino acids (circled in orange) have pLDDT >70 (high confidence) and yellow highlighted amino acids (circled in orange) have pLDDT >50 (low confidence). The signal peptide is not shown (which had a pLDDT <50, i.e. very low confidence). 33

Figure 1.5. The skeletal structures of common polyphenolics (phenolic acids and flavonoids) and the R groups represent organic groups. 52

Figure 1.6. The non-enzymatic conversion of DHA to MGO (in blue). 56

Figure 1.7. Reaction pathways that form methylglyoxal in honey (from sources other than dihydroxyacetone), (a) Formation of MGO through a Schiff base intermediate from a hexose, (b) Formation of MGO from a 1,2-ene-diol intermediate from the autoxidation of hexoses.[119] 58

Figure 3.1. The effect of time on the absorbance reading (λ_{660}) post-reaction of the IHC Schade method on the reaction mixes containing type 1 water with different starch substrate concentrations. 74

Figure 3.2. Michaelis-Menten plots of clover (left) and mānuka (right) honeys from the inter-day method validation showing overlapping within error (standard deviations shown as error bars). 76

Figure 3.3. The correlation between the diastase activity (DN number as determined by an external lab using the Phadebas method) to the residual starch solution (mg of starch/mL/min/g of honey) determined in-house using the enzyme kinetics analysis at the set starch substrate concentration 2.22 mg/mL. 77

Figure 4.1. Generic Michaelis-Menten plot showing the positioning of $V_{\max}$ and K_{M}...... 79

Figure 4.2. Increase in HMF concentration normalised to day 0 (mg/kg) vs time for mānuka 1 and 2 and clover control and spiked honey samples stored at 27 °C. The error bars were set to the standard deviation of the duplicates at each time point and fitted with linear regression. 83

Figure 4.3. (a) Normalised DHA concentration (mg/kg) vs time (days) and (b) normalised MGO concentration (mg/kg) vs time (days) for the time trial samples (mānuka honeys, clover control and spiked clover honeys). The errors bars were set to the standard deviation of the duplicates at each time point. 85

Figure 4.4. Concentration of 3PLA (mg/kg) vs time (days) in various time trial honeys stored at 27 °C. 87

Figure 4.5. The concentrations of 2MAP, 2MBA and 4HPLA in mānuka honey 1 (a) and mānuka honey 2 (b) over time when stored at 27 °C... 87

Figure 4.6. Normalised diastase activity decreasing in mānuka honeys 1 and 2 and clover honey (control and spiked) samples stored at 27 °C, over ~200 days. 91

Figure 4.7. Normalised diastase activity decreasing in clover control and spiked honeys stored at 27 °C, over 200 days.. 92

Figure 4.8. Normalised changes in $V_{\max}$ (a) and K_{M} (b) of diastase in various honeys stored at 27 °C.. 93

Figure 4.9. Correlation between the K_{m} and $V_{\max}$ of aged honeys (mānuka honeys 1 and 2, clover control and spiked honeys) at 27 °C for all four time points.................... 98

Figure 5.1. A 10% SDS-PAGE gel with 32% (w/v) diluted honeys run in lanes 1 and 2 and Precision Plus Protein™ standard ladder (labelled). Lane A (left) contains a clover honey and lane B (right) contains a mānuka honey. The gel bands excised for LC-MS/MS protein identification are labelled 1-6... 100

Figure 5.2. The 10% SDS-PAGE gel of time trial honeys. Day 0 and ~200 samples of each mānuka honey (1 and 2), clover control and clover spiked samples. The distinct banding observed for all day 0 samples as compared to the smeared band for end point samples (i.e. day 200 or 203) is illustrated with the example of the 3PLA+DHA spike (using the white brackets).. 101

Figure 5.3. The distinct banding observed for all day 0 samples as compared to the smeared band for the end point samples (i.e. day 200 or 203) is illustrated with the example of the 3PLA+DHA spike (using the white brackets) on a 10% SDS-PAGE gel. This is an expansion of Figure 5.2. 102

Figure 5.4. The *A. mellifera* diastase with active site residues highlighted red in stick format (left) and same structure (right) with the His (yellow), Cys (orange), Lys (green) and Arg (cyan) residues in stick format. These highlighted surface residues present potential modification sites. 105

Figure 5.5. Examples of advanced glycation end products (AGEs) formed from MGO reactions with amino acid residues (cysteine, lysine, arginine and histidine) mentioned in literature.[119, 166, 298-307, 346-352] 106

Figure 5.6. The proposed structures for MGO-chain modifications on cysteine, based on known modifications (such as hemithioacetal or CEC). In blue is the modification, black and red text is used to differentiate different isomeric forms that give the same mass shift. 112

Figure 5.7. The proposed structures for MGO-chain modifications on lysine, based on known modifications (such as MGO-Lys and CEL). In blue is the modification, black and red text is used to differentiate different isomeric forms that give the same mass shift. 113

Figure 5.8. The proposed structures for MGO-chain modifications on arginine, based on known modifications (such as CEA and MG-H1). The blue part of the structure is the modification and the black part is arginine. 114

Figure 5.9. The structure of the DHA-Lys modification (proposed mechanism of formation from the reaction of DHA to lysine is given by Sun *et al*).[310] In blue is the DHA modification and in black is the lysine residue. 115

Figure 5.10. The proposed structures for MGO-chain modifications on histidine, based on known modifications (such as MGO-Lys). In blue is the modification. 115

Figure 5.11. The structure of the 2-caffeic acid (2-CCA) modification on cysteine,[358, 359] with the modification shown in blue. 116

Figure 5.12. The potential structures of Cys-MGO-phenolic compound modifications. The modification from MGO is depicted in blue. 117

Figure 5.13. The coverage map of the amino acid sequence for diastase (UniProt accession number Q8N0N7), (a) mānuka 1 day 0 and (b) mānuka 1 day 203. Peptides detected by LC-MS/MS are highlight grey with blue underlines; detected modifications are shown as small coloured boxes with letters identifying oxidation (aqua) and

carbamidomethylation (pink), active site residues are identified by red circles and the signal peptide is highlighted yellow. Trypsin cleavage sites are identified with triangles red for arginine C-terminal end and orange for lysine C-terminal end. 122

Figure 5.14. The diastase sequence (UniProt accession no Q8N0N7) peptides that were present for the day 0 samples but absent for day 200/203 time trial samples for all the honeys (mānuka 1 and 2, clover control and clover spiked honeys). These represent the peptides where modifications, not in the databank, occurred over the course of the time trial. The signal peptide is highlighted yellow, the active site residues are identified by the red downwards triangles, clover control peptides are highlighted grey while all other honey peptides were assigned a colour according to the legend. 123

Figure 5.15. The diastase sequence peptides, identified on the surface of the AlphaFold model of *A. mellifera* α-amylase (UniProt accession no Q8N0N7 without the signal peptide), that were present for the day 0 samples but absent for end point samples for all honeys (mānuka 1 and 2, clover control and clover spiked honeys). The active site residues are coloured red, while peptide change for each sample was recorded by a colour legend. The skeletal structure of diastase is shown on the right without the surface or any peptide changes. The arrows indicate unique peptide changes not observed for the clover control. 124

Figure 5.16. The correlation between the diastase activity (expressed DN as evaluated using the Phadebas method) and the number of peptides detected for diastase using LC-MS/MS for end point time trial honeys (blue). A higher number of peptides detected correlates to lower modification levels due to the incomplete nature of the current modification library. Day 0 samples (orange) follow the trend of the aged samples which indicates that denaturation is not a significant contributor to activity loss. 126

Figure 5.17. The modifications detected on MRJP1 from the various time trial honey samples. (a) Comparison of the modifications detected between the day 0 and day 200/203 samples of mānuka honeys to the clover control. (b) Comparison of the modifications detected between day 0 and day 200 of the clover control and spiked (MGO, 3PLA, DHA, 3PLA+MGO and 3PLA+DHA spikes) samples. Note that the number of modifications only reflects those in the databank and hence DHA-specific modifications are not accounted for. 128

Figure 8.1. The Michaelis-Menten fitting of time trial honey data for 4 different time points (days, 7, 41/43, 112 and 200/203). Days 43 and 203 for the mānuka honeys and

days 41 and 200 for the clover control and spiked honeys. (a) Mānuka honey 1, (b) Mānuka honey 2, (c) clover control, (d) clover +MGO, (e) clover +3PLA, (f) clover +DHA, (g) clover +3PLA+MGO and (h) clover +3PLA+DHA honey............. 163

Figure 8.2. The unique diastase sequence peptides, identified on the surface of the AlphaFold model of *A. mellifera* α-amylase (UniProt accession no Q8N0N7 without the signal peptide) rotated away from the active site cleft, that were present for the day 0 samples but absent for end point samples of mānuka honeys 1 and 2, clover control and clover spiked with MGO and 3PLA+DHA. The active site residues are coloured red, while peptide change for each sample was recorded by a colour legend. The skeletal structure of diastase is shown on the top left without the surface or any peptide changes.. 164

List of Tables

Table 1.1. Variation requirements for NZ exported honeys to NZ's seven highest honey consuming countries, NR = not reported. 21
Table 1.2. Proteins (including enzymes) found in honey (of *A. mellifera* origin) and their functions, EC numbers are given for proteins which are enzymes in the table. 25
Table 1.3. Comparison of the main diastase activity determination tests (specific for honey).................................... 37
Table 1.4. Factors influencing the presence of diastase and the enzyme activity in honey. 40
Table 1.5. Parameters for the NZ MPI definition of monofloral and multifloral Mānuka honey.[10] 43
Table 1.6. Sugars found honey.[36-38, 123, 125, 126, 154-156] 45
Table 1.7. Concentrations of various phenolic compounds in mānuka, clover and other honeys, µg/g (ND = not detectable and tr = trace).* 53
Table 2.1. Composition of spike standard solutions for the 3in1 method. 65
Table 2.2. The composition each solution type for the quantification of DHA, MGO and HMF (3in1 method). 65
Table 2.3. Gradient conditions for the 3in1 method. 67
Table 2.4. Composition of starch substrate solutions (SS 1-8) for diastase activity analysis. 68
Table 2.5. The composition of various reagents used for protein modification identification. 70
Table 3.1. The R^2 values from the calibration curves and Michaelis-Menten model fits of the method validation of clover and mānuka honey. 75
Table 4.1. The concentrations of MGO, DHA, HMF, 3PLA, 2MAP, 2MBA and 4HPLA (mg/kg) in the mānuka honeys for the time trial experiment....... 80
Table 4.2. Variation in the theoretical spiked vs measured concentration of spiked analytes in the initial analysis of time trial clover honeys....................... 81
Table 4.3. Summarised chemical composition (MGO and DHA concentrations, mg/kg), diastase activity (DN) and kinetics ($V_{\max}$ and K_{M}) of the time trial honeys (mānuka, clover control and clover spiked honeys).* 95

Table 5.1. Most abundant proteins in bands 1-6 (Figure 5.1) and the instances of α-amylase (bands 3 and 4). There are two entries in UniProt for α-amylase (Q9U8X5 and Q8N0N7). 101

Table 5.2. Literature and proposed surface modifications resulting from reactions of MGO, DHA and phenolic compounds with amino acid residues (Cys, Arg, Lys and His). 108

Table 8.1. The standard abbreviations (3-letter and 1-letter codes) given to all 20 amino acids. 162

Table 8.2. The concentrations of MGO, DHA, HMF, 3PLA, 2MAP, 2MBA and 4HPLA (mg/kg) in the mānuka honeys for the time trial experiment (provided by the supplier). 162

List of Abbreviations

−10lgP	−10 logarithm of a *p*-value
2MAP	2’-Methoxyacetophenone
2MBA	2-Methoxybenzoic acid
3PLA	3-Phenyllactic acid
4HPLA	4-Hydroxyphenyllactic acid
ACN	Acetonitrile
AGE	Advanced glycation end product(s)
AH	Artificial honey
ALE	Advanced lipoxidation end products
AOAC	Association of Official Analytical Collaboration
APS	Ammonium persulfate
BLASTp	Basic Local Alignment Search Tool (Protein)
DHA	Dihydroxyacetone
DIN	Deutsches Institut für Normung
DN	Diastase number
DNS	3,5-Dinitrosalicyclic acid
DTT	Dithiothreitol
E-*p*NP-G7	4,6-Ethylidene-(G7)-1-4-nitrophenyl-(G1)-alpha-D-maltoheptaside
EU	European Union
FAO	Food and Agriculture Organisation
GH	Glycoside hydrolase
HA	Hydroxyacetone
HMF	Hydroxymethylfurfural (5-Hydroxymethylfurfural)
HPLC	High performance liquid chromatography
IANZ	International Accreditation New Zealand
IHC	International Honey Commission
LC	Liquid chromatography
MeOH	Methanol
MGO	Methylglyoxal
MPI	Ministry for Primary Industries
MRJP	Major royal jelly protein(s)
MS	Mass spectrometry

MS/MS	Tandem mass spectrometry
MStd	Mixed calibration standard(s)
NCBI	National Centre for Biotechnology Information
ND	Not detectable
NR	Not reported
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDA	Photodiode array detector
PDB	Protein Data Bank
PFBHA	*O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine
pI	Isoelectric point
PI	Potassium iodide
pLDDT	Predicted local distance difference test
*p*NP	*p*-nitrophenol
QC	Quality control
RMSD	Root mean square deviation
RSD	Relative standard deviation
SDS	Sodium dodecyl sulphate
SS	Starch substrate solution
TEMED	*N*, *N*, *N'*,*N'*-Tetramethylethylenediamine
tr	Trace
UniProt	Universal Protein Resource
UV	Ultra-violet
WHO	World Health Organisation

Modification Abbreviations

2-CCA	2-S-Cysteinyl caffeic acid
CEA	Carboxyethyl arginine
CEC	Carboxyethyl-cysteine
CEL	Carboxyethyl-lysine
MG-H1	5-Methylimidazol-4-one/MGO imidazolone, MG-H/ hydroimidazolone isomer 1/ Nδ-(5-methyl-4-oxo-5-hydroimidazo-linone-2-yl)-1-ornithine
MG-H2	Hydroimidazolone isomer 2/ 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazilon-1-yl)pentanoic acid
MG-H3	Hydroimidazolone isomer 3/ 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid
MGO-Cys	Hemithioacetal/ Hemimercaptal

1 Introduction and Literature Review

1.1 Introduction

Diastase activity is an important honey quality parameter imposed on exported New Zealand honey by most receiving countries. The diastase activity test along with the 5-hydroxymethylfurfural (HMF) concentration test ensure honeys have not been exposed to excessive heating or prolonged storage periods and, thus, is of good quality. Unfortunately, mānuka honey, which makes up a large proportion of New Zealand's multi-million dollar honey export revenue,[1] fails the diastase test more often than any other floral type.[2] New Zealand exported 10,250 tonnes of honey by June 2024 and had an export revenue of $419 million for that year.[3] Mānuka honey is stored for extended periods of time before sale to meet the demands of consumers for high MGO (MGO concentration increases over time and diastase activity declines over time). However, high grade mānuka honeys with acceptable HMF levels can still fail the diastase activity test, preventing their export.

A variety of bioactive compounds [methylglyoxal (MGO), dihydroxyacetone (DHA) and phenolic compounds] have been implicated as potential causes for low diastase activity in mānuka honey.[4] Therefore, it is important to investigate how MGO, DHA and phenolic compounds are affecting diastase activity by uncovering whether diastase activity reduction is solely due to the protein denaturing over time or (as is predicted) due to inhibition caused by modifications by MGO, DHA and phenolic compounds to the protein surface. Previously, MGO-specific[5] and phenolic compound modifications[6, 7] have been reported in literature and since mānuka honey has high levels of MGO, DHA and phenolic compounds as compared to other honeys (such as clover)[8] it is suspected that there are significantly more modifications occurring in mānuka honey than in other honeys. Modifications are known to impact the structure of proteins (in the absence of denaturation) and can lead to enzyme activity inhibition.[9]

This thesis aims to explore the extent and diversity of modifications (caused by MGO, DHA and phenolic compounds) on diastase, the locations of these modifications and from this, infer if such modifications are an unaccounted factor in the reduction of diastase activity. The findings will provide insight into the suitability of diastase activity as a measure of honey quality for the complex matrix of mānuka honey.

1.2 Regulations of NZ honey for domestic and international sale

The general requirements of NZ honey to be exported include safety checks, labelling and traceability requirements.[10, 11] For honey to be sold in NZ (and exported out of NZ) it also must meet the CODEX definition of honey (strictly reducing sugar content must be >60% and moisture content <21%).[12, 13]An internationally recognized standard for the components of honey was developed by the CODEX Alimentarius Commission which comprises of the United Nation's Food and Agriculture Organization (FAO) and World Health Organization (WHO).[14, 15] The CODEX standards are used as a guidance for nations to develop their own regulatory standards of honey. The majority of NZ's honey produced is exported (82% in 2023 and 59% in 2024 as per the Ministry for Primary Industries, MPI[3]) in descending order to the United States, China, United Kingdom, Australia, Japan, Germany, Singapore and various other countries according to TrendEconomy.[16] In addition to meeting the general food safety standards (Table 1.1), different regulations are set for imported honeys by individual buyers, typically as limits on the minimum DN value (diastase enzymatic activity) and maximum HMF content. The US[17, 18], UK[19, 20], European Union (EU)[19, 21], Canada[22, 23], and Saudi Arabia[24] require the diastase number, DN, to be >8 (the US allows low enzyme content honeys to have DN >3 and Canada and Saudi Arabia allow DN >3 if HMF is <15 mg/kg). The EU is composed of 27 countries that work on guidelines, laws and other shared values through three main bodies (the European Commission, European Parliament and the Council of the European Union). Other countries, such as China[22, 25, 26] and India[27] have a lower DN requirement (DN >4 and >3 respectively) with no additional conditions.[22, 25-27]

Table 1.1. Variation requirements for NZ exported honeys to NZ's seven highest honey consuming countries, NR = not reported.

Country	Diastase (DN)	HMF (mg/kg)	Fructose + Glucose (reducing sugars)	Sucrose	Moisture	Free acid	Water-insoluble content
United States[17, 18]	>8 (>3 for low enzyme honey)	NR	NR	NR	NR	NR	NR
China[22, 25, 26]	>4	<40	≥60%	≤10 or 5%	<20% (for grade B <24%)	<4 mL/100 g	NR
United Kingdom[19, 20]	>8 (>3 for low enzyme honey)	<40 (honeys from tropical climates or blends of honeys from tropical climates <80)	>60 g/100 g (blossom honey) >45 g /100 g (honeydew and related blends)	<5 g/100 g	<20%	<50 milliequivalents/1 kg	<0.1 g/100 g (<0.5 g/100 g for pressed honey)
Australia[12, 28]	NR	NR	≥60%	NR	≤21%	NR	NR
Japan[22, 29]	NR	<59 (fair trade) <15 (health and nutrition first grade) <40 (health and nutrition)	>60%	<5%	NR	NR	NR

Country	**Diastase (DN)**	**HMF (mg/kg)**	**Fructose + Glucose (reducing sugars)**	**Sucrose**	**Moisture**	**Free acid**	**Water-insoluble content**
Germany[19, 22]	>8 (>3 for low enzyme honey)	<40 (honeys from tropical climates or blends of honeys from tropical climates <80)	>60 g/100 g (blossom honey) >45 g /100 g (honeydew and related blends)	<5 g/100 g	<20%	<50 milliequivalents/1 kg	<0.1 g/100 g (<0.5 g/100 g for pressed honey)
Singapore[30, 31]	NR	NR	NR	8% (w/w)	20% (w/w)	NR	NR

Invertase activity can also be used as a honey freshness/quality indicator since activity is lost at temperatures above room temperature and rapidly lost above 50 °C. Invertase is considered to be more sensitive to temperature and thus a better honey quality indicator than diastase and so is included in the honey standards of some European countries (Germany, Poland, Turkey, Spain and Belgium).[32, 33] In honey, the invertase activity is advised to be >64 units/kg (units are the enzyme activity that can transform 1 µM of substrate in 1 minute under optimal conditions[34]) although some exceptions to this exist (e.g. naturally low enzyme activity honeys are advised to have invertase activity of >45 units/kg).[33, 35] Glucose oxidase activity is also an indicator of honey freshness but is not used as a regulatory test in any country.[36]

1.3 Diastase (α-amylase)

Diastase is a common name given to various amylases (including α- and β-amylase), however, it is interchangeably used for α-amylase when discussing honey in the honey industry because it is consistently reported as the most abundant honey amylase enzyme [exact percentage not reported in literature and *Apis mellifera* β-amylase is not found in the Universal Protein Resource (UniProt)].[36-38] For this reason, the term diastase will be used interchangeably with α-amylase (EC 3.2.1.1) in this research. The diastase enzyme catalyses the breakdown of starch (α-amylose and amylopectin biopolymer) into simple sugars (e.g. mono- and disaccharides).[4, 36-38] Diastase does this by hydrolysing the internal α-D-(1→4) glycosidic bonds in starch and related polysaccharides (e.g. amylopectin, amylose and glycogen) to produce dextrin.[2, 39, 40] The glycosidic bond connects the hemiacetal group of a saccharide molecule to the hydroxyl group of another organic compound.[41] Comparatively, β-amylase (EC 3.2.1.2, previously called saccharogenic amylase) produces maltose by hydrolysing the α-D-(1→4) glycosidic bonds from the nonreducing end of starch (saccharification of starch) and is often preceded by α-amylase in the liquefaction of starch.[42, 43]

Diastase is one of many proteins found in honey (Table 1.2). Floral and geographical origins influence diastase content in honey, however, secretions from the salivary gland of honey bees is the major source of diastase in honey.[38, 39, 44]

There is no literature evidence of the expression of β-amylase in *A. mellifera* and hence, this confirms that α-amylase is the predominant amylase in honey. The bee secretions are added to nectar when it is ripened into mature honey.[44, 45] The predicted role of secreted *A. mellifera* diastase is to degrade any trace/contaminating complex carbohydrates from nectar (which generally does not contain starch) during honey maturation or from pollen to maintain a stable hive environment and to exploit diverse food sources for nutritional needs (nectar and pollen are the primary nutrition sources for honey bees).[46-50] Trace levels of plant diastases are possible in floral honeys as genes for plant α- and β-amylases are switched on prior to anthesis (to convert starch into simpler sugars in the nectaries).[46, 51] However, this is unlikely for mānuka honey as mānuka flowers have green nectaries which have been speculated to obtain sugars for nectar from photosynthesis (instead of stored starch).[52] Other factors that can affect diastase content in honey include pH, nectar flow, and the foraging pattern of bees.[44]

Table 1.2. Proteins (including enzymes) found in honey (of *A. mellifera* origin) and their functions, EC numbers are given for proteins which are enzymes in the table.

Protein (EC number)	**UniProt Primary accession no.**	**No. of amino acids**	**Mass (kDa)**	**Function and importance in honey bees**
Acid phosphatase (EC 3.1.3.2)	Q5BLY5	338	45.4	Acid phosphatase catalyses the hydrolysis of organic phosphates (e.g. ATP, phosphatidic acid and phosphate monoester) to generate inorganic phosphates by the removing a phosphate group from organic phosphate.[38, 45, 53] It is predominantly found in bee venom and acts as an allergen.[54]
α-Amylase (EC 3.2.1.1)	Q8N0N7	493	56	α-Amylase catalyses the conversion of starch into maltose and other smaller sugars through the hydrolysis of internal α-D-(1→4) glycosidic bonds.[36, 38, 45, 55] This is added to nectar during enzyme-loading to ripen nectar to honey.[2]
β-Amylase (EC 3.2.1.2)	Not available	-	-	β-Amylase catalyses the breakdown of starch through the hydrolysis of α-(1→4) glycosidic bonds to form maltose and maltotriose.[38, 45, 56]
Apisimin	Q8ISL8	78	7.9	Apisimin (structural protein) functions to hold queen larvae on the royal jelly surface by forming long fibrous structures (at pH 4.0) by forming the apisin oligomer and contributes to the high viscosity of royal jelly.[57, 58]
Catalase (EC 1.11.1.6)	A0A7M6UWJ0, A0A7M7L745	513, 597	58.0, 67.6	Catalase is an iron-dependent (Fe^{+3}) enzyme which catalyses the degradation of two hydrogen peroxide molecules to form two water molecules and one oxygen molecule.[38, 45]

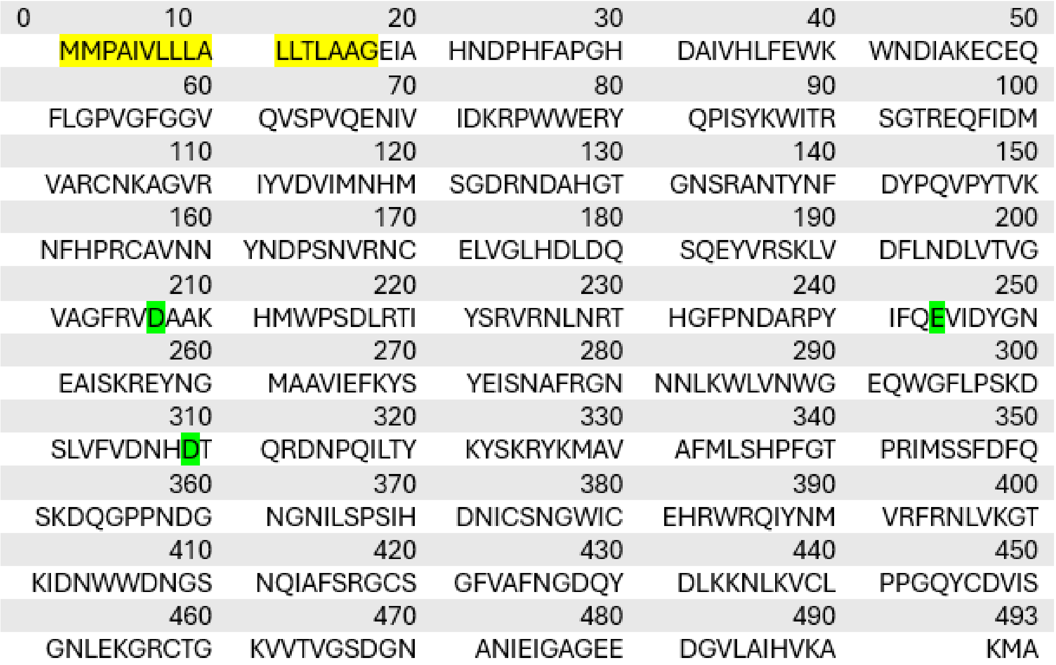
Protein (EC number)	**UniProt Primary accession no.**	**No. of amino acids**	**Mass (kDa)**	**Function and importance in honey bees**
Ceramide glucosidase, Glucocerebrosidase, Glucosylceramidase (EC 3.2.1.45)	A0A7M7GZU6, A0A7M7IFB4	511, 522	58.0, 59.3	Glucosylceramidase is a type of β-glycosidase that forms glucose and ceramide by catalysing the hydrolysis of glucosylceramide.[45, 59] Glucosylceramidase deficiencies can cause neurodegeneration and a shorter honey bee life span.[45, 60]
Chymotrypsin (EC 3.4.21.1)	A0A7M7GKE2	312	34.3	Chymotrypsin is a serine protease (endopeptidase) and forms short peptides by catalysing the cleavage of peptide bonds of carboxyl groups of leucine and aromatic amino acids (tyrosine, phenylalanine and tryptophan).[45, 61]
Elastase (EC 3.4.21.36)	Not available	-	-	Elastase is a serine endopeptidase and forms short peptides by catalysing the cleavage of peptide bonds of short aliphatic amino acids (glycine, alanine and valine).[45, 61]
Esterase (EC 3.1)	Various	-	-	Esterases catalyse the formation of an alcohol and an acid through the degradation of ester-containing compounds.[62, 63] In honey bees, this enzyme can allow esterase-mediated detoxification of pesticides.[62, 64]
Glucose oxidase (EC 1.1.3.4)	Not available	-	-	Glucose oxidase catalyses the conversion of glucose to gluconolactone which is later converted to gluconic acid (one of the prominent acids in honey) and hydrogen peroxide (by-product, inhibitor of glucose oxidase and gives honey antibacterial activity).[36, 38, 45, 65]
α-Glucosidase, Invertase, Saccharase, Sucrase (EC 3.2.1.20)	Q17058	567	65.6	Invertase catalyses the hydrolysis of sucrose (from nectar or honeydew) into glucose and fructose by cleaving the non-reducing terminal α-(1→4) glycosidic bond.[36, 38, 45]

Protein (EC number)	**UniProt Primary accession no.**	**No. of amino acids**	**Mass (kDa)**	**Function and importance in honey bees**
β-Glucosidase (EC 3.2.1.21)	Not available	-	-	β-Glucosidase catalyses the hydrolysis of β-(1→4) glycosidic bonds in cellulose and cellobiose to produce glucose.[45, 66]
MRJP1, Apalbumin 1, Apisin, Bee-milk protein, Defensin-1, Royalactin, Royalisin.	O18330	432	48.9	MRJP1 employs the Egfr-mediated signalling pathway, is an important factor in the differentiation of honey bee larvae into queens and makes up 31-66% of protein content in royal jelly.[57, 67-69] It has also been suggested that tryptic digestion of MRJP1 causes the production of jelleine-II and further modification may result in jelleines-I and -IV (jelleines-I and -II have been shown to have antimicrobial activity).[68, 69] Royalactin is the monomeric form of MRJP1, apalbumin is an oligomer of the MRJP1 gene product (420 kDa) and apisin is an oligomer of royalactin with apisimin and 24-methylenecholesterol.[57, 67]
MRJP2, Apalbumin 2	O77061	452	51.7	MRJP2 is used as a nitrogen reserve for nutrition[57], regulates phenotypic plasticity in honey bees[70] and has several immunoregulatory functions.[57, 69]
MRJP3, Apalbumin 3	Q17060	544	61.7	MRJP3 is involved in honey bee nutrition[57], stabilises RNA and protects it from degradation by binding to RNA and forming extracellular ribonucleoprotein granules (honey bees participate in the horizontal transfer of RNA to promote social immunity).[71]
MRJP4	Q17061	464	52.9	MRJP4 is important in honey bee nutrition and has antimicrobial activity.[57, 72]
MRJP5	O97432	598	70.2	MRJP5 is important in honey bee nutrition as a nitrogen-rich source, is added to royal jelly and is predicted to have other functions as well (due to evidence of its presence in forager bees).[57, 73]
MRJP6	Q6W3E3	437	49.8	MRJP6 is important in honey bee nutrition and in adult foraging bees.[57, 73]

Protein (EC number)	UniProt Primary accession no.	No. of amino acids	Mass (kDa)	Function and importance in honey bees
MRJP7	A0A8B6WZ41	443	50.5	MRJP7 has antibacterial activity and is proposed to have functions in the bee brain as it is highly expressed in the nurse bee brain instead of the hypopharyngeal glands (hence is only a very small component of royal jelly).[57, 73]
MRJP8	B3GM11	415	47.0	MRJP8 is an ancestral protein from which MRJP1-7s are derived, it is not a food protein and is speculated to have a function independent of sex, caste and tissue in honey bees (due to equal expression in all parts and all honey bees).[73]
MRJP9	A0A8B6WZ33	423	48.7	MRJP9 is an ancestral member of the MRJP family of proteins, it is not a food protein and is assumed to have a function irrespective of body section (due to high expression in a variety of bee body sections).[73]
Trypsin (EC 3.4.21.4)	A0A7M7G1K4	552	57.0	Trypsin is a serine protease (endopeptidase) that catalyses the cleavage of peptide bonds of carboxyl groups of arginine and lysine to form short peptides.[45, 61] This enzyme is involved in digestive and developmental processes, immune and defence against pathogens.[61]

1.3.1 Diastase (α-amylase) structure

A. mellifera diastase has 493 amino acids (Figure 1.1), *a* mass of 56,009 Da, an equal number of positively and negatively charged amino acid residues (arginine + lysine = 53 and asparagine + glutamine = 53) and a theoretical isoelectric point (pI) of 7.22 (electrically neutral at a pH of 7.22). This enzyme primarily belongs to the glycoside hydrolase family 13 (GH13) but can also be classified under GH57, GH119 and potentially GH126 due to its broad substrate preferences.[74] The GH13 family encompasses a variety of enzymes (hydrolases, transferases and isomerases) and non-enzymatic proteins (heteromeric amino acid transporters and 4F2 antigens).[74]



0 10	20	30	40	50
MMPAIVLLLA	LLTLAAGEIA	HNDPHFAPGH	DAIVHLFEWK	WNDIAKECEQ
60	70	80	90	100
FLGPVGFGGV	QVSPVQENIV	IDKRPWWERY	QPISYKWITR	SGTREQFIDM
110	120	130	140	150
VARCNKAGVR	IYVDVIMNHM	SGDRNDAHGT	GNSRANTYNF	DYPQVPYTVK
160	170	180	190	200
NFHPRCAVNN	YNDPSNVRNC	ELVGLHDLDQ	SQEYVRSKLV	DFLNDLVTVG
210	220	230	240	250
VAGFRVDAAK	HMWPSDLRTI	YSRVRNLNRT	HGFPNDARPY	IFQEVIDYGN
260	270	280	290	300
EAISKREYNG	MAAVIEFKYS	YEISNAFRGN	NNLKWLVNWG	EQWGFLPSKD
310	320	330	340	350
SLVFVDNHDT	QRDNPQILTY	KYSKRYKMAV	AFMLSHPFGT	PRIMSSFDFQ
360	370	380	390	400
SKDQGPPNDG	NGNILSPSIH	DNICSNGWIC	EHRWRQIYNM	VRFRNLVKGT
410	420	430	440	450
KIDNWWDNGS	NQIAFSRGCS	GFVAFNGDQY	DLKKNLKVCL	PPGQYCDVIS
460	470	480	490	493
GNLEKGRCTG	KVVTVGSDGN	ANIEIGAGEE	DGVLAIHVKA	KMA

Figure 1.1. Amino acid sequence of *A. mellifera* diastase (UniProt primary accession no. Q8N0N7), yellow-highlighted amino acids form the signal peptide and green-highlighted amino acids are the catalytic triad (full forms of each amino acid given in the Appendix in Table 8.1).

Generally, the GH13 proteins contain three domains [Figure 1.2 (a)]; β-strands surrounded by α-helices (8 of each) arranged in a barrel structure (domain A), loop connecting β3 and α3 (domain B, protrudes out of the barrel and is of variable length) and anti-parallel β-sandwich at the C-terminal (domain C).[74, 75]

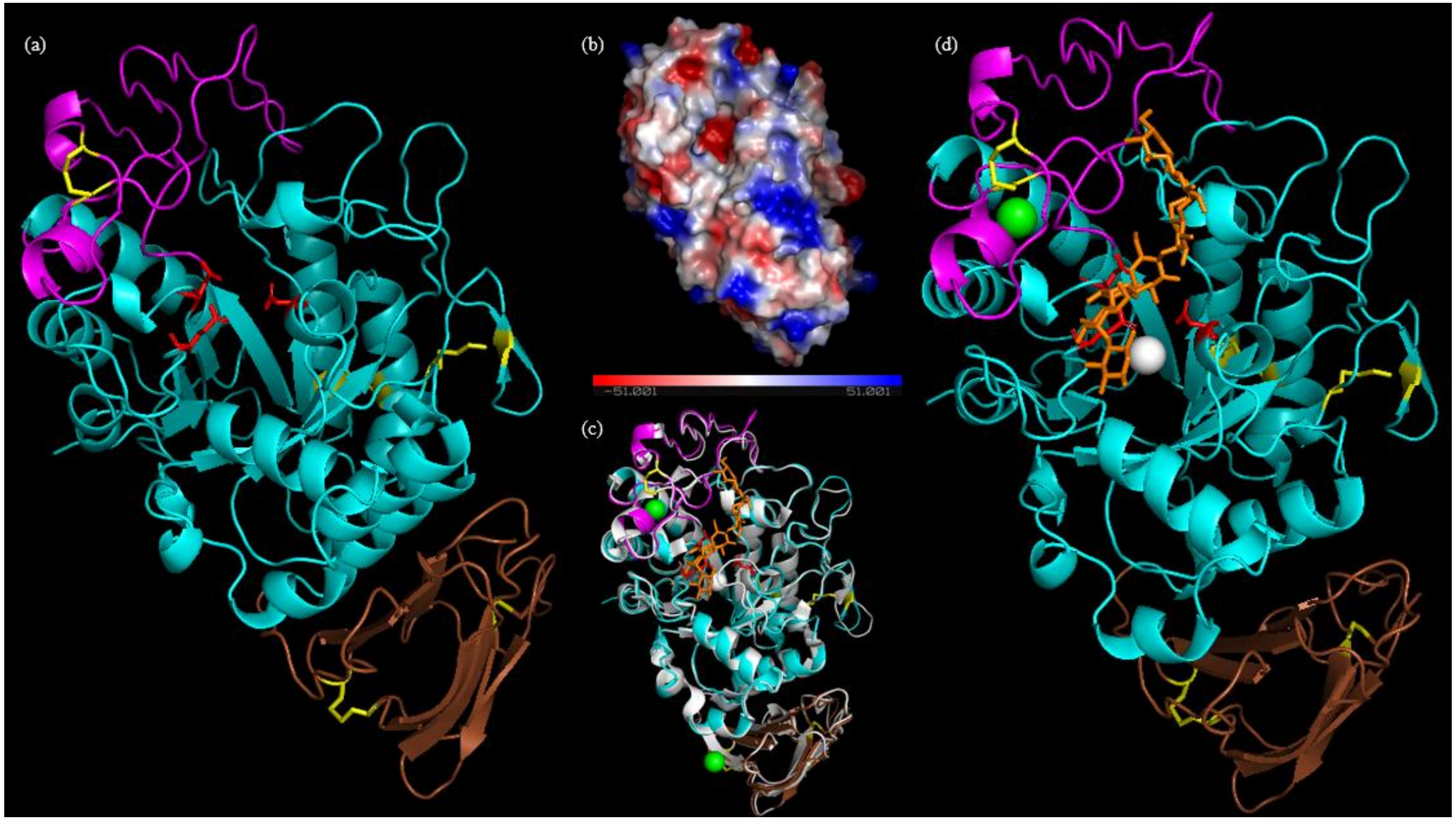


Figure 1.2. (a) *A. mellifera* diastase (AlphaFold model, UniProt ID: Q8N0N7) with domains highlighted (cyan = domain A, magenta = domain B and brown = domain C), catalytic triad amino acids highlighted red and disulfide bonds highlighted yellow. (b) Surface charge distribution of diastase. (c) *A. mellifera* diastase (UniProt ID: Q8N0N7) aligned to *D. melanogaster* diastase (PDB ID: 8ORP). (d) *A. mellifera* diastase with substrate (orange) positioned in the active site based on overlay with *D. melanogaster* diastase (c; PDB ID: 8ORP).

The active site is composed of a catalytic triad of two aspartic acids (207D and 309D) and glutamine (243Q) located in the catalytic cleft [Figure 1.2 (a)]. The general mechanism of action (double displacement) involves one of the aspartic acids acting as a catalytic nucleophile, glutamic acid as a proton donor and the other aspartic acid for stabilizing the transition-state (Figure 1.3).[74, 75] In *Drosophila melanogaster* α-amylase [very similar structure to *A. mellifera* α-amylase, Figure 1.2 (c)], the aforementioned roles are carried out by the same amino acids at different sequence locations 186D, 223Q and 288D respectively.[76] Important features of diastase, other than the active site, include the surface binding site, chloride and calcium ion binding sites and five disulfide bonds stabilising the structure.[74-76]

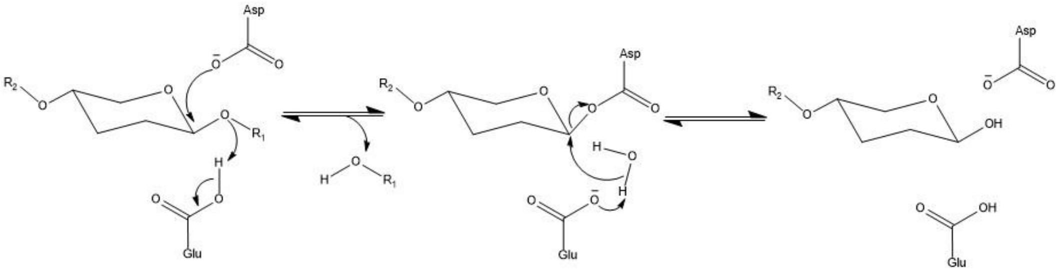


Figure 1.3. The general mechanism for the double displacement of starch by diastase modified from Lim and Oslan (2021).[4, 77]

Five unique entries of diastase sequences are available through the National Centre for Biotechnology Information (NCBI) database and UniProt. Each entry had a 100% query coverage when put through NCBI's basic local alignment search tool for protein sequences (BLASTp) indicating complete homology. The *Drosophila melanogaster* α-amylase had the highest sequence similarity with a 98% query cover score (% of a query sequence found in the matched sequence, higher % indicates a greater identical sequence match) and 60.16% identification (% of identical amino acid residues in the same alignment position of the query sequence to the matched sequence) to *A. mellifera* α-amylase upon analysis through BLASTp. Other species with high similarity to *A. mellifera* α-amylase included *Tenebrio molitor*, *Sus scrofa*, *Homo sapiens*, and *Oryzias latipes* with query cover scores of 95-98%, 95%, 95% and 94% respectively (~60%, ~51%, ~49% and ~50%

identification respectively). Solved structures of α-amylase are available in the Protein Data Bank (PDB) for the aforementioned species but not for *A. mellifera*.

There are several commercially available α-amylases primarily from microbial species (such as *Aspergillus oryzae*, *Bacillus amyloliquifaciens*, *B. licheniformis*, *B. subtilis*) most of which are mainly used in the sugar industry to produce high fructose sugar syrups used as sweeteners.[4, 78-80] There are other α-amylases, from for example human saliva and porcine pancreas, but there are no purified *A. mellifera* α-amylases available commercially.[4, 79, 81] The *A. mellifera* diastase has previously been purified from honey and honey bees through native techniques but it has not been recombinantly expressed and purified and no crystal structures are available.[82-85] Instead, the AlphaFold structure of *A. mellifera* diastase can be studied to predict mechanisms of action described earlier (Figure 1.3). These mechanisms can also be assumed to be highly similar to *D. melanogaster* α-amylase mechanisms as when the *D. melanogaster* α-amylase (PDB ID 8OR6 and PDB ID 8ORP) is aligned to *A. mellifera* diastase, extremely low root mean square deviation (RMSD) values are generated indicating high structure-wise similarity (i.e. RMSD is 0.513 Å and 0.411 Å respectively). This high similarity is because the AlphaFold structure is a predicted structure (with high confidence, Figure 1.4) formed from the amino acid sequence of *A. mellifera* diastase (Figure 1.1) and uses a deep learning algorithm that considers the multi-sequence alignments and physical and biological knowledge of the protein.[86] The confidence of the AlphaFold structure is depicted by a per-residue model confidence score formed from the predicted local-distance difference test (pLDDT).[86]

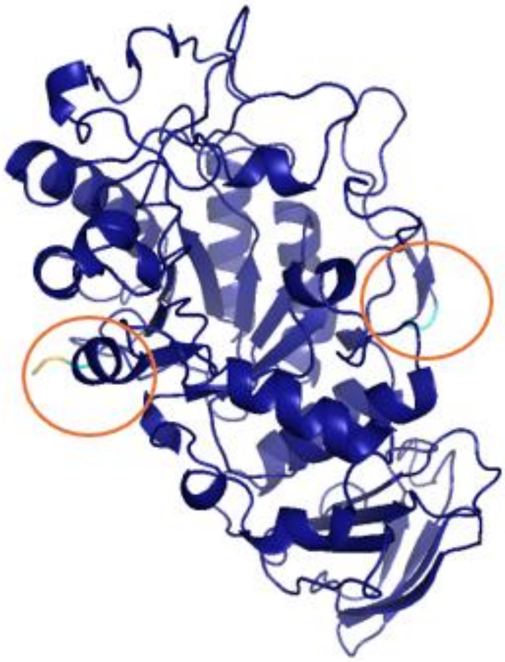


Figure 1.4. ***A. mellifera*** **diastase (UniProt ID: Q8N0N7) AlphaFold structure depicting the level of confidence of the model by the colour scheme; blue highlighted amino acids have a pLDDT >90 (very high confidence), cyan highlighted amino acids (circled in orange) have pLDDT >70 (high confidence) and yellow highlighted amino acids (circled in orange) have pLDDT >50 (low confidence). The signal peptide is not shown (which had a pLDDT <50, i.e. very low confidence).**

Overlay of the AlphaFold model with *D. melanogaster* α-amylase confirmed the positioning of the substrate in the active site with the catalytic triad positioned in proximity for subsequent catalytic function [Figure 1.2 (d)]. The *D. melanogaster* α-amylase structure with acarbose (PDB ID: 8ORP) incorporates a heptasaccharide due to transglycosylation (a side-reaction that α-amylase also carries out) instead of the pseudo-tetrasaccharide acarbose ligand. Hence, the PDB structure 8ORP depicts the α-amylase structure in a transition state-like conformation with the heptasachharide as part of the side-reaction, transglycosylation, instead of representing the method of inhibition of acarbose in α-amylase.[76] This is likely due to the crystallisation conditions (i.e. low water content and high localised sugar concentration near the active site in the crystal arrangement). The alignment of the 8ORP structure to the predicted structure of *A. mellifera* diastase (Figure 1.2) shows that *A. mellifera* might also have the ability to carry out this side-reaction due to the high similarity of the proteins, and highly conserved amino acid sequence. This side-reaction is seen for other α-amylases as well (e.g. α-amylases from human saliva and pancreas) and appears to be a conserved mechanism across the GH13 family among species.[74] The exact mechanism for the transglycosylation process of α-amylases is unclear and is considered thermodynamically unfavourable unless in a nonaqueous environment or in large concentrations of substrate.[87, 88] It is predicted that oligomeric species (such as sugars) could act as alternative nucleophiles (in place of water) which then cause extended chain of sugars to form. Silva *et al* (2019) showed the occurrence of non-enzymatic transglycosylation in honey models.[87-89] However, it is unlikely that *A. mellifera* diastase in honey catalyses transglycosylation because its primary function is hydrolysis.[74]

The *D. melanogaster* α-amylase structure also shows two strontium ions [these would be calcium ions under native conditions, substituted by strontium due to crystallisation conditions, Figure 1.2 (d)] of which one shows the site of the conserved calcium ion (coordinated by three water molecules, domain A and domain B residues) and the other shows the non-conserved calcium ion (coordinated by six water molecules and only one coordination to the enzyme to a glutamine residue which is not well conserved in *Drosophila*). The conserved chloride ion is found in the active site and is coordinated by various amino acid residues through direct and indirect interactions (one of which is the catalytic glutamine residue).[76] The conserved ion binding sites are likely to be present in *A. mellifera* diastase in similar locations and with similar interactions (based on conserved protein structure and sequence alignment). The role of the calcium ion is to contribute to the structural integrity of the active site (by holding the barrel and domain B structure close) while the chloride ion acts as an activator ion (promotes catalytic activity by adjusting the properties of neighbouring amino acid residues).[76, 90]

1.3.1.1 Diastase activity

Diastase activity is used by various countries to test for honey quality using the standardised (by the International Honey Commission, IHC) Schade or Phadebas methods. Honeys that have been exposed to harsh storage conditions (such as high temperatures or long storage times) result in lower diastase activity for the honeys over time.[36] However, activity rapidly decreases for mānuka honey (a phenomenon thought to be influenced by the complex matrix of mānuka honey). Diastase activity is expressed as DN and is defined as the quantity of enzyme that converts 0.01 g of starch to the prescribed end-point at 40 °C in 1 hour under the test conditions.[39, 91, 92] Since diastase is used as a broad term for α-amylase found specifically in honey, diastase activity assays come under the broader α-amylase assays. The most commonly used methods for determining diastase activity in honey include the Schade, Phadebas, Association of Official Analytical Collaboration (AOAC) 958.09 and more recently the *p*-nitrophenol method.[36, 93] These are colorimetric methods that measure the absorbance at a wavelength between 600-660 nm, are specific for honey samples and are generally stopped assay methods (the Schade method is a pseudo-continuous assay method) which measure the end-point of an assay reaction rather than directly following the assay progression over time.

The Schade method uses a dilute KI and I_2 resublimed solution to stop the reaction of diastase in dissolved honey solution which is added to a fixed amount of starch solution. This is incubated in a 40 °C water bath for 15 minutes, then terminated periodically (first time point at 5 minutes, the rest determined by the readings being between 0.155-0.456, i.e. within the linear range) post-addition of starch solution to the honey solution. The calibration of starch in this method is carried out to test the suitability of starch solution at various dilutions and is not used to generate a standard curve.[94] This method works via the active diastase (in the honey) degrading the added starch and then iodine binding to unreacted starch polymers to form a starch-triiodide complex (in a 3D structure), the formation of which is measured.[95]

The Phadebas method uses commercially available Phadebas tablets (1 per sample) and works in a similar way to the Schade method. However, this does not require KI and resublimed I_2; instead the tablets (composed of insoluble blue starch cross-linked polymers) are degraded, releasing the dye and the absorbance is measured.[94, 96] The Phadebas method also does not generate a standard curve for diastase activity.

Recently, a new German Deutsches Institut für Normung (DIN) 107520-2 method (also termed the *p*-nitrophenol method) was developed that uses the 4,6-ethylidene-(G7)-1-4-nitrophenyl-(G1)-alpha-ᴅ-maltoheptaoside (E-*p*NP-G7) chemical as the substrate, and requires a coupling enzyme (microbial α-glucosidase) for detection.[97] The first reaction is the hydrolysis of E-*p*NP-G7 to smaller products by α-amylase in honey and the second reaction converts the products into *p*-nitrophenol (*p*NP) which is measured at an absorbance of 405 nm. For the second reaction, a given concentration of microbial α-glucosidase is added to the reaction and the diastase activity is taken as directly proportional to the rate of formation of *p*NP. This method requires a certified reference material with known amylase activity (U/I, units are the enzyme activity that can transform 1 μM of substrate in 1 minute under optimal conditions[34]) to form a linear calibration curve. The German DIN method is more specific for α-amylase (over β-amylase and γ-amylase, i.e. exoenzymes) than the aforementioned methods due to the ethylidene residue (which prevents exoenzymes from degrading the substrates, hence only measuring the α-amylase activity).[97, 98]

The AOAC 958.09 method uses the same procedure as the Schade method, however, the AOAC method offers more flexibility in regards to the wavelength, spectrophotometer, choice of dilutions for the calibration of starch and has a smaller honey sample requirement (5 g instead 10 g).[99]

All the methods are standardised, stopped-assay methods (except for the Schade method) and have a range of advantages and disadvantages (Table 1.3). Stopped-assay techniques measure activity after stopping the reaction and are ideal for high-throughput screening as is required in the honey industry (because typically a value for the measure of current activity in the honey is needed). These methods, however, utilise a single substrate concentration and thus do not account for the complexity of substrate-enzyme binding interactions which greatly affect rates.[100, 101] This is a disadvantage for honeys with complex matrices (such as mānuka honey) as it overlooks any effects on diastase activity because of the matrix itself.

Continuous assays, as opposed to stopped assays, are an advantage in enzyme characterisations as they directly follow a reaction progression, minimising handling steps and associated errors, and the linear fitting of a greater number of timepoints reduces errors associated with any one measurement.[101] A non-standardised diastase continuous assay has been reported in literature for honey using diastase purified from honey before activity analysis. This adds steps to the method and requires a large sample size (300 g), making it non-ideal for high throughput testing.[85] Since the activity is then tested in buffers, the activity of the protein can be influenced by the purification process, buffers and does not account for activity differences that would occur if measured in the native honey sample.[85] There is no continuous assay method for the analysis of diastase activity in honey present in the literature.

Pseudo-continuous assays (such as the Schade method) can approximate a continuous stream of data points but since the Schade method only uses one starch (i.e. substrate) concentration for the reaction, it also does not account for the complexity of substrate-enzyme binding interactions.[101]

Table 1.3. Comparison of the main diastase activity determination tests (specific for honey).

Method	Advantages	Disadvantages
AOAC 958.09	-AOAC standardised method -Low cost -Readily available reagents and equipment -Could be scaled down -Can utilise various starch solutions -Readily available equipment	-Moderate honey requirement (5 g) -Labour-intensive (large volumes) -Requires a regulated temperature (40.0 ± 0.2 °C)
German DIN 107520-2	-DIN standardised method -Generates a standard curve -Specific for α-amylase (i.e. activity unaffected by β- and γ-amylase) -Could be scaled down -Readily available equipment	-High honey requirement (10.00 ± 0.01 g) -Labour-intensive (large volumes) -High cost (due to specific E-*p*NP-G7 substrate, microbial α-glucosidase enzyme and certified calibration standard) -Requires regulated temperature (37.0 ± 0.2 °C)
Phadebas	-Low honey requirement (1 g) -High-throughput (fewer steps) -IHC standardised method -Readily available equipment	-Labour-intensive (large volumes) -High cost due to commercial Phadebas tablets -Cannot be scaled down (due to the specific structure of Phadebas tablets) -Requires regulated temperature (40 °C)

Method	Advantages	Disadvantages
		-Fast method
Schade	-IHC standardised method -Low cost -Readily available equipment -Could be scaled down	-High honey requirement (10 g) -Low-throughput (many steps) -Labour-intensive (large volumes) -Requires regulated temperature (40.0 ± 0.2 °C) -Requires a highly specific starch

Other assays available for measuring α-amylase activity (non-specific for diastase activity in honey) can be categorised into three types; specific for purified amylase samples (stopped assay), specific for certain food products other than honey (generally stopped assays) and those that use less common techniques (stopped and continuous assays). The first and third types can overlap. The first type (purified amylase samples) utilise methods that typically measure absorbance of 3-amino-5-nitrosalicylic acid at 540 nm, are continuous assays (quantity of formed product is measured over time) and require 3,5-dinitrosalicyclic acid (DNS) stopping agent which is reduced by the maltose (a product from the degradation of starch) to form 3-amino-5-nitrosalicylic acid.[102-107] This first type of assay is not ideal for honey samples as the largely reducing-sugar matrix can produce a large background signal that could interfere with the actual diastase activity measurements (particularly for low activity honeys). The second type incorporates the Amylase SD and Ceralpha methods.[108, 109] Both methods are specific for flour, have extensive steps, measure absorbance between 400-405 nm and use the same chemical (E-*p*NP-G7) as the German DIN method to terminate the reaction.[108, 109] Using these methods provides no advantage over the four standardised diastase activity determination methods due to the method being specialised for flour samples. The third category of amylase assays use various substrates (e.g. BODIPY®FL-DQ™ which is a continuous assay substrate and Trizma base solution which is a stopped assay substrate) in spectrophotometric settings or use simple acetic acid triiodide solutions coupled to direct potentiometric measurement techniques (stopped assay) to test for activity in honey and other food products.[92, 95, 110, 111] The third type of methods are more complex than the four standardised methods for diastase activity determination as they can involve high cost substrates (e.g. BODIPY®FL-DQ™), a large number of steps, complex instrumental techniques and data processing.

1.3.1.2 Diastase activity in honey

In 1999, the IHC conducted a review of the EU honey directive and CODEX Alimentarius Standard for Honey and found that the EU imposed stricter and more severe regulations than the CODEX guidelines for diastase activity in honey. Bogdanov *et al* (1999) explained that the EU refers to the entire life of retail honey whereas the CODEX refers to honey after processing and blending.[32] The CODEX recommends diastase activity levels of >8, however, gives an exception to low enzyme content honey for which it recommends DN values of >3.[112] It does not give any examples of low enzyme content honey and also does not specify whether the low enzyme content refers to the total enzyme content in honey or just diastase content. The EU directive goes a step further to give the example of citrus honeys as a naturally low enzyme content honey and also specifies that the HMF content of such honeys should not exceed 15 mg/kg in order for the diastase exception of >3 to be implemented.[19] An example of citrus honey is orange blossom honey.[113] Several articles in the literature have also claimed other honeys are naturally low diastase activity honeys such as black locust, clover and honey from tropical regions.[38, 44, 93, 114]

Diastase has been suggested to be unsuitable as a honey quality test because several factors can influence amount and activity of diastase in honey; these factors are summarised in Table 1.4.

An earlier study proposed that α-glucosidase activity or a ratio of α-glucosidase: α-amylase would be a better honey quality indicator. Aldcorn *et al* (1985) discussed how α-glucosidase would be present at more consistent levels in honey as it has a functional role to play in honey (i.e. to hydrolyse sucrose which is a major component of nectar). As well as this, the literature review by Aldcorn *et al* (1985) showed that α-glucosidase had a higher susceptibility to heat abuse than diastase.[114] Hence, α-glucosidase activity would be a better depiction of honey quality.

Table 1.4. Factors influencing the presence of diastase and the enzyme activity in honey.

Factor	Type of factor	Reason
Bee age and role	Honey-related	Forager bees (worker bees 21+ days after emergence[115, 116], involved in foraging for nectar, pollen, water or propolis)[116] express α-amylase in the hypopharyngeal gland and secrete this during nectar ripening.[82, 117]
Ionic conditions/ Metal ions	Honey-related	Purified diastase (*A. mellifera*) has been shown to be inhibited by $CuCl_2$, $MgCl_2$, and $HgCl_2$.[85] The Ca^{2+} and Cl^- ions are required as activators.[76] Various metal ions are found in honey and can play roles in activation and inhibition through different methods.
Microbial contributions	Honey-related	Honey bee gut microbiota (such as *Bacillus subtilis* which is found in nectar, the honey sac of honey bees and fresh honey) can contribute to diastase activity in honey as gut microbiota diastase is mixed with nectar and honey bee diastase while the nectar is stored in the honey sac of honey bees.[118]
Reactive species	Honey-related	Highly reactive compounds (e.g. MGO and phenolic compounds) can modify certain reactive amino acid residues of proteins which could inhibit diastase activity through competitive or non-competitive inhibition.[119, 120]
pH	Enzyme-related	Diastase (*A. mellifera*) has an optimal pH between 4.0-5.5 while the pH of honey can be lower (i.e. slightly acidic, between e.g. 3.5-5.5).[83, 85, 121, 122] Deviations from the optimal pH can result in reduced enzyme activity due to changes in enzyme conformation and surface charge.
Temperature	Enzyme-related	Deviations from the optimal temperature of diastase (*A. mellifera*), 55 °C, can result in slower activity (at lower temperatures) or complete loss of activity (at extremely high temperatures).[4, 85] Hence, diastase activity is impacted by the storage temperature of honey.

Diastase activity diminishes upon storage and mānuka honey is stored for extended periods of time before sale to meet the demands of consumers for high MGO (MGO concentration increases over time). Bell and Grainger (2023) stated that mānuka honey had naturally low levels of diastase activity upon harvesting compared to other floral honeys and was more rigorously tested than other honeys.[2] The rigorous testing is one of the reasons why mānuka honey fails diastase testing on export honey more often than other floral types.[2] Bell showed that diastase activity decreased more rapidly in mānuka honey compared to other floral types by carrying out several storage time trial experiments at 20 and 27 °C for 160-198 days.[4] This work showed negative correlations between the changes in diastase activity to DHA and MGO concentrations in aged mānuka honey.[4] Clover honey spiked with MGO and 3-phenyllactic acid (3PLA) caused diastase activity to decrease faster than the control.[4] Hence, it is important to investigate the complexities of a mānuka honey matrix (as compared to other honeys) to understand all the potential factors (i.e. various compounds) involved in interfering with diastase activity.

1.4 Honey composition

Honey is a naturally sweet product with a variable consistency, colour, flavour and aroma.[123] It is produced by honey bees from plant nectar, plant secretions or insect excretions of plant sucking insects. Once the nectar/secretions/excretions are collected, they are modified with honey bee substances (i.e. carbohydrate-digesting enzymes such as diastase), dehydrated (regurgitated in the mouth of honey bees and evaporated) and stored in wax cells.[36, 37, 112, 123-127] Honey is capped for long-term storage once the water content is less than 20%.[125, 128] Honey made from plant nectar is referred to as floral honey while honeydew honey is made from the secretions of the extrafloral parts of plants or from plant sucking insects.[36, 38, 112, 125] Nectar contains carbohydrates, water, inorganic ions, amino acids, enzymes, lipids, volatile substances, and antioxidants.[46] In some cases toxic compounds may also be present.[46]

Honey bees store large amounts of honey in anticipation of the winter period, adverse climate and in case of nectar source fluctuations to provide a constant and reliable supply of food.[125, 129, 130] The EU also specify honey for human consumption should be collected from *A. mellifera* species (the most distributed and domesticated honey bee species).[19, 93] In New Zealand *Apis mellifera carnica* (known as the Carniolan honey bee) and *Apis mellifera linguistica* (Italian strain) are the main honey bee species used for honey production.[131-133] Various monofloral, and multifloral honeys are produced in NZ including native honeys (kāmahi, kānuka, mānuka, pōhutukawa, rātā, rewarewa and tawari). Honeydew predominantly from West Coast Beech forests and non-native honey (clover and thyme) are also produced in NZ.[134, 135]

Honey is comprised of mainly sugars (>80%) and water (~17%).[38] Other compounds found in honey include minerals, vitamins, proteins (including enzymes), free amino acids, acids, hydrogen peroxide, phenolic compounds, pigments and volatile compounds.[36, 125] The proportions of these depends on the source of the honey, the process by which honey bees modify it, the environment (e.g. climate, season, flora, altitude and botanical diversity) and the length of time that honey remains in the hive.[36-38]

1.4.1 Mānuka honey

Mānuka honey is produced in New Zealand by *A. mellifera* from the native mānuka tree (*Leptospermum Scoparium*) and is recognized for its non-peroxide antibacterial activity (i.e. retained antibacterial activity in the absence of hydrogen peroxide).[131, 136, 137] New Zealand's MPI has a strict definition of mānuka honey (both monofloral and multifloral, Table 1.5) to verify the authenticity of any honey marketed as mānuka honey and to maintain consistency of mānuka honey labelling across the industry, MPI and overseas authorities.[138]

Table 1.5. Parameters for the NZ MPI definition of monofloral and multifloral Mānuka honey.[10]

Parameter	Monofloral Mānuka honey	Multifloral Mānuka honey
2'-Methoxyacetophenone (2MAP)	≥5 mg/kg	≥1 mg/kg
2-Methoxybenzoic acid (2MBA)	≥1 mg/kg	≥1 mg/kg
4-Hydroxyphenyllactic acid (4HPLA)	≥1 mg/kg	≥1 mg/kg
3-Phenyllactic acid (3PLA)	≥400 mg/kg	≥20 mg/kg, <400 mg/kg
DNA from Mānuka pollen	<Cq 36 (~3 fg/µL)	<Cq 36 (~3 fg/µL)

The term mānuka or kahikātoa is a Māori term used to describe the tea-tree, scientifically known as *Leptospermum scoparium.*[139-141] This species is part of the Myrtaceae family that contains 133 genera and is split in 2 broad sub-families including Leptospermoidae and Myrtoidae (differentiated by their capsular and fleshy-fruit respectively).[140, 142] Leptospermum is a genus that comprises of 86 recognized species, one of which is found in New Zealand and 83 in Australia.[142, 143] Mānuka is considered the most widespread shrub species in NZ and has been classified by different varieties including *L. scoparium var. scoparium*, *var. extimium*, *var. incanum*, *var. linifolium*, *var. prostatum*, *var. myrtifolium*, and *var. Parvum*.[140, 144] Mānuka is indigenous to NZ and earlier studies report it as not endemic because it is also found on the southern coast of New South Wales and in Tasmania Australia.[140, 142] However, a recent study reported that the Tasmanian *L. scoparium* was genetically distinct from NZ mānuka and suggested it be recognised as a separate endemic Australian species.[145] This indigenous shrub/small tree grows to a height of 2-10 m and is found all over NZ (through North, South and Stewart Island) in a range of habitats such as wetlands, river gravels and dry hillsides.[140, 146, 147] Mānuka typically has white, solitary flowers in clusters but they can be pink or red and generally flower between October to February depending on latitude.[140, 147]

Mānuka has green nectaries which contain chloroplasts instead of amyloplasts and hence contain little to no starch prior to or following anthesis (time period in which the flower is fully open and functional).[52, 148] Nectar in mānuka flowers is produced 14 days following anthesis.[52] As a result, most of the nectar sugars are predicted to be derived from the photosynthesis that occurs in the green nectaries. In contrast, accumulation of starch (from the phloem supplied sugar) in the non-green or pale green nectaries is typically seen for the well-studied, short-lived and rapid nectar secreting *Arabidopis* and *Nicotiana* species.[46, 149] The accumulation of starch occurs in the early phases of nectary maturation (insoluble form of glucose used for storage in plants) and the starch is degraded as nectar sugars are secreted. Due to no accumulation of starch in mānuka flower nectaries, it is reasonable to assume that no α-amylase ends up in nectar which otherwise may be present in starch-accumulating nectaries.[46, 52, 148-151]

The compounds 2MAP, 2MBA, 4HPLA and 3PLA are found at higher concentrations in mānuka honey (than most other honeys) and hence are used as chemical markers.[8, 10, 152] McDonald *et al* (2018) examined the four compounds as unique mānuka markers and observed that only 2MAP was exclusively found in mānuka nectar and honey. Hence, 2MAP could be used to differentiate between mānuka honey from other NZ honeys and could also distinguish between monofloral and multifloral honeys. 2MBA, 4HPLA and 3PLA were not exclusive to mānuka plants, however, could still be used to differentiate between mānuka honey and other NZ honeys and between monofloral and multifloral mānuka honey depending on the concentration. 2MAP, 2MBA, 4HPLA and 3PLA were also observed to be stable (relatively) over temperature and time. These qualities make the four compounds appropriate chemical markers for mānuka honey along with DNA analysis.[8, 10, 153]

1.4.2 Sugars

Sugars make up a significant proportion of honey (>80%) and the monosaccharides glucose (~28% of honey[38]) and fructose (~39% of honey[38]) make up the main proportion.[36-38, 123] A variety of other sugars may also be present in honey (Table 1.6).

Table 1.6. Sugars found honey.[36-38, 123, 125, 126, 154-156]

Sugar	Type	Main	Minor/Trace
Monosaccharide	Reducing	Glucose Fructose	
Disaccharide	Reducing	Maltose Isomaltose Kojibiose Turanose	Laminaribiose Gentiobiose Cellubiose Nigerose Isomaltulose Leucrose Melibiose
	Nonreducing	Sucrose	Trehalose
Trisaccharide	Reducing	Panose Maltotriose	
	Nonreducing		Melezitose Raffinose Planteose

Monosaccharides are more abundant in floral honeys than disaccharides.[123, 157] Honeydew honey also contains a high percentage of oligosaccharides because of the excretion of excess modified sugar and water content from homopteran insects.[46] Honey bees show a preference for sucrose over fructose and glucose, which is the main sugar found in nectar.[51, 158-162] Nectar is released by nectaries and the carbohydrates in it come from the degradation of stored starch and/or photosynthesis.[51, 159, 163, 164] As honey granulates, glucose is the main sugar that crystallizes as it has a lower solubility in water than fructose.[123, 165] High sugar content in honey allows honey to have antibacterial activity due to an osmotic effect (water becomes inaccessible to microorganisms in honey).[38, 126, 157]

1.4.3 Water content

Water content is reduced by the honey bees to avoid spoilage and is less than 18% in sealed honey combs.[22, 38, 125] Honey with moisture content of more than ~17% is considered more likely to ferment if sufficient yeast spores are present. The official moisture content limit is set by the CODEX at 20%.[112] Water content can range between 13-25% as it is affected by the original water content in nectar, the storage process after extraction and the weather conditions while nectar is ripening.[125] Water content influences the reactions that occur in honey, such as the hydrolysis of starch by diastase or the conversion of the DHA dimer to the monomer.[4, 166] Moisture content also affects honey quality (due to the risk of fermentation), viscosity and granulation.[123, 167] Granulation is dependent on the glucose: water and fructose: glucose ratios. When the glucose: water and fructose: glucose ratios are >1.7 and <1.33 respectively, crystallization occurs whereas when they are >2 and <1.1 respectively, the crystallization process occurs more rapidly.[36, 38, 157]

1.4.4 Minerals

Minerals make up ~0.4% of honey and the main elements observed include potassium (most abundant, ~33-70% of the total mineral content[168, 169]), sodium, calcium and magnesium.[38, 127] Other elements can vary more significantly and include (but are not limited to) aluminium, boron, manganese, rubidium, zinc, chlorine and iron.[38, 127] The mineral content in honey mainly originates from the absorption from soil by the plant and transportation to the nectar.[38, 170] Other sources could be environmental pollution, geological location and contamination during the processing of honey and beekeeping practices.[38, 170] Elemental composition can be used as a fingerprint tool to identify the geological origin of the honey.[127, 170] Minerals are present in higher quantities in honeydew honey than in floral honey.[121, 171] Hence honeydew honey also has higher conductivity.[121] A recent study suggested honey bees may be seeking out specific nutrients such as potassium and sodium (based on their increased foraging activity after the consumption of potassium and sodium in the winter and summer seasons).[172] Potassium and sodium have important roles in biological processes in *A. mellifera*, while other trace elements such as heavy metals can pose health risks to bees and humans.[38, 170, 172-175] Mineral content in honey has also been associated with honey colour. Lighter coloured honey tends to have lower mineral content than darker honey.[176, 177]

1.4.5 Vitamins

Vitamins make up <0.1% of honey and can include vitamin C (ascorbic acid), vitamin B2 (riboflavin), vitamin B3 (niacin), pyridoxine, and vitamin B1 (thiamine) depending on the floral source.[37, 38] Certain vitamins such as α-tocopherol (a form of vitamin E) and vitamin C act as antioxidants in honey and their effects decrease when honey is heat treated.[38] Some of these vitamins are water-soluble and content in honey predominantly originates from pollen grains (which are present in suspension), but content also depends on the floral type of the honey and the geographical region.[37, 38, 46, 178-182]

1.4.6 Proteins and Enzymes

Proteins make up 0.1-1.6% of the total honey mass and come from both floral (nectar and pollen) and insect (salivary, digestive and hypopharyngeal glands of the honey bee) origins.[36, 37, 61, 125, 126, 183-187] Proteins detectable in honey are of two types; carbohydrate metabolising enzymes [e.g. glucose oxidase, invertase (α-glucosidase), diastase (α- and β-amylase, covered in depth in section 1.3)] and royal jelly characteristic proteins (MRJPs 1-9). The royal jelly characteristic proteins have antibacterial activity to prevent spoilage and are suggested to be a part of the honey ripening process.[57, 188] The main family of proteins expressed in honey bees comprises of nine proteins which are known as major royal jelly proteins 1-9, (MRJP1-9).[57, 189-191] These MRJP proteins are added to honey by the honey bees during honey ripening.[188, 192] The MRJP proteins are expressed in all honey bee castes, sexes and sections of the body which supports the multifunctional role of these proteins. Buttstedt *et al* (2013), showed significant expression of MRJP1-7 occurred in worker heads while MRJP8-9 (considered ancestral proteins) were highly expressed in the thorax and abdomen.[73] The heads of honey bees contain the hypopharyngeal glands which are involved in the production of royal jelly.[73, 193, 194]

Different castes of honey bees also show different levels of protein expression. There are three castes of bees: queen bee, drone bees and worker bees. These emerge from eggs in vertical comb cells which vary in size (smallest is for worker bees and biggest is for drone bees). The queen bee is an adult mated female that lays eggs to produce more bees and can live more than 1-2 years but are replaced by beekeepers (to ensure optimal colony health and honey production).[115, 195] Drone bees are male bees that come from unfertilised eggs, mate with queen bees and live 21-32 days. Worker bees are unmated female bees that live 30-60 days in spring and longer (150-200 days) in winter. They carry out a variety of roles in the hive as they age transforming from a house bee (1-5 days after emergence) into a nurse bee (6-18 days after emergence) then into a guard bee (19-21 days after emergence), and finally into forager bees (21+ days after emergence).[115, 116, 196] Nurse bees feed the brood, so have high expression of proteins (specifically MRJPs) and larger hypopharyngeal glands than foragers.[73, 192, 197-199] Evidence of the role of MRJPs as secretory proteins is their 16-20 amino acid N-terminal signal peptides.[69] As well as this, foragers express different proteins (carbohydrate metabolising proteins such as α-glucosidase III) than nurse bees.[200-202]

The most abundant and functionally active protein is MRJP1.[38, 44] The monomeric form of MRJP1 is known as royalactin and has been used in studies investigating its potential as a longevity promoting natural agent.[57, 203, 204] The oligomeric form of MRJP1 (known as apisin) is most prevalent and the crystal structure has been solved with apisimin.[57, 205-207] Apisimin is a small, acidic peptide found in royal jelly which interacts with MRJP1.[208, 209] MRJP1, specifically, is observed to be highly expressed in the mushroom bodies (involved in learning and memory) found in honey bee brains.[192, 210] These MRJPs are closely related to the yellow protein of *Drosophila melanogaster* evolution-wise and are also called bee-milk proteins except for MRJP6 and MRJP7.[192, 211, 212] The yellow protein in *D. melanogaster* is involved in the production of black melanin and male sexual behaviour.[213-215] In *A. mellifera* orthologous yellow proteins have been found that have similar function to the same protein in *D. melanogaster*.[191, 209] Other MRJPs have various polymorphic forms depending on the *Apis* species of honey bee they are expressed in (due to differences in post-translational modifications).[68]

The bee-secreted carbohydrate metabolising enzymes are responsible for processing honey. These carbohydrate metabolising enzymes include invertase (also known as α- and β-glucosidase and sucrase), diastase (α- and β-amylase), glucose oxidase and glucosylceramidase. Invertase makes up 50% of the total protein content in the hypopharyngeal gland of the forager bee while diastase and glucose oxidase make up 2-3% each.[38] Some studies consider β-glucosidase separately from invertase due to its slightly different mechanism of action of a similar function.[36, 45]

Other enzymes such as catalase, acid phosphatase, proteases and esterases are also found in honey.[37, 38, 45] Catalase is involved in the breakdown of hydrogen peroxide. The level of catalase and glucose oxidase and their rates of activity will determine how much hydrogen peroxide is in honey. This is important as hydrogen peroxide is responsible for antibacterial properties in most honeys.[38, 45] The main source of catalase in honey does not arise from the addition by the honey bee but from microorganisms, plant nectar and pollen.[38, 45, 216-221] Acid phosphatase content in honey is from mainly pollen but also nectar.[38, 222] Honey proteases can come from nectar, pollen or the secretions of honey bees (from the cephalic glands).[61] The most common proteases found in honey bees and honey include the serine proteases, trypsin, chymotrypsin and elastase.[45, 61, 184] Serine proteases have serine in their active site and form short peptides and amino acids by cleaving peptide bonds between certain amino acid residues.[45, 223] These proteases can affect the composition of honey and have been proven to degrade MRJPs.[61] Moritz and Crailsheim (1987) found nurse bees had the highest proteolytic activity and this was confirmed by Jimenez and Gilliam (1988).[224, 225] Proteases have been detected in the midgut, cephalic glands and hypopharyngeal glands of adult honey bees.[61, 184, 226-230] Esterase activity increases in honey bees with age and is involved in detoxification.[62-64]

Factors that affect enzyme levels include nectar composition and concentrations, the age of bees, physiological period of the colony, whether there is a high-flow sugar-concentrated nectar (if it is concentrated, honey will contain a low enzyme content due to less processing required[34, 39]) and the intensity of the nectar flow.[39, 231]

1.4.7 Free amino acids

Proline is the most abundant amino acid in honey (50-85% of total amino acids in honey[38, 232]), amino acids make up ~1% (w/w) of honey and their sources include nectar, pollen and honey bees.[37, 38] Due to the abundance of proline, it has been used as a honey maturation test parameter because the amino acids decrease in quantity over time.[37] Proline is produced by the salivary gland of the honey bee during the conversion of nectar to honey.[37] Other free amino acids found in honey include glycine, alanine, valine, leucine, isoleucine, lysine, arginine, histidine, serine, tyrosine, phenylalanine, aspartic acid and glutamic acid.[36, 38] These amino acids (through the Maillard reaction with reducing sugars) form various products and darken the colour of honey (giving it a brown-like appearance).[37, 38, 46, 123, 233]

1.4.8 Pollen

Pollen is the male gametophyte produced by plants for sexual reproduction by the transfer of pollen grains via pollinators (who are offered rewards such as nectar) to the female part of plants.[38, 46, 47] Pollen is the main food source of larvae after storage and modification in the hive as a protein-, lipid-, sterol-, mineral- and vitamin-rich source.[47, 234] Pollen is stored separately in the hive from nectar, however, small amounts of pollen can end up in honey during collection.[125] In honey, pollen can give an indication of which plant species the honey bees collected their nectar, and the geographical origin of the honey.[22, 125]

1.4.9 pH and acids

Acids in honey contribute to its flavour, aroma and pH.[125] Honey contains amino acids (aforementioned in section 1.4.7) and organic acids which make up ~0.5-0.6% of honey.[38] The major acid found in honey is gluconic acid which is formed from the oxidation of glucose and the transformation of the intermediary product (gluconolactone) by the enzyme glucose oxidase (hydrogen peroxide is made as a side product).[38, 123, 125] Organic acids in honey come from nectar, or through reactions during the transformation of nectar to honey by enzymes from the honey bees (as with glucose oxidase).[36-38] A range of organic acids can be found in honey such as acetic, butyric, citric and galacturonic acid.[36, 38] Due to the acid content, honey is slightly acidic with pH values that can range between 3.2-6.5.[36, 37, 123] This acidity prevents the growth of bacteria.[36, 37]

1.4.10 Phenolic compounds

Phenolic compounds make up 0.02-0.2% of honey and are the secondary metabolites of plants with antioxidant and antibacterial properties (they allow plants to overcome biotic and abiotic stresses). They are characterised with having one or more aromatic rings, at least one hydroxyl group and can also contain a distinctive functional group. Phenolic compounds comprise of phenolic acids, flavonoids, tannins, lignans and coumarins.[38, 235, 236] In plants, phenolic compounds function as antioxidants, ultra-violet (UV) protection barriers and are involved in defence, colouration (darken honey) and signalling. In honey, phenolic compounds come from the nectar collected by honey bees.[37, 38, 126, 237-239]

Phenolic compounds are formed in plants through the shikimic acid pathway and the malonate-acetate (polyketide) pathway.

The shikimic pathway produces the tryptophan, tyrosine and phenylalanine aromatic amino acids. The phenylalanine is modified to form hydroxycinnamic acids which includes all the cinnamic acids (cinnamic, *p*-coumaric, caffeic and ferulic acid) and *p*-hydroxybenzoic acid. The malonate-acetate pathway produces flavonoid compounds from the reaction between malonyl-CoA, acetyl-CoA and *p*-coumaric acid. In more detail, a phenolic ring structure is formed from the cyclization of a polyketide chain. The polyketide chain is formed by the sequential additions of malonyl-CoA (similar to fatty acid synthesis). A combination of shikimic and malonate-acetate pathway products can also produce various phenolic compounds.[240-244]

Phenolic acids are (structure-wise) simple polyphenols and contain a benzene ring, carboxylic, hydroxyl, and methoxyl groups. Common phenolic compounds are derived from benzoic or cinnamic acid structures (Figure 1.5).[239, 245, 246] Benzoic acid derived phenolic acids include 4-hydroxybenzoic, protocatechuic, gallic, vanillic and syringic acid. Other acids can include phenylacetic, mandelic and homogentisic acid. Comparatively, the basic flavonoid skeleton contains 15 carbon atoms arranged in two aromatic rings connected by an oxygenated, 3-carbon, heterocyclic ring.[247, 248] Flavonoids can be divided into several subgroups (depending on the basic structure, Figure 1.5) including isoflavones, neoflavonoids, flavones, flavonols, flavanones, catechins, anthocyanins and chalcones.[247, 249, 250] Flavanones comprise of hesperetin, pinocembrin and naringenin. Flavones comprise of chrysin, apigenin, luteolin and tricetin. Pinobanksin is an example of dihydroflavonols and other flavonoids include galangin, kaempferol, quercetin, isorhamnetin and myricetin.

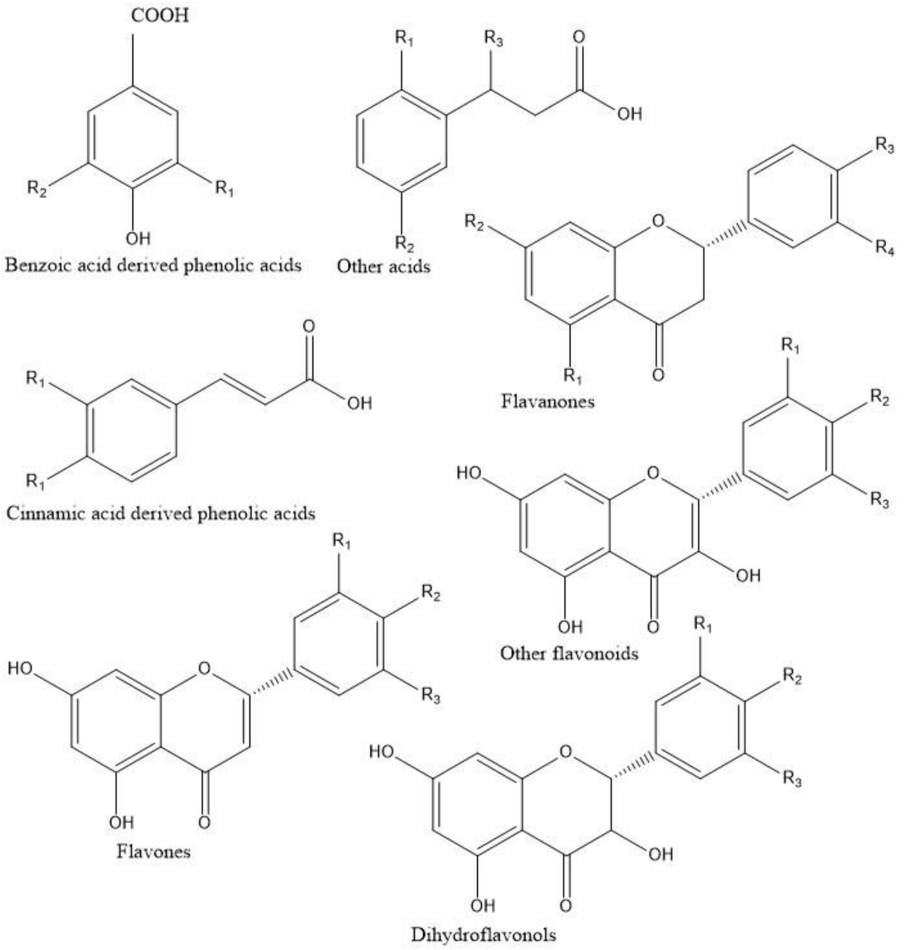


Figure 1.5. The skeletal structures of common polyphenolics (phenolic acids and flavonoids) and the R groups represent organic groups.

A range of phenolic compounds have been quantified in various honeys. A summary of compounds present in mānuka, clover and other honeys is presented in Table 1.7 to demonstrate the wide range of compounds and their concentrations. Mānuka and clover have been presented separately due to the focus of mānuka in this research and clover due it being used as a matrix for storage trials.

Honeys can have a very diverse phenolic content. Phenolic compounds found at concentrations <1 µg/g (in most honeys) include flavonoids such as acacetin[251], alpinetin[252], astragalin[253], epicatechin gallate[253], fisetin[254], hispidulin[255], hyperoside[256], isoliquiritigenin[253], sakuranetin[251], tangeretin[257] and phenolic acids such as benzyl caffeate[252], 2,4-dihydroxybenzoic acid[258], 3,4-dimethoxycinnamic acid[252, 254, 259], isoferulic acid[252], γ-resorcylic acid[260] and 2,4,6-trihydroxybenzoic acid monohydrate.[260]

Table 1.7. Concentrations of various phenolic compounds in mānuka, clover and other honeys, µg/g (ND = not detectable and tr = trace).*

Compound name	Cas no.	Type of phenolic compound	Mānuka honey (µg/g)	Clover honey (µg/g)	Other honeys (µg/g)*
Aesculetin[255]	305-01-1	Coumarin			1.68-4.83
Apigenin[253, 257, 259-266]	520-36-5	Flavonoid	0.39	ND-0.41	ND-34.83
Biochanin A[267]	491-80-5	Flavonoid			11.9-18.1
Catechin[253, 258, 262, 268]	154-23-4	Flavonoid	0.043-25.14	ND	ND-71.63
Chrysin[251, 252, 257, 259-261, 263, 264, 266, 269, 270]	480-40-0	Flavonoid	0.68-1.23	0.76-1.23	ND-12.21
Epicatechin[262, 271]	490-46-0	Flavonoid		ND	ND-28.4
Epigallocatechin gallate[253, 268]	989-51-5	Flavonoid	ND		ND-9.67
Eriodictyol[255]	552-58-9	Flavonoid			0.14-0.25
Galangin[252, 255, 257, 259, 261, 263-267]	548-83-4	Flavonoid	1.24	0.36	ND-133.4
Gallocatechin[267]	970-73-0	Flavonoid			175.6-238.9
Hesperetin[254, 257, 258, 261]	69097-99-0	Flavonoid	0.36-0.66	1.68	ND-7.46
Hesperidin[254, 257, 263, 272, 273]	520-26-3	Flavonoid	4.7	ND-0.34	ND-26.4
Isorhamnetin[253, 255, 262]	480-19-3	Flavonoid		ND	ND-3.28
Kaempferol[251-253, 255, 257-264, 267, 269, 270, 274, 275]	520-18-3	Flavonoid	0.21-1.95	ND-0.5	ND-32.7
Luteolin[251, 254, 255, 259, 261, 263-265, 267, 269, 273, 274]	491-70-3	Flavonoid	1.07-5.19	0.76	ND-60.2
5-Methoxy pinobanksin[252]	119309-36-3	Flavonoid			0.37
Myricetin[253, 257, 259, 261, 263]	529-44-2	Flavonoid	ND	ND	ND-1.125
Naringenin[253-255, 257, 263, 265, 266, 272, 273]	67604-48-2	Flavonoid	1.18	1.27	ND-34.95
Naringin[254, 257]	10236-47-2	Flavonoid		ND	ND-11
Pinobanksin[251, 252, 261, 269, 273]	548-82-3	Flavonoid	2.62	3.71	0.53-16.22
Pinocembrin[251-255, 259, 261, 264-266, 269, 274]	480-39-7	Flavonoid	1.51-7.82	11.59	0.01036-12.33

Compound name	**Cas no.**	**Type of phenolic compound**	**Mānuka honey (µg/g)**	**Clover honey (µg/g)**	**Other honeys (µg/g)***
Quercetin[251, 253-255, 257-264, 269, 270, 272-275]	117-39-5	Flavonoid	0.14-1.25	ND-2.98	ND-320.27
Quercitrin[255, 272]	522-12-3	Flavonoid			0.23-1.78
Rutin[253-256, 258, 259, 262, 263, 267, 269, 272-275]	153-18-4	Flavonoid	ND-0.07	ND-5.37	ND-538.62
Spiraeoside[267]	20229-56-5	Flavonoid			39.9
Taxifolin[253, 263, 267]	480-18-2	Flavonoid			0.0001-61.9
Leptosperin[276]	486-23-7	Glycoside of methyl syringate	398		
Benzoic acid[252, 269, 272]	65-85-0	Phenolic acid	0.48	0.46	0.21-2.34
Caffeic acid[252-264, 269-273, 275, 277, 278]	331-39-5	Phenolic acid	0.78-17.9	1.03-17.9	ND-26.78
Caftaric acid[260, 267]	67879-58-7	Phenolic acid			0.05-252
Chlorogenic acid[253-255, 257, 258, 260, 262-265, 267, 268, 272, 277, 278]	327-97-9	Phenolic acid	ND-16.5	ND	ND-365.4
Cinnamic acid (*trans*-cinnamic acid)[252, 254, 258, 272, 278]	140-10-3	Phenolic acid	1.49	0.25	ND-2.29
o-Coumaric acid[254, 272]	614-60-8	Phenolic acid		1.07	0.01-2.9
p-Coumaric acid[252-256, 258, 260-265, 267-274, 277, 278]	501-98-4	Phenolic acid	ND-8.41	ND-0.98	ND-40.73
3,4-Dihydroxybenzoic acid[253-255, 257-260, 262-265, 271-273]	99-50-3	Phenolic acid	1.66-49.7	ND-2.75	ND-744.6
3,5-Dihydroxybenzoic acid[254, 260]	99-10-5	Phenolic acid			ND-2.28
Ellagic acid[251, 253-255]	476-66-4	Phenolic acid			0.12-3.15
Ferulic acid[252, 254, 256, 258-263, 265, 272, 273, 277, 278]	537-98-4	Phenolic acid	0.43	ND-0.70	ND-9.35
Gallic acid[152, 253, 254, 257-260, 262-265, 267-269, 273, 275-278]	149-91-7	Phenolic acid	0.23-186.06	ND-30.6	ND-992.6
Gentistic acid[257, 260, 263]	490-79-9	Phenolic acid	30.4	9.2	ND-26.4
3-Hydroxybenzoic acid[272]	99-06-9	Phenolic acid			0.26-12.37
p-Hydroxybenzoic acid[251, 252, 254, 256-258, 260, 262, 263, 265, 269, 271, 272, 275-279]	99-96-7	Phenolic acid	3.28-9.5	ND-4.05	ND-55.26
3-Hydroxyphenylacetic acid[260]	621-37-4	Phenolic acid			4.2-140
4-Hydroxyphenylacetic acid[277]	156-38-7	Phenolic acid			0.17-1.86

Compound name	**Cas no.**	**Type of phenolic compound**	**Mānuka honey (µg/g)**	**Clover honey (µg/g)**	**Other honeys (µg/g)***
2-Methoxybenzoic acid[152, 276]	579-75-9	Phenolic acid	1.2-27.67	tr	tr-0.7
4-Methoxybenzoic acid[152, 276]	100-09-4	Phenolic acid	tr-3	tr	ND-4
4-Methoxyphenyllactic acid[152, 276]	104-01-8	Phenolic acid	7.4-48.325	5	tr-402
Methyl syringate[152, 258, 276]	884-35-5	Phenolic acid	9.4-158.08	1.7	1.7-158.08
3-Phenyllactic acid[152, 258, 276]	828-01-3	Phenolic acid	330-1314		20-940
Rosmarinic acid[259, 267]	20283-92-5	Phenolic acid			0.085-20.8
Salicylic acid[263, 265, 272, 273]	69-72-7	Phenolic acid		15.95	1.41-243.1
Sinapic acid[258, 259, 272, 278]	530-59-6	Phenolic acid	0.58		0.126-8.02
Syringic acid[152, 254-258, 261-263, 265, 268, 272, 273, 275, 277-279]	530-57-4	Phenolic acid	ND-40	ND-5.01	ND-87.7
2,3,4-Trihydroxybenzoic acid[257]	610-02-6	Phenolic acid	24	ND	ND-28.6
Trimethoxybenzoic acid[152]	118-41-2	Phenolic acid	26-106	2	2-27.2
Vanillic acid[252, 256-262, 265, 267, 271, 272, 275, 277-279]	121-34-6	Phenolic acid	ND-0.85	ND-0.31	ND-41.5
Veratric acid[260]	93-70-2	Phenolic acid			1.1-1.8
Coniferaldehyde[273]	458-36-6	Phenolic aldehyde			3.27
4-Hydroxybenzaldehyde[260]	123-08-0	Phenolic aldehyde			0.28-1.5
Protocatechualdehyde[257, 260]	139-85-5	Phenolic aldehyde	18.2	13.1	0.12-21.7
Vanillin[257, 263]	121-33-5	Phenolic aldehyde	ND	ND	ND-52
Carnosol[266]	5957-80-2	Terpenoid			9.52

*Other honeys include: acacia, astragalus, basil, black bee, blackberry, beech forest, bergamot blossom, blue borage, buckwheat, Changbai snow, chaste, chestnut, chrysanthemum, citrus, common eryngo, dendropanax dentiger, eucalyptus, forest, gallberry, goldenrod, heather, honeydew, honeysuckle, hypericum, jarrah, Jerusalem tea, jujube, Kāmahi, Kānuka, koromiko, lavender, lemon, lime, linden, litchi, locust, longan, loquat, lychee, marruca, mint, motherwort, mountin coptis, multifloral, NZ ling, oak, oilseed rape, orange, oregano, pagoda tree flower, palmetto, phacelia, pine, Pōhutukawa, rape, rātā, rewarewa, rhododendron, rosemary, schisandra, sulla, sunflower, thistle, thyme, tilia tree, tupelo, unspecified, vitex, wild carrot, wild hawthorn, wild flower, wild osmanthus, wild rose, willow honeydew, wolfberry, zaohua and *Ziziphus Spina-christs* honey.

Phenolic compounds 3-phenyllactic acid, methyl syringate, trimethoxybenzoic acid, gallic acid and leptosperin had concentrations >100 mg/kg in mānuka honey.

The majority of the phenolic compounds were at elevated levels in mānuka honey (as compared to clover honey) except for apigenin, hesperetin, naringenin, pinobanksin, pinocembrin, quercetin and rutin. 3PLA and 2MBA are found at significantly higher concentrations in mānuka honey (330-1314 and 1.2-27.67 µg/g respectively) than other honeys (20-940 and trace-0.7 µg/g respectively). Since phenolic compounds are known to be reactive and their high concentrations in certain honeys (e.g. mānuka honey) indicate that they could have a role in forming modifications on proteins found in honey directly or through an interaction with MGO.[119] Bell (2022) reported that 3PLA influence quickened the loss of diastase activity over time using a clover honey matrix spiked with 3PLA as compared to clover honey spiked with other compounds (such as methyl syringate) and the control sample.[4]

1.4.11 Dihydroxyacetone (DHA) and Methylglyoxal (MGO)

Mānuka nectar contains varying amount of dihydroxyacetone (DHA).[280, 281] The concentration of DHA varies between trees and seasons and is converted to MGO during honey maturation through a non-enzymatic dehydration reaction (Figure 1.6).[281-283] DHA and MGO are two important distinguishing and unique compounds of mānuka honey.

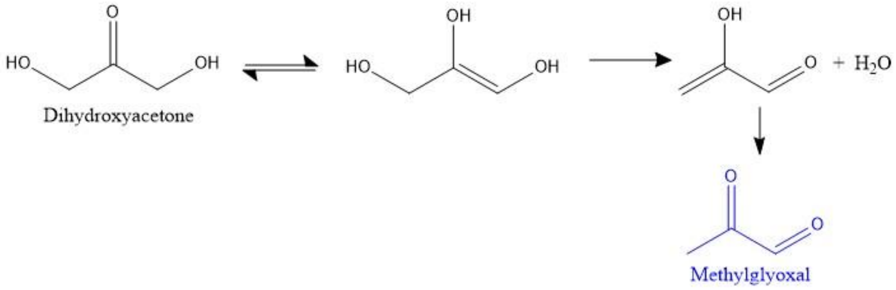


Figure 1.6. The non-enzymatic conversion of DHA to MGO (in blue).

MGO is present at elevated levels in mānuka honey. It is a 2-oxoaldehyde (dicarbonyl derived from propanal), is polar (due to the 2 carbonyl groups and short non-polar carbon chain) and dissolves in a range of polar solvents.[284] MGO is commercially supplied as a clear, yellow liquid.[285, 286] Medicinal grade mānuka honeys have high concentrations (i.e. 1000+ mg/kg or unique mānuka factor, UMF, 25+).[287] These high MGO honeys have much higher antibacterial activity than MGO alone and this is suggested to be the result of the synergy between MGO and honey's other components.[288] Alternatively, it could be due to the diffusion of MGO in a water matrix versus a sugar matrix. MGO exhibits antibacterial activity by limiting bacteria adherence and mobility through alterations in the bacterial fimbriae and flagella.[289] The antibacterial activity property of mānuka honey is highly sought as it improves the efficiency of preventing and treating common microorganism infections (better than other honeys) especially hospital-acquired medically-resistant ones such as *S. aureus*.[290-292] Most other honeys show antibacterial activity solely due to the presence of hydrogen peroxide (levels of which are dependent on the glucose oxidase and catalase activity in honey); whereas, mānuka honey contains MGO, providing antibacterial activity even in the presence of high catalase environments (such as in wounds).[289, 293]

MGO, as well as forming from the conversion of DHA (Figure 1.6), is also produced from glycolytic intermediates during the Maillard reaction in honey (Figure 1.7).[119, 137, 281, 294] These Maillard reaction products are also known as advanced glycation end products (AGEs).[233] Since high MGO concentrations are only observed for mānuka honey, it is safe to assume that MGO-forming reaction processes (other than the conversion of DHA) produce minimal to nil MGO concentrations.

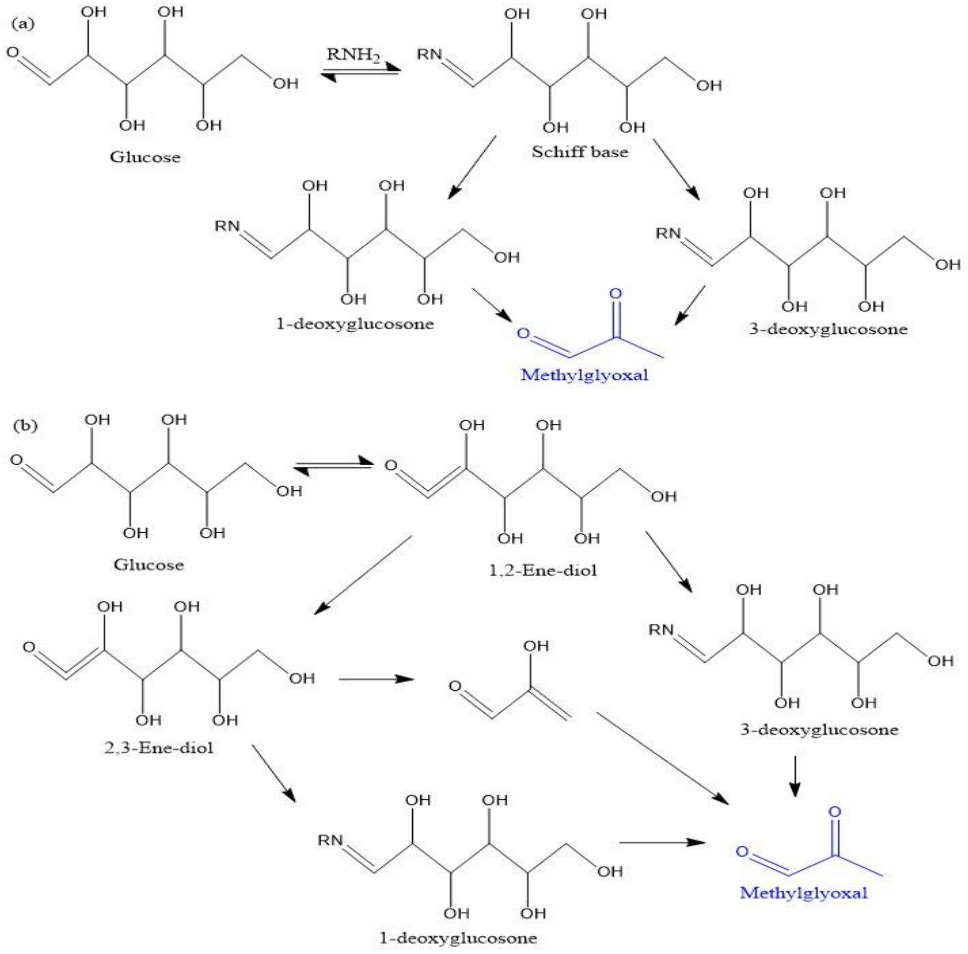


Figure 1.7. Reaction pathways that form methylglyoxal in honey (from sources other than dihydroxyacetone), (a) Formation of MGO through a Schiff base intermediate from a hexose, (b) Formation of MGO from a 1,2-ene-diol intermediate from the autoxidation of hexoses.[119]

Adams *et al* (2009) did not find MGO in mānuka nectar while Stephens *et al* (2010) found measurable quantities of MGO in mānuka nectar and Grainger *et al* (2016) postulated that observations from Stephens *et al* (2010) may have resulted due to extended storage of nectar before analysis.[152, 281, 282] A later study by McDonald *et al* (2018) confirmed that MGO was not present in mānuka nectar.[153] Grainger *et al* (2016) also described the conversion of DHA to MGO as a first order kinetic reaction in honey.[282] However, first order was only obeyed in the initial MGO formation period in mānuka honey due to a subsequent loss of MGO at later times. The study observed that MGO increased to a certain point after which it began decaying until a DHA: MGO ratio of 0.6: 1 was observed for mānuka honeys.

This decay was associated with the loss of MGO through increasing MGO consuming reactions occurrence as the DHA to MGO conversion slowed.[282]

MGO is a highly reactive glycating compound which reacts with side chains of certain amino acids residues and amino-containing lipids to form advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs) respectively (i.e. protein modifications).[137, 295] The most reactive amino acid residues include cysteine, lysine, arginine, tyrosine, methionine, histidine and tryptophan.[296, 297] Their corresponding chemically reactive side chains include thiol/sulfhydryl, butylammonium, guanidinium, tyrosyl, S-methyl thioester, imidazolyl and indole groups.[296]

MGO is known to react with cysteine residues to produce hemithioacetal compounds (MGO-Cys), carboxyethyl-cysteine (CEC), MGO-di-Cys, MGO-tri-Cys and Cys-MGO-Arg.[119, 166, 298-300] MGO reacts with lysine residues to produce MGO-Lys, putative crosslink, carboxyethyl-lysine (CEL), MGO-lysine dimer (MOLD) and MGO-lysine-arginine dimer (MODIC).[119, 166, 298, 299, 301-305] MGO reacts with arginine residues to produce Cys-MGO-Arg, MODIC, 5-hydro-5-methylimidaxol-4-one, 5-methylimidaxol-4-one/MGO imidazolone, argpyrimidine, hydroimidazolone isomer 1 (MG-H1), hydroimidazolone isomer 2 (MG-H2), hydroimidazolone isomer 3 (MG-H3) and carboxyethyl arginine (CEA).[119, 299, 300, 304, 306, 307] MGO reacts with histidine to form MGO-His.[119, 308, 309] All of these modifications could be occurring on proteins (such as diastase) in honey and potentially causing inhibition through various methods. Since high MGO concentrations are unique to mānuka honey, the effects of MGO-based modifications are more likely to affect diastase activity of mānuka honey to a greater extent than the activity of other honeys.

DHA can exist as a dimer or monomer in honey, however, it is the monomeric form that then forms MGO. Grainger suggested a possible reaction for converting dimeric DHA to monomeric form involving protonation to catalyse this process and predicted this was a rate-determining step in the conversion of DHA to MGO.[166] As well as forming MGO, DHA can also react with amino acids, hence is able to modify proteins (such as diastase). There is extensive literature highlighting DHA's role as the main active agent in commercial sunless tanning products.[310-312] In such products, DHA undergoes Maillard reactions with amino acids (with the amino groups) of epidermal proteins found in the skin to produce brown pigments (melanoidins).[310, 311] Since DHA primarily reacts with the amino groups this means that all free amino acids are able to react (through the amino group adjacent the carboxylic group), but lysine, arginine and histidine can also react whilst in protein sequences using the amino group on their side chains. Sun *et al* proposed reaction mechanisms for the formation of DHA melanoidin products on the side chain amino group of lysine (DHA-Lys) and on the amino group adjacent the carboxylic group of arginine (DHA-Arg) and histidine (DHA-His).[310] From their proposed structures, only DHA-Lys is plausible as a modification on a protein whereas DHA-Arg and DHA-His would only occur on either the N-terminal arginine or histidine or require breaking the polypeptide chain to form the modification. A recent study investigated the reaction of DHA with the thiol group of cysteine and found that this reaction inhibited the colour formation of the reaction of DHA with the amino group of cysteine.[311] The exact structures of the melanoidins formed on proteins remains an unexplored part of literature that has the potential to explain the changes occurring on proteins and understanding if these are occurring on diastase in mānuka honey and affecting its activity.

1.5 Research aims

New Zealand’s multi-million dollar honey industry exports over 10,000 tonnes (10,250 tonnes between June 2023 to June 2024) of honey annually and mānuka honey alone generates a large proportion of the export revenue.[1, 313] However, due to a rigorous testing regime, and a unique matrix, mānuka honey fails the diastase test more often than other floral honeys, limiting exports.[2] The diastase test is one of the quality tests imposed on NZ’s exported honeys by receiving countries and is used to check whether honey has been exposed to high temperatures or extended storage periods. Unfortunately, high grade mānuka honey can fail this test despite acceptable HMF levels, suggesting that something other than time and heat that are contributing to low diastase activity. This urges the need to investigate a variety of bioactive compounds (MGO and phenolic compounds) for their potential roles in decreasing diastase activity in mānuka honey. This research will build on recently reported research[4] by using enzyme kinetics and modelling techniques to investigate the potential surface level modification of honey proteins, and how this effects testing results. Evidence of protein modification and inhibition by the unique matrix of mānuka honey has implications for further work to allow the exemption of mānuka honey from diastase testing, potentially increasing NZ honey exports.

This research aimed to investigate the interactions of 3PLA, MGO and DHA with diastase to determine if they were causing modifications to diastase over time which impact diastase activity.

1.6 Thesis outline

This thesis has been separated into the following chapters:

The **Methods** chapter details the chemical and biological methodology used for this study. The **Method Validation** chapter examines the validity of the in-house enzyme kinetics analysis method.

Fresh mānuka honey and clover honey spiked with MGO, DHA, 3PLA and combinations of these were stored at 27 °C for ~200 days and periodically tested for MGO, DHA, HMF and 3PLA (and mānuka markers 2MAP, 2MBA, 4HPLA) concentrations to determine the impact on diastase activity and enzyme kinetics of diastase over time in the **Time Trial Results and Discussion** chapter.

The initial and final time points (day ~200) from the time trial were examined for surface protein modifications on diastase and other honey proteins in the **Protein Modifications Occurring in Honey** chapter.

The main findings were summarised, and recommendations for future research on this topic were discussed in the **Conclusion and Future work** chapter.

2 Methods

2.1 Honey samples

Two mānuka honey samples were supplied by Prolife Foods (concentrations of key compounds in both honeys were provided by the supplier, Appendix Table 8.2). Clover honey from the same company was purchased from a commercial outlet, several jars with the same batch number were combined and homogenized to form one clover honey batch. This honey was sub-portioned for the storage trial. All honeys were stored in the freezer (−23 °C) when not used for analysis. A separate clover and mānuka honey were used as quality control (QC) honeys for the enzyme kinetics analysis.

2.2 Solvents, standards, reagents, consumables

2.2.1 Solvents

Type 1 water (i.e. Milli Q water) was obtained using a Millipore Milli Q Reference water purification system (18.2 mΩ resistivity). Acetonitrile (ACN, gradient grade for LC) was obtained from Sigma and methanol (MeOH, HPLC grade) was obtained from Scharlau.

2.2.2 Standards and reagents

2.2.2.1 Time trial

Methylglyoxal solution (MGO, ~40%) was obtained from Fluka. 5-hydroxymethyl-2-furfuraldehyde (HMF, 99%) and 1,3-dihydroxyacetone dimer (DHA, 97%) obtained from Sigma.

2.2.2.2 Quantification of DHA, MGO and HMF (3in1 method)

Methylglyoxal solution (MGO, ~40%) was obtained from Fluka. 5-hydroxymethyl-2-furfuraldehyde (HMF, 99%), 1,3-dihydroxyacetone dimer (DHA, 97%), hydroxyacetone (HA, 90%), ᴅ-(+)-glucose (≥99.5%), sucrose (ACS grade) and D-(-)-fructose (≥99%) were purchased from Sigma. *O*-(2,3,4,5,6-Pentaflurobenzyl)hydroxylamine hydrochloride (PFBHA, 99+%) was purchased from Alfar Aesar and citric acid (>99%) was purchased from Ajax FineChem.

2.2.2.3 Enzyme kinetic analysis

Potassium iodide (PI, >99%) and starch soluble iodometric indicator (>99%) were purchased from Ajax Chemicals. Iodine resublimed (99.5%) was from BDH and sodium chloride (≥99.5%), sodium acetate (≥99%) and glacial acetic acid (100%) were purchased from Sigma.

2.2.2.4 Protein modification identification

Tris(hydroxymethyl)aminomethane (ACS reagent, Ph Eur), calcium chloride (ACS reagent, Ph Eur), sodium chloride (≥99.5%), ammonium sulfate (ACS, ISO reagent, Ph Eur), ammonium persulfate (APS, ≥98%), *ortho*-phosphoric acid (85%), methanol (≥99.9%), glycine and *N,N,N',N'*-Tetramethylethylenediamine (TEMED, 99%) was purchased from Sigma. Sodium dodecyl sulphate (SDS, ≥99%) and 30% Acrylamide/Bis 37.5:1 purchased from BIO-RAD. Coomassie blue G-250 was purchased from BDH.

2.3 General methods

2.3.1 Apparatus temperature management

An oven, incubator and water baths were used for method analysis, storage trials or to dry glassware. The temperature was set accurately for each equipment using a thermocouple and all equipment held the temperature over the testing period (± 0.5 °C). The incubator temperature (27 °C) was checked at each time point of the storage trial.

2.3.2 Glassware cleaning

All glass vials were washed before use. The glass vials were washed with detergent, well rinsed with tap water, placed in a concentrated nitric acid bath overnight, thoroughly rinsed with tap water (to remove acid) and then oven dried at 100 °C for a minimum of 3 hours.

2.3.3 Image generation, spectrophotometric and statistical analysis

Analysis of data and graphical visualisation was carried out using Microsoft Excel (Version 2505) and GraphPad Prism (Version 10.5.0). ChemDraw (Version 21.0.0) was used to produce chemical structures, diagrams and reaction schemes. Spectral data was obtained from the external liquid chromatography tandem mass spectrometry (LC-MS/MS) and was externally processed using PEAKS Studio 11 bioinformatics software. Spectrophotometric data was acquired using HALO VIS-20 Dynamica alphatech and SpectraMax M4 microplate reader (Molecular Devices) and SoftMax® Pro data acquisition and analysis software (Version 7.1). Gel images were obtained using Invitrogen^{TM} iBright^{TM} Imaging Systems FL1000 (Thermo Fisher Scientific).

2.3.4 Honey storage time trial

Two mānuka honeys (1 and 2) were selected to study in this time trial. Clover honey was portioned into six samples (300 g each) consisting of the control sample and 5 spike samples. The five spikes included MGO, 3PLA, DHA, 3PLA+MGO and 3PLA+DHA and 10 mL spike standards (33.3 mg/mL) were made. The clover honey aliquots (300 g) were spiked with spike standards (9 mL) for a final concentration of ~970 mg/kg for each analyte of interest. Both mānuka honeys, clover control and all five clover spike honeys were stored at 27 °C for ~200 days (203 days for the mānuka honeys and 200 days for all clover honeys including control and spikes). Sub-samples were taken periodically during the time trial (total of 18-19 times).

2.4 Quantification of DHA, MGO and HMF (3in1 method)

2.4.1 Matrices, reagents and Standards

The matrices [artificial honey solution (AH) and dilute QC honey sample], reagents (dilute QC honey sample, stock HA, HA:ACN, PFBHA and citrate buffer solutions) and standards (stock individual standards and mixed calibration standards, MStds) were prepared as described by Bell (2022).[4] Spike standard solutions were made (Table 2.1) using the individual stock standard solutions of DHA, MGO and HMF. All solutions were stored between 4-6 °C.

Table 2.1. Composition of spike standard solutions for the 3in1 method.

Stock standard solution	High spike solution (mL)	Low spike solution (mL)
DHA	1.50	0.075
HMF	1.80	0.30
MGO	6.45	0.50

2.4.2 Sample preparation

Honey (1.0 ± 0.1 g) was dissolved in type 1 water (4.4 mL) and homogenized. The reaction was carried out in 96 well deep well plates. Each well contained analyte solution (150 µL) to which HA:ACN (0.7 mL, 1:19) solution was added and then PFBHA (0.1 mL) solution. The composition of the solution depended on the solution type (Table 2.2). Wells were sealed with a silicon lid, the plate was inverted several times for thorough mixing and incubated at 50 °C for 60 ± 10 min. The plate was then left to cool at room temperature and after cooling, the samples were analysed by high-performance liquid chromatography (HPLC).

Table 2.2. The composition each solution type for the quantification of DHA, MGO and HMF (3in1 method).

Solution type	Composition	Volume (µL)	Total volume (µL)
Sample	Diluted sample	150	150

MStd	Artificial honey (AH) solution	50	150
	MStd	100	
AH blank	AH solution	50	150
	T1 H_2O	100	
Water blank	T1 H_2O	150	150
Final spiked solution	High/low spike solution	50	150
	Dilute QC honey sample	100	

2.4.3 Instrument method

The HPLC system used for the 3in1 method was the Waters Alliance 2695 HPLC system fitted with a pump, degasser, autosampler and column oven. Detection was carried out using a 2996 photodiode array detector (PDA, 240-400 nm) and the system was operated using the Empower^{TM} 3 Chromatography Software.

Separation was performed on a Kinetex® C18 150x4.6 mm, 5 µm column. Mobile phase A was 30% ACN in type 1 water (v/v) and mobile phase B was 100% ACN. Total analysis time was 10.5 minutes using the gradient stated in Table 2.3. The flow rate was 0.8 mL/min, injection volume was set to 10 µL and column oven and sample chamber temperatures were set to 30 °C and 8 °C respectively. The individual 2D detection channels were set at 260 nm (DHA, HA and PFBHA), 273 nm (HMF) and 244 nm (MGO). Retention times for quantification for DHA, HA, PFBHA, HMF and MGO isomers 1 and 2 were 4.09, 4.80, 4.45, 4.97, 7.5 and 7.98 min respectively.

Table 2.3. Gradient conditions for the 3in1 method.

Time (min)	% Mobile phase A (30% ACN in water)	% Mobile phase B (100% ACN)
0	90	10
2.50	15	85
5.50	15	85
7.00	90	10
10.50	90	10

2.4.4 Data work-up

The concentrations of each analyte (DHA, MGO and HMF) in honey were calculated as carried out by Bell (2022).[4]

2.5 Diastase activity analysis

The diastase activity results are expressed in diastase number (DN). Analysis was carried out by an International Accreditation New Zealand (IANZ) accredited laboratory using the Phadebas method.[94]

2.6 Enzyme kinetic analysis

This method was developed from the official IHC Schade method and adapted for an enzyme kinetic experiment.[94]

2.6.1 Reagents and standards

Sodium chloride solution (100 mL, 0.029 g/mL) was made. Iodine stock solution was made by dissolving iodine resublimed (11.0 g) and potassium iodide (22.0 g) in 30-40 mL of type 1 water and then making it up to 500 mL with type 1 water. Acetate buffer solution (pH 5.3) was made by dissolving sodium acetate (43.5 g) in ~200 mL type 1 water, adjusted the pH to 5.3 with glacial acetic acid (~ 5 mL) and made to 250 mL with type 1 water. The iodine stock solution was stored in the dark and all other solutions were stored at room temperature.

On the day of analysis, fresh starch stock solution (0.02 g/mL) was made to a final volume of 20 mL. This was done as per the IHC Schade method.[94] Dilute iodine solution was also made fresh on the day of analysis (and stored in an amber coloured bottle) by dissolving potassium iodide (0.6 g) and iodine stock solution (0.06 mL) in type 1 water, made to 15 mL.

Starch substrate solutions (SS 1-8) were prepared in 15 mL falcon tubes (Table 2.4) where SS 1 was a water blank. A separate dilute starch solution (6.7 mg/mL) was made with a composition of 2 mL of type 1 water to 1 mL of stock starch solution (0.02 g/mL).

Table 2.4. Composition of starch substrate solutions (SS 1-8) for diastase activity analysis.

	Stock starch solution (0.02 g/mL), mL	Type 1 water, mL	Final starch substrate solution concentration (mg/mL)
SS 1	0	5.000	0.0
SS 2	0.375	4.125	1.7
SS 3	0.567	3.933	2.5
SS 4	0.75	3.750	3.3
SS 5	1.500	3.000	6.7
SS 6	2.250	2.250	10.0
SS 7	3.000	1.500	13.3
SS 8	4.500	0.000	20.0

Each batch of samples were analysed with a QC clover honey sample with a known DN number obtained from an IANZ accredited laboratory using the Phadebas method.

2.6.2 Sample preparation

Honey (1.000 ± 0.001 g) was dissolved in type 1 water (1.5 mL) and acetate buffer solution (0.5 mL). To the homogenized mixture, sodium chloride solution (0.3 mL) and type 1 water (2 mL) were added to get a final volume of 5 mL. Once prepared, this diluted sample was tested within 3 hours.

The spectrophotometric measurements for the final λ660 readings were carried out in 200 µL volume 96 well plates. The calibration curve was generated from the first column (in each plate used) using the dilute starch solution (6.7 mg/mL) with final concentrations between 0.17 to 0.02 mg/mL. This was tested in the SpectraMax M4 microplate reader (Molecular Devices) at 660 nm and analysed with the SoftMax® Pro data acquisition and analysis software (Version 7.1).

The reaction of samples was carried out in 96 well deep well plates by pipetting 0.4 mL of each honey down the 8 well column, to this 0.2 mL of SS 1-8 were added down the column. These were homogenized and at 5 min intervals 3 µL of each reaction mix was added to 200 µL 96 well plates (containing 157 µL type 1 water and 3 µL dilute iodine solution) down the column, homogenized and instantly measured at 660 nm. Assays were done in duplicate.

2.6.3 Data work-up

The calibration curve was used to calculate the residual starch concentration (mg/mL) post-reaction (and factoring in the mass of the honey sample used) for each starch substrate concentration for each honey.

From the calibration curve, the concentration of starch post-reaction for each reaction was calculated (Equation 1).

Equation 1

$$Concentration\,of\;starch\;\left(\frac{mg}{mL}\right)=\frac{Absorbance\;at\;\lambda_{660}-intercept}{gradient}$$

For each starch substrate concentration, the concentration of residual starch (mg/mL) was plotted against time. The gradient resulting from this for each starch substrate solution was divided by the mass of the honey sample (g) and multiplied by a factor of 1000 (Equation 2) to give rates of enzymatic activity in mg of starch/mL/min/g of honey. This was plotted against the known starting starch concentrations (mg/mL) to generate plots that were fitted to the Michaelis-Menten equation (Equation 3) in GraphPad Prism (Version 10.5.0). The Michaelis-Menten fitting plotted the rates of reactions of different substrate concentrations as the y-axis against the starting substrate concentration as the x-axis to generate key parameters, such as $V_{\max}$ and K_{M} values.

Equation 2

$$Starch\ concentration\ (mg\ of\ starch/mL/min/g\ of\ honey) = \frac{Slope\ of\ concentration\ of\ starch\ \left(\frac{mg}{mL}\right) against\ time\ (\mathrm{min})}{mass\ of\ honey\ (g)} \times 1000$$

Equation 3

$$Reaction\ rate = \frac{V_{max} \times [substrate\ concentration]}{K_M + [substrate\ concentration]}$$

2.7 Mānuka marker analysis

The mānuka marker analysis (quantification of 3PLA, 2MAP, 2MBA and 4HPLA) was carried out by an IANZ accredited laboratory using the MPI mānuka marker LC-MS/MS analysis method.[314]

2.8 Protein modification identification

2.8.1 Reagents

The compositions of the various reagents made for protein modification identification are recorded in Table 2.5. All reagents were made to 1 L except for Q4 dye which was made to 10 mL.

Table 2.5. The composition of various reagents used for protein modification identification.

Reagent	Composition
Buffer solution	20 mM Tris pH 8.5, 300 mM NaCl, 2 mM $CaCl_2$
Q4 dye	1 M Tris pH 6.8, 20% glycerol, 0.25% SDS, 10% 2-mercaptoethanol, 40% bromophenol blue
1X SDS running buffer	25 mM Tris, 250 mM glycine, 0.1% SDS w/v
Fixing solution	50% v/v methanol, 2% v/v phosphoric acid
Colloidal Coomassie stain	17% w/v ammonium, 2% w/v phosphoric acid, 34% w/v methanol, 0.06% w/v Coomassie blue G-250
Destaining solution	5% v/v methanol

Five 10% polyacrylamide gel electrophoresis (PAGE) gels were made by homogenizing 30% acrylamide (10.00 mL), resolving buffer (7.5 mL of 1.5 M Tris, pH 8.8), APS (0.15 mL), TEMED (0.015 mL) and type 1 water (12.35 mL). This was poured into the gel caster and left to set for ~30 minutes after adding a layer of isopropyl alcohol (~1 mL) over each gel slot. Once set, the isopropyl alcohol was decanted. The stacking gel layer was made by homogenizing 30% acrylamide (2.125 mL), stacking buffer (1.6 mL of 1 M Tris, pH 6.8), APS (0.063 mL), TEMED (0.0063 mL) and type 1 water (8.625 mL). This was poured equally over each slot in the gel caster, combs inserted to create sample wells and left to set for ~30 minutes. Once set, the gels were removed from the gel caster and stored at 3.5 °C in a damp cloth in a plastic bag until use.

2.8.2 Sample preparation

Honey (1.132 ± 0.001 g) was dissolved in buffer solution (2.5 mL) and homogenized. Then Q4 dye (10 µL) was added to the diluted honey sample (30 µL) in polymerase chain reaction (PCR) tube strips (Axygen® PCR-0208-C). The Q4: diluted honey sample (10 µL, 1:3) was injected into a single lane of a 10% SDS-PAGE gel. Each unique honey sample was separated by an empty lane in the gel and each gel's first lane contained a BIO-RAD Precision Plus$^{\text{TM}}$ Standard ladder (5 µL). In some instances, the ladder was also added to the last lane of the gel (to ensure the bands ran at the same level on the gel). The gel(s) with 1X SDS running buffer were run between 80-120 V using the BIO-RAD PowerPac™ Basic, with water cooling. The gel(s) were fixed with 3x 30 min washes of fixing solution (<100 mL per wash) and rinsed with tap water. Colloidal Coomassie stain (<100 mL) was added to the gel(s) to stain overnight, gels were left with stain on the shaker (IKA® KS 130 Basic at 160 rpm). The unused stain was then poured off the gel(s), destaining solution (<100 mL) was added, left for 10 min in destaining solution, washed 3x with MilliQ water and gel(s) left in MilliQ water. The gel(s) was imaged and sent to MS3 for further prep and LC-MS/MS analysis.

An external laboratory (MS3 Solutions) processed samples for protein modification identification. At MS3 Solutions the gel band(s) of interest were cut out, each band was cut further into smaller pieces, digested with sequence-grade trypsin, incubated overnight with residual stain removal solution (2 mL of 25 mM ammonium bicarbonate in 50% acetonitrile), washed with fresh buffer and dehydrated with acetonitrile. The gel band(s) were then incubated in dithiothreitol (DTT) wash solution (10 mM DTT in 25 mM ammonium bicarbonate), then in iodoacetamide wash solution (55 mM iodoacetamide in 25 mM ammonium bicarbonate) and were then digested overnight in trypsin (10 µL of 0.1 µg/µL trypsin in 25 mM ammonium bicarbonate and 5% acetonitrile) at 37 °C. The digested peptides were extracted from the gel pieces by sonication (10 min), two washes of extraction solution (0.2% formic acid in 40% acetonitrile), then washed with acetonitrile, the wash steps were combined for lyophilisation and the dried peptide sample was analysed by LC-MS/MS after resuspension in suspension solution (50 µL of 0.2% formic acid in 5% acetonitrile).

2.8.3 Data work-up

At the external laboratory, processed samples for protein modification identification were analysed by LC-MS/MS. The data from the LC-MS/MS was analysed using PEAKS Studio 11 bioinformatics software which compared identified peptides to sequences of interest and detected specified modifications.

3 Method Validation for Enzyme Kinetics

The enzyme kinetic analysis method was validated before use and is detailed in this chapter. The 3in1 method was validated in the laboratory prior to this work commencing in the Grainger lab by Bell (2022).[4] The analysis for the protein modification method was sent to a commercial laboratory. The analysis for diastase activity (DN results) and mānuka markers (3PLA, 2MAP, 2MBA and 4HPLA) were carried out in IANZ accredited laboratories.

3.1 Method development for enzyme kinetics analysis

The diastase activity enzyme kinetic analysis was modified from the official IHC Schade method.[94] This method was adapted to use less sample and reagents, produce Michaelis-Menten plots instead of diastase numbers (DN) to gain more information about the enzyme kinetics of *A. mellifera* diastase, and collect pseudo-continuous assay data with time. This modified method was a pseudo-continuous assay (similar to the official IHC Schade method) whereas the honey industry uses the Phadebas method (stopped assay) as the most common method for diastase activity determination to generate DN numbers (quantity of enzyme that converts 0.01 g of starch to the pre-scribed end-point at 40 °C in 1 hour under the test conditions).[94] However, assays at a set substrate concentration overlook the affinity of the enzyme for the substrate due to different honey matrices and enzyme variations. Comparatively, a pseudo-continuous assay involves stopping the reaction at different time points to approximate a continuous stream of data and is more accurate as it can show if the results are within the linear range over time.

Initially, an appropriate starch had to be selected that fit the requirements of the IHC Schade method. Out of a selection of starches from various companies (BDH, Analar, Sigma, and UNIVAR® AJAX chemicals) that were analysed, only one starch (UNIVAR® AJAX chemicals iodometric starch) showed absorbance readings at 660 nm that were within the IHC Schade method limits.

The λ_{660} readings were initially recorded manually (strictly within 30 sec of stopping the reaction) using a HALO VIS-20 Dynamica Alphatech spectrophotometer. Time was an important variable because the IHC Schade method stated to "immediately read the absorbance" after adding honey to the starch mix.[94] Time was investigated as a parameter by taking absorbance readings (λ_{660}) using a HALO VIS-20 Dynamica Alphatech spectrophotometer at 0.5, 1, 5, 60 and 120 minutes after stopping the reaction. Various dilutions of starches were tested as per the IHC Schade method. A change was observed for all concentrations of starch after 30 seconds [i.e. a logarithmic increase in the absorbance readings (λ_{660}), Figure 3.1]. Hence, all samples were tested within 30 seconds of stopping the reaction for consistency.

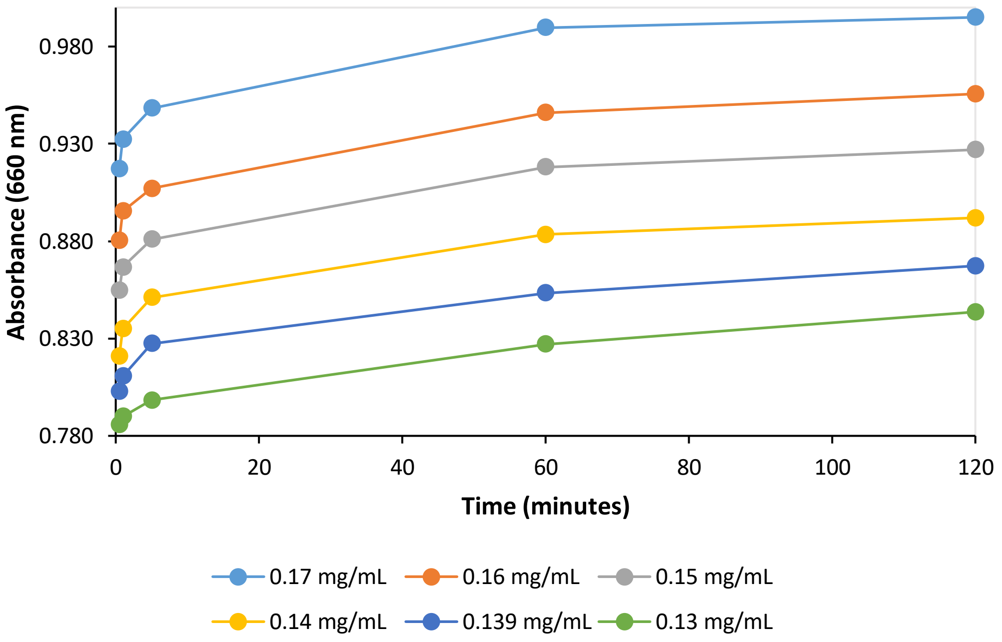


Figure 3.1. The effect of time on the absorbance reading (λ_{660}) post-reaction of the IHC Schade method on the reaction mixes containing type 1 water with different starch substrate concentrations.

The volumes were scaled down to a final volume of 190 µL to perform assays in a 200 µL 96 well plate and the starch concentrations were varied to suit the purpose of this study. The official IHC Schade method selects one dilution of starch solution to add to honey based on the results of testing various starch concentrations. However, to study the enzyme kinetics, the starch concentrations added to honey needed to vary to gain information of enzyme interaction strength with the substrate, and hence the various concentrations of starch were selected based on their fitting to the Michaelis-Menten plot. The plate system used a SpectraMax M4 microplate reader which allowed higher throughput and generated less liquid waste.

3.2 Validation of the enzyme kinetic method

The diastase activity analysis method was validated using a clover and a mānuka honey, chosen for their high DN (22 and 32 respectively). Using the enzyme kinetic analysis method (section 2.6), six replicate samples of each honey were analysed on three consecutive days. The same process was repeated a few weeks later to obtain a total of 36 replicates for each honey. Data was assessed with a 95% confidence interval, data impacted by human error was removed, and outliers were removed using the GraphPad Rout method (which identifies outliers using the false discovery rate) with the Q value set to 1%. R^2 values (from data fits with the Michaelis-Menten model) of 0.78 and 0.81 were achieved for the clover and mānuka honey respectively (on average, Table 3.1). Calibration curves from all method validation days generated R^2 value of >0.95 on average (Table 3.1).

Table 3.1. The R^2 values from the calibration curves and Michaelis-Menten model fits of the method validation of clover and mānuka honey.

		R^2 values (data fit to Michaelis-Menten model)	
Day	**R^2 values (Calibration curve)**	**Clover honey**	**Mānuka honey**
1	0.8824	0.7128	0.7523
2	0.9610	0.8962	0.7834
3	0.9780	0.9103	0.8889
4	0.9788	0.8068	0.7972
5	0.9568	0.6529	0.7825
6	0.9508	0.6930	0.8668

The reproducibility (inter-day precision), given as the relative standard deviation (RSD), was tested by comparing the six replicate samples tested independently over six days and for the clover and mānuka honeys and was 19.5 and 6.6% for V_{max} and 24.4 and 25.9% for K_{M} (Figure 3.2). While high for analytical chemistry, this variability is in line with that expected for this analysis, where increased errors are incurred from minor variability in enzyme additions and multiparameter fitting (for example RSD values as high as 28.7% have been reported for K_{M} in literature[315]). With the Michaelis-Menten fit, the replicates within both honey samples had overlapping 95% confidence intervals for V_{max} and K_{M} indicating that the difference between the replicates was not statistically significant across the independent assay measurements. Hence, the statistically significant agreement of fitting parameters across validation assays made this method fit for obtaining enzyme kinetics results of diastase in honey.

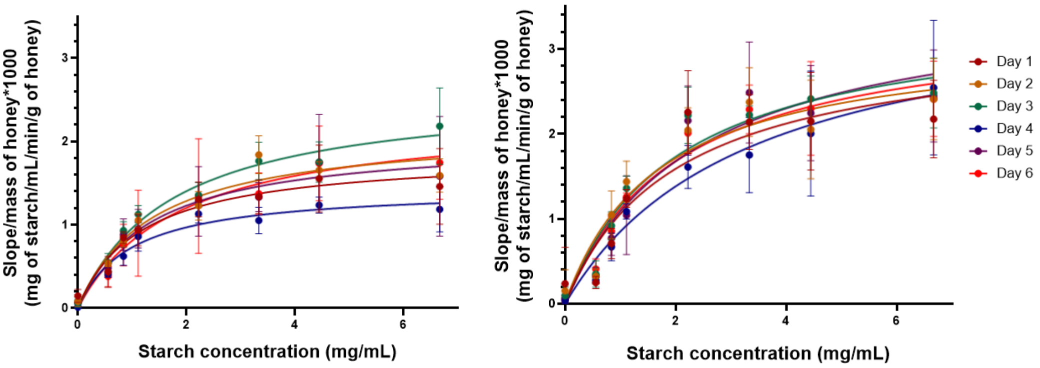


Figure 3.2. Michaelis-Menten plots of clover (left) and mānuka (right) honeys from the inter-day method validation showing overlapping within error (standard deviations shown as error bars).

Several honey samples from the time trial (along with the QC clover honey) were also tested externally for the diastase activity (DN number) using the Phadebas method and this was related back to this in-house diastase activity method (Figure 3.3). A weak positive correlation was observed between the DN number (from Phadebas diastase activity analysis) and the residual starch solution from enzyme kinetics analysis at the starch substrate concentration of 2.22 mg/mL. Low to no correlation was observed between the two parameters at starch substrate concentrations above and below 2.22 mg/mL.

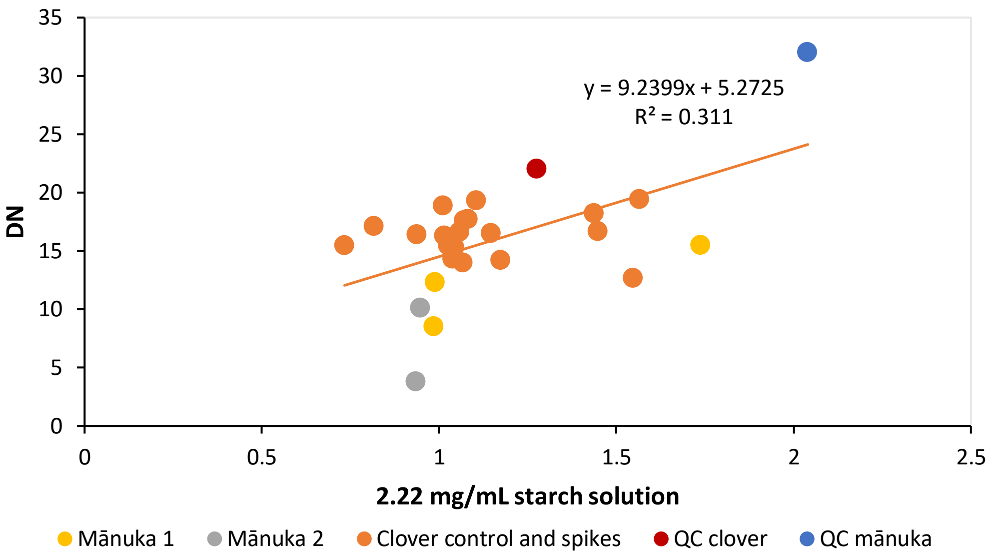


Figure 3.3. The correlation between the diastase activity (DN number as determined by an external lab using the Phadebas method) to the residual starch solution (mg of starch/mL/min/g of honey) determined in-house using the enzyme kinetics analysis at the set starch substrate concentration 2.22 mg/mL.

4 Time Trial Results and Discussion

Mānuka honey fails the diastase test more often than other honeys when exported due to a rigorous testing regime and a unique matrix that contains high levels of MGO, DHA and 3PLA. These compounds have previously been studied for their role in rapidly decreasing diastase activity levels.[4] This chapter investigates the changes in enzyme kinetics (V_{max} and K_{M}) of diastase due to 3PLA and changing MGO, DHA and diastase activity (DN number) in the honey matrix over time.

Enzyme kinetics is the study of enzymatic reactions (how these are affected by different conditions) and are critical to understanding how enzymes (such as diastase) are being affected in a honey matrix. A core aspect of enzyme kinetics is the Michaelis-Menten curve which involves characterising enzyme activity across increasing substrate concentrations, characterising both the binding affinity between enzyme and substrate, and the enzymes fastest catalytic rate when substrate is supplied at non-limiting concentration.[100] Previously, there have been three instances of enzymatic studies of *A. mellifera* diastase which focused on purified enzyme (from multifloral, persimmon, and honeydew honey) under steady-state conditions with Michaelis-Menten fitting.[83, 85, 316] Of the three, only one outlined a method for determining enzyme kinetics in which they used a *p*-nitrophenol analogue as the substrate and observed enzyme kinetics for diastase after purifying it from a multifloral honey matrix to identify how certain metals impacted the activity.[85] No study, thus far, has observed the change in enzyme kinetics of *A. mellifera* diastase in honey over time, if compounds in honey affect the enzyme kinetics or how this relates to the loss of enzyme activity in honey.

Generally, enzymes are studied under steady-state conditions which assume the rate of formation and the rate of disappearance of the enzyme-substrate complex is balanced allowing the concentration of the enzyme-substrate [ES] complex to be held constant.[100] Most environmental conditions (thermodynamically) favour the formation of the product from the substrate and reaction progress curves reflect this by showing an initial increase in the formation of product which plateaus near the end of the assay. From the curve of a Michaelis-Menten plot, key parameters such as V_{max} and K_{M} can be derived (Figure 4.1). The V_{max} is the maximum reaction velocity at infinite substrate concentration (i.e. when all available enzyme active sites are occupied with bound substrate) and K_M is the dissociation constant which reflects the concentration of substrate required to occupy half of the available enzyme active sites under steady-state conditions.[317]

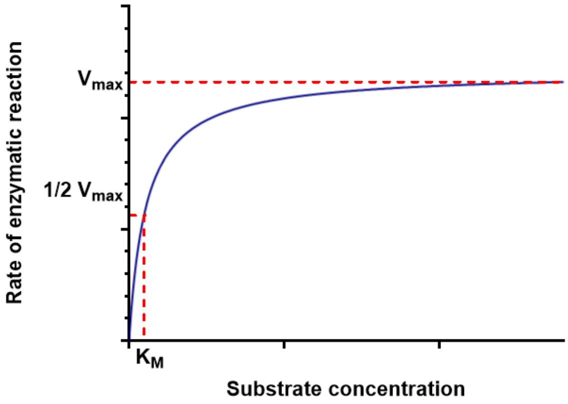


Figure 4.1. Generic Michaelis-Menten plot showing the positioning of V_{max} and K_M.

This study investigated changes in enzymatic kinetics to *A. mellifera* diastase in mānuka honey compared to clover honey to investigate the effect of the more complex mānuka matrix on enzyme activity. Additionally, the effect of the characteristic mānuka compounds (MGO, DHA and 3PLA) on diastase activity over time was tested by creating controlled environments where compounds were added in known quantities into clover honey (which does not contain the compounds of interest). For this, two mānuka honeys and six clover honeys (control sample and several spiked samples) were tested over a period of ~200 days at 27 °C to determine the changes in the enzyme kinetics of diastase, diastase activity (DN number) and concentrations of MGO, DHA, 3PLA and mānuka markers (2MAP, 2MBA and 4HPLA).

4.1 Time trial set-up

Two mānuka honeys were selected to study in this time trial. The initial concentrations of key compounds found in the mānuka honeys provided by the supplier were analysed (Table 4.1).

Table 4.1. The concentrations of MGO, DHA, HMF, 3PLA, 2MAP, 2MBA and 4HPLA (mg/kg) in the mānuka honeys for the time trial experiment.

Honey	MGO	DHA	HMF	3PLA	2MAP	2MBA	4HPLA
Mānuka 1	107	895	11.3	650	13	11	8.3
Mānuka 2	154	1971	0.1	2200	52	21	17

The classification of mānuka honeys 1 and 2 as monofloral mānuka honeys by the suppliers was confirmed by the four mānuka honey chemical markers (3PLA, 2MAP, 2MBA and 4HPLA) concentrations in Table 4.1 (i.e. concentrations greater than 400, 5, 1 and 1 mg/kg respectively). Mānuka honeys 1 and 2 were suitable for the time trial experiment as both had been extracted in early 2024, shortly before commencement of this experiment. Additionally, they contained low HMF indicating they were fresh and had not been exposed to heating post-extraction.[112] Both honeys also had significant differences in the concentrations of all other compounds (Table 4.1) which provided the opportunity to compare how these compounds could influence diastase activity and modifications on proteins (see chapter 5). To isolate whether these compounds directly impacted diastase activity, they were added into clover honey (which does not naturally contain these compounds) and stored at 27 °C.

To isolate the effects of MGO, 3PLA, DHA and combinations of these on honey proteins (specifically diastase activity), clover honey was used as the matrix because there are no commercially available *A. mellifera* α-amylases (discussed in chapter 1) which can be added to spiked artificial honey to observe diastase activity changes over time.[4] The advantage of using clover honey as the matrix is that the sugar and water content as well as pH will be similar to mānuka honey and it does not contain DHA, MGO or 3PLA.[4] This approach has previously been used in the literature.[4, 166]

Clover honey was portioned into a control sample and five spike samples. The concentrations of the compounds added to the matrix were analysed before commencing the time trial experiment (Table 4.2). A large disparity was observed between the measured and theoretical concentrations of MGO, in line with previous spiking studies.[4, 166] This is likely due to the loss of MGO through the rapid interaction with compounds in the matrix (e.g. reaction with amino acid residues in proteins to form advanced glycation end products, AGEs) as it is a highly reactive compound.[4, 137, 166, 295] Comparatively, the theoretical and measured DHA concentrations were less variable due to DHA being less reactive than MGO. Large disparities were observed for 3PLA between the theoretical and measured concentrations (Table 4.2). It is likely that the honey had not been sufficiently homogenized when the 3PLA spike was added and that the day 0 sub-sample for all 3PLA spike samples came from a pocket of high 3PLA spike solution. Mixing occurred at every time point which would aid the distribution of the spiked compound throughout the course of the experiment.

The spike samples chosen for the time trial (Table 4.2) were based on an earlier study (Bell, 2022) which observed that clover honey spiked with the single compounds 3PLA or MGO resulted in a significantly faster rate of decline in diastase activity.[4] In this time trial, clover honey was also spiked with a combination of 3PLA and MGO to determine if the combined effects of mānuka compounds confounded diastase activity to a greater extent than the individual compounds. Additionally, 3PLA and DHA were added to clover honey to simulate naturally aging mānuka honey (DHA conversion to MGO) and the interaction of these compounds with 3PLA.

Table 4.2. Variation in the theoretical spiked vs measured concentration of spiked analytes in the initial analysis of time trial clover honeys.

Spike sample	Spike compound	Theoretical spiked concentration (mg/kg)	Measured concentration (mg/kg)	Difference in concentration (%)
MGO	MGO	971	239	-75.3*
3PLA	3PLA	971	1700	+75.2†
DHA	DHA	971	911	-6.2
3PLA+MGO	3PLA	971	1700	+75.2†
	MGO	972	251	-74.2*
3PLA+DHA	3PLA	970	1500	+54.6†
	DHA	970	822	-15.3

*Rapid loss of MGO occurred as MGO is a highly reactive compound that participates in many Maillard reactions.[119]

†Inefficient mixing of 3PLA spiked clover honeys caused the measured 3PLA concentrations to be higher than the theoretical concentrations for data point 0.

The time trial honeys (mānuka and clover) were incubated at 27 °C for approximately 6 months (200-203 days). Samples were taken weekly for the first 70 days, then fortnightly until day ~140; after this, samples were taken monthly (producing 19 time points for the mānuka honeys and 18 time points for the clover honeys). Samples were collected more frequently at the start (first 70 days) of the time trial to capture any changes that occurred quickly due to the reactivity of MGO. A storage temperature of 27 °C was selected based on previous research[4, 166] which showed the reaction occurred in a timely manner and is a realistic storage temperature. At the completion of the time trial, samples from all time points were analysed for concentrations of HMF, DHA and MGO and samples from four time points (spaced across the storage time) were analysed for enzyme kinetics, diastase activity and phenolic compounds (3PLA, 2MAP, 2MBA, 4HPLA) due to time constraints. The changes in these compounds were tracked over time to correlate the losses in any free compounds of the honey composition to additions to the protein surface capable of impacting diastase activity.

4.2 Changes in HMF over time

HMF formation naturally occurs in honey over time from the degradation of reducing sugars and this formation can be enhanced in honey through processing or long storage times.[318] HMF concentrations increased over the ~200 days of incubation at 27 °C for all of the time trial honeys; however, none exceeded the 40 mg/kg maximum limit required for export. HMF results were plotted relative to the day 0 concentration (normalised) to better visualise changes in HMF relative to the starting concentrations (Figure 4.2). The largest changes in the HMF concentrations were observed for the clover honeys spiked with 3PLA+MGO, 3PLA+DHA, MGO and mānuka honey 2. HMF is formed from the caramelisation and Maillard reaction in honey and thus its concentrations will increase in honeys over time.[318] Heating honey can accelerate the formation of HMF. Since HMF levels can indicate if honey has been exposed to extended storage times or heating (which is undesirable), the CODEX set the limit for HMF in honey to 40 mg/kg and 80 mg/kg for honey originating from tropical regions.[112, 318, 319]

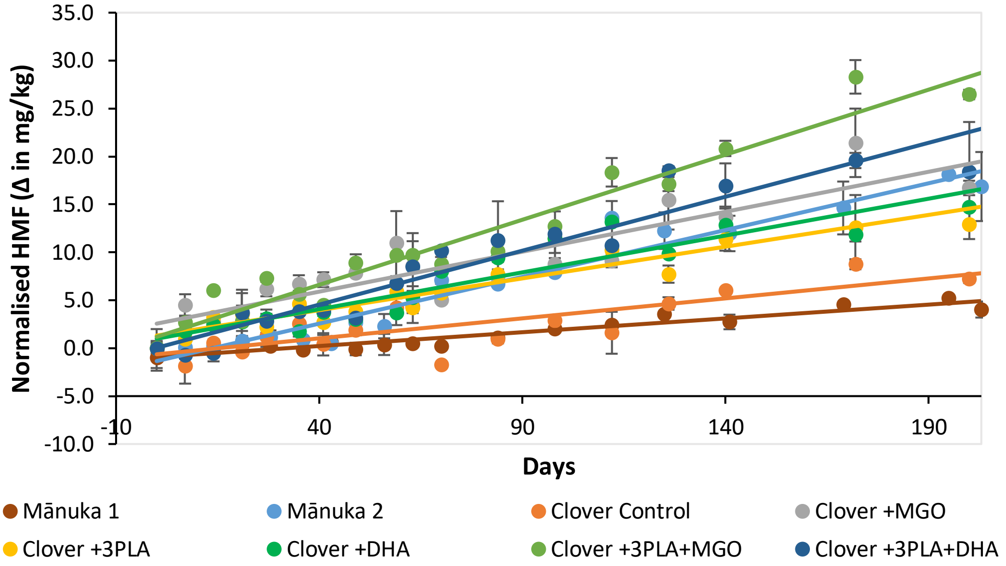


Figure 4.2. Increase in HMF concentration normalised to day 0 (mg/kg) vs time for mānuka 1 and 2 and clover control and spiked honey samples stored at 27 °C. The error bars were set to the standard deviation of the duplicates at each time point and fitted with linear regression.

The findings from the work by Bell (2022) showed there was a weak negative correlation between the HMF concentrations and diastase activity level of various floral honeys and aged honeys.[4] HMF concentration increased while diastase activity decreased which, due to the time and storage temperature, indicates honey quality degradation; however, the processes are unrelated.[4, 320] Unlike this study, Bell (2022) showed HMF concentrations decreased then plateaued over time for all spiked clover honey samples except the MGO spiked sample. The observations were likely due to the initial HMF concentrations of the clover honey being significantly high (116 mg/kg, i.e. well above the 40 mg/kg limit). The plateauing behaviour of initially high HMF content honeys was similar to a different study[321] in which honeys were spiked with excess HMF (~120-250 mg/kg).[4] In general, Bell[4] reported that the HMF concentration increased for all of the time trial mānuka honeys (at 27 °C) after the incubation period of ~200 days and this was consistent with the findings from this study. Since HMF levels remain below export limits, any decline in diastase activity below limit (DN <8) indicates that the honey remains export worthy (HMF is a more universal honey quality test).

4.3 Changes in DHA and MGO over time

DHA and MGO were spiked into clover honey because they are found at high concentrations in mānuka honey and are predicted to cause inhibition to diastase. MGO concentrations in mānuka honeys can exceed 1000 mg/kg whereas MGO is not found in clover honey.[322-324] MGO is formed from a chemical reaction from DHA, which has been reported up to 2700 mg/kg.[8, 325] As expected, DHA decreased [Figure 4.3 (a)] in samples containing DHA and MGO concentration increased over time for samples containing DHA [Figure 4.3 (b)].

The largest changes in DHA and MGO were observed for mānuka honey 2, where DHA concentration decreased and MGO concentration increased. The change in DHA for clover honeys spiked DHA, and 3PLA+DHA followed the same trend as both mānuka honeys (as is expected). DHA converts to MGO and this conversion is a first-order reaction overall. Literature reports deviations from the linearity of the first-order kinetics of honey at later time points as it matures if stored at 37 °C.[282] Due to the conversion of DHA to MGO, the loss of MGO is not as noticeable while there is a high concentration of DHA. However, as DHA becomes depleted, the loss of MGO (in reactions such as Maillard reactions) becomes more noticeable; this generally occurs when the ratio of DHA to MGO is 2:1. The DHA and 3PLA+DHA spiked honeys had a DHA: MGO ratio of ~6:1 at the end of the time trial (i.e. day 200). Mānuka honeys 1 and 2 reach a DHA: MGO ratio of ~2.3:1 and 3:1 (respectively) by day 203. Hence, why the loss of MGO (to Maillard or other reactions) is not observed for these honeys [Figure 4.3 (b)]. Losses in MGO content is observed for the MGO and 3PLA+MGO spiked clover honeys as these were not spiked with DHA and hence, the loss of MGO to MGO-consuming reactions are observed in isolation (i.e. are not masked by the formation of MGO from DHA).

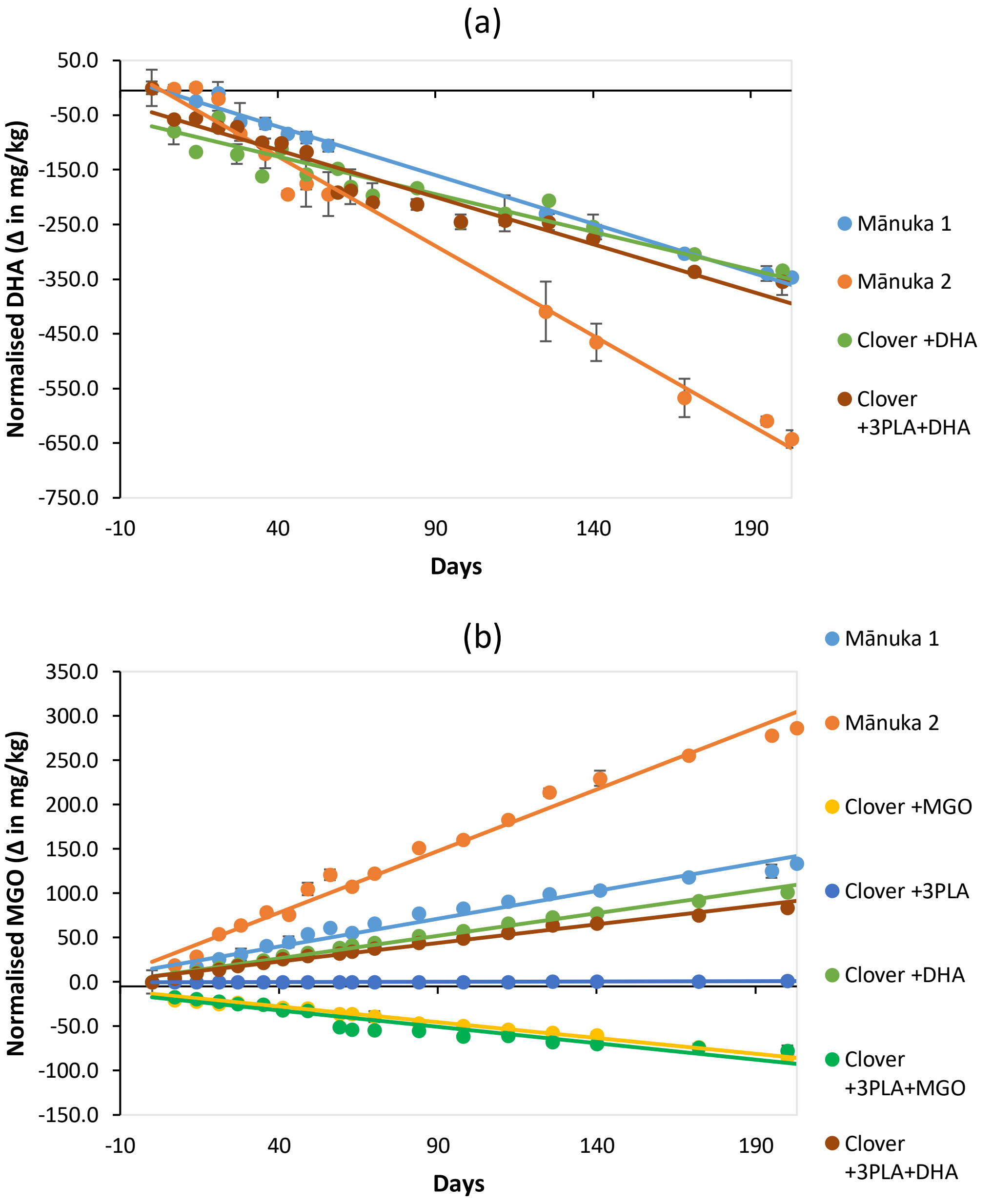


Figure 4.3. (a) Normalised DHA concentration (mg/kg) vs time (days) and (b) normalised MGO concentration (mg/kg) vs time (days) for the time trial samples (mānuka honeys, clover control and spiked clover honeys). The errors bars were set to the standard deviation of the duplicates at each time point.

In contrast, a previous study reported an increase in the MGO concentration before a rapid decline was observed for a clover honey spiked with MGO. The increase was due to a single data point in the previous study whereas this study observed seven time points within the first 50 days of the time trial and did not observe an increase in the MGO concentration. The single data point reported in the previous literature could be due to inefficient mixing at the start of the trial.[4]

The decrease in DHA concentration is not 1:1 to the increase in MGO concentration (as observed for both mānuka honeys 1 and 2 and clover honeys spiked with DHA and 3PLA+DHA). More DHA is lost than MGO formed for all samples which can be attributed to DHA being in the dimeric form and the participation of DHA in reactions other than the conversion to MGO. DHA consuming reactions include modification reactions on amino acids to form melanoidin products. These reactions contribute to the colour of the honey and could be occurring on diastase.[310]

4.4 Changes in mānuka markers and phenolic compounds over time

3-Phenyllactic acid is a phenolic acid and is found in significantly higher concentrations in mānuka honey than other honeys;[4, 276] it is one compound used to define mānuka honey[326] (Table 1.5). Kānuka and NZ ling honeys are two other honeys that have been reported to contain high levels of 3PLA.[4, 276] The concentration of 3PLA was below detection levels for clover honey spiked with MGO or DHA (as expected).

3PLA is used in the honey industry as one of four mānuka markers because its concentration remains stable in honey over time.[153] In the present study, the concentration remained stable for both mānuka honeys and clover honeys spiked with 3PLA, 3PLA+MGO or 3PLA+DHA (Figure 4.4). The other three mānuka markers (2MAP, 2MBA and 4HPLA) are also found in elevated concentrations in mānuka honey compared to other honeys (mānuka marker data generated from single replicates analysed by an IANZ laboratory).[276] Their concentrations remained stable over the time trial for both mānuka honeys (Figure 4.5) and were below detection levels for all of the time trial clover honeys as expected. Therefore, they are unlikely to be binding to proteins themselves to form modifications. However, since 3PLA, 2MBA and 4HPLA are phenolic compounds, they might act as proton donors and could be involved in acid catalysed or protonation-dependent modification reactions.

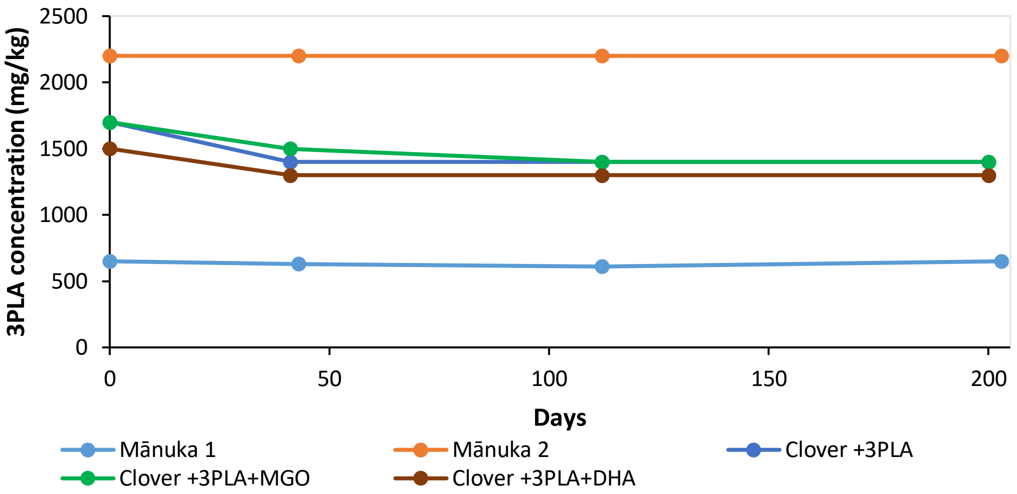


Figure 4.4. Concentration of 3PLA (mg/kg) vs time (days) in various time trial honeys stored at 27 °C.

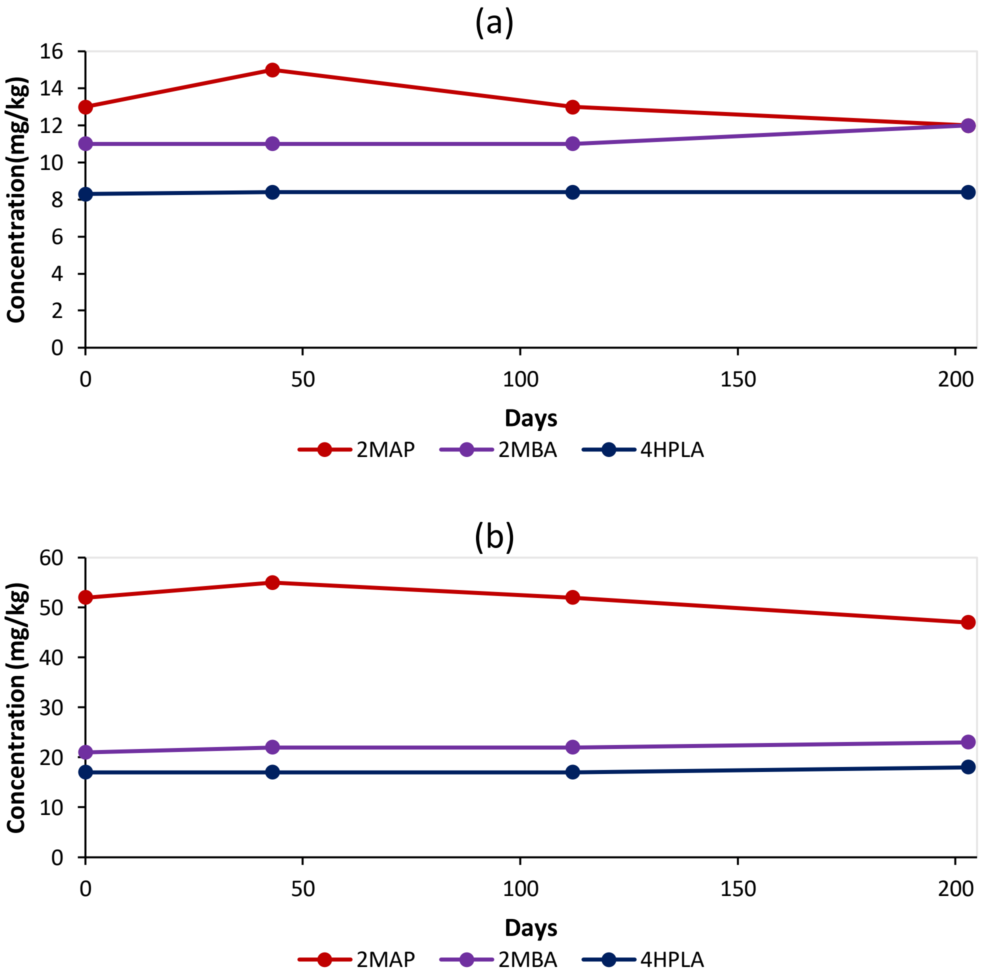


Figure 4.5. The concentrations of 2MAP, 2MBA and 4HPLA in mānuka honey 1 (a) and mānuka honey 2 (b) over time when stored at 27 °C.

Phenolic compounds (e.g. quercetin, caffeic acid) are known to react with MGO (which is a highly reactive compound known to modify proteins) to form adducts and can modify proteins.[119] They do this through reversible noncovalent (such as hydrogen bonds, ionic interactions and π-π stacking) and irreversible covalent (mainly by forming reactive quinones through oxidation of phenolic compounds, followed by coupling reactions) interactions with proteins.[6] Rohn *et al (*2002) showed commercially bought α-amylase and trypsin (EC 3.2.1.1 and EC 3.4.21.4, both from porcine pancreas), and lysozyme (EC 3.2.1.17 from hen egg white) displayed decreased enzymatic activity as the concentrations of phenolic compounds (such as chlorogenic, ferulic and gallic acid) was increased.[81] Other studies demonstrated similar results for the phenolic compounds *p*-hydroxybenzoic acid, ferulic acid, caffeic acid and chlorogenic acid and found that due to the small size of *p*-hydroxybenzoic acid, it had greater inhibitory activity against porcine pancreatic α-amylase.[327, 328] Phenolic compounds have been shown to bind to both active site and non-active sites of porcine pancreatic α-amylase influencing its activity through competitive and uncompetitive inhibition by forming covalent and non-covalent interactions.[329] Since these affects are occurring on porcine pancreatic α-amylase, it is highly likely that they are also occurring on *A. mellifera* diastase. 3-Phenyllactic acid was also found to have inhibitory effects on a different enzyme (tyrosinase) and had the most favourable binding energetics and its inhibitory effects were not caused by environmental acidification.[7] This literature highlights the potential role of phenolic compounds in inhibiting honey proteins through modifications on the proteins. Since 3PLA concentrations remained stable for both mānuka honeys and all 3PLA spiked clover honeys, it is unlikely that 3PLA is forming irreversible modifications on diastase which could impact the catalytic ability of diastase. Instead, it is predicted that reversible noncovalent modifications of phenolic compounds on proteins are forming or that phenolic compounds are acting as proton donors, facilitating protonation or acid catalysis requiring modification reactions of other compounds on proteins.

The antioxidant properties of phenolic compounds allow them to readily donate protons. Phenolic compounds (such as 3PLA) are known to act as antioxidants in foods through hydrogen atom transfer, single electron transfer, sequential proton loss electron transfer and transition metal chelation.[330] Of these, hydrogen atom transfer and sequential proton loss are of particular interest because they highlight the ability of phenolic compounds to act as hydrogen donors. Phenolic compounds can act as proton donors due to the presence of hydroxyl groups and after donating a proton they form resonance stabilised structures due to the delocalisation of the negative charge of the oxygen across multiple atoms.[330, 331] Often MGO consuming reactions (e.g. self-condensation, argpyrimidine formation, MG-H1 formation) and other reactions (e.g. formation of N-fructosyl lysine, Amadori rearrangement) that form modifications on proteins in honey require acid catalysis or protonation.[5, 332, 333]

Mānuka honey contains more phenolic compounds than most other floral honeys (other honeys such as buckwheat, Malaysian and goldenrod honeys can also have high total phenolic content[334]) and these could be involved in the rapid decline in diastase activity due to their role in modifying the protein or acting as acid catalysts. The phenolic content in honeys is influenced by the floral source, geographic region, environmental/seasonal factors and nectar collection process by honey bees.[257, 335] The total phenolic content (TPC) and total flavonoid content (TFC) has been reported as 250-985 mg/kg and 14.7 mg/kg respectively in mānuka honey in a recent review article.[8] Another study compared total phenolic and total flavonoid contents of various honeys and found mānuka honey had approximately double the levels of both TPC and TFC than clover honey.[269]

The TPC method commonly uses the Folin-Ciocalteu reagent, however, it is important to note that this reagent is not specific for phenolic compounds and can be affected by other reducing agents found in honey (e.g. reducing sugars or MGO).[336] Hence, the TPC test is better described as a reducing content test. Instead, more accurate methods of analysis, such as LC-MS or HPLC-UV techniques that detect individual phenolic compound levels are a better indication of phenolic compound content in honeys. The following phenolic compounds are found at higher concentrations (approximately 2-fold or more) in mānuka honeys than clover honey; carnosol, chlorogenic acid, cinnamic acid (*trans*-cinnamic acid), confieraldehyde, *p*-coumaric acid, 3,4-dihydrobenzoic acid, galangin, gallic acid, gentistic acid, hesperidin, 4-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, 3-phenyllactic acid, kaempferol, leptosperin, luteolin, 2-methoxybenzoic acid, 4-methoxybenzoic acid, 4-methoxyphenyllactic acid, methyl syringate, syringic acid, 2,3,4-trihydroxybenzoic acid, trimethoxybenzoic acid and vanillic acid (literature sources reported in Table 1.7). Of these, leptosperin, gentistic acid, 2-methoxybenzoic acid, 3-phenyllactic acid and trimethoxybenzoic acid are found at elevated levels in mānuka honey compared to other honeys (literature sources reported in Table 1.7). Different phenolic compounds have different antioxidant abilities and hence, different proton-donating abilities. Since mānuka honey has double the concentration for most compounds (as listed earlier) than clover honey, it is expected that a higher number of protonation or acid catalysis requiring modification reactions occur in mānuka honey than in clover honey. Due to limited time, changes in concentrations of these compounds were not investigated but this could be a future avenue of study as potential participators in modifications of proteins.

4.5 Changes in diastase activity over time

Diastase activity was assessed for the time trial honeys using the honey-industry standard Phadebas method (single replicates externally assessed by an IANZ accredited laboratory). All honeys showed a decrease in diastase activity over the 200 days (Figure 4.6), however this decrease was more pronounced for both mānuka honeys 1 and 2 and clover honeys spiked with 3PLA+MGO, and 3PLA+DHA (consistent with previous data).[4] Since 3PLA concentrations remain stable, this suggests that MGO and DHA are important compounds involved in forming modifications and this is enhanced in the presence of 3PLA.

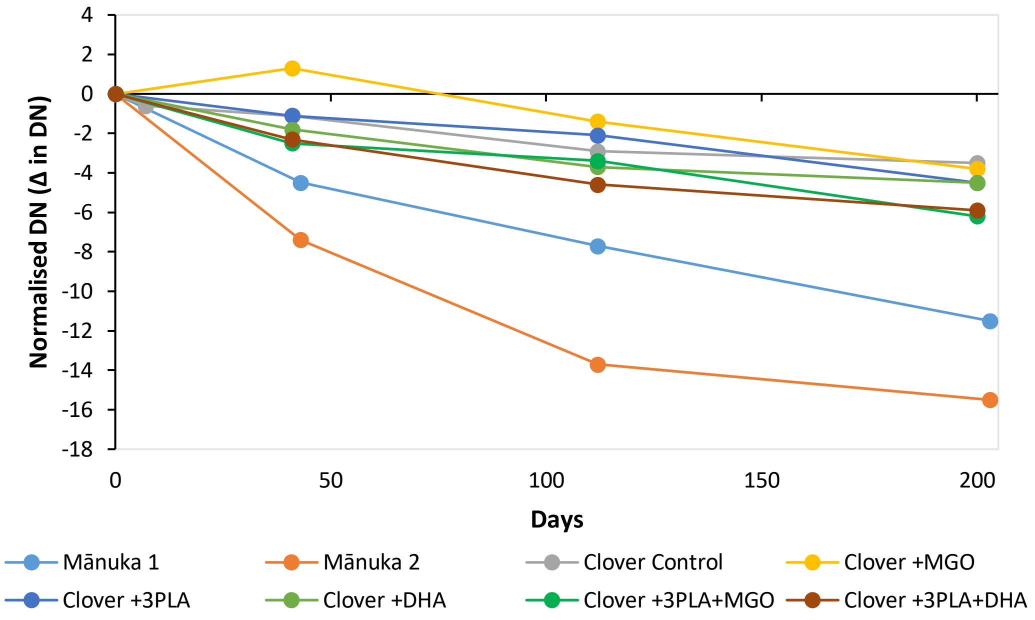


Figure 4.6. Normalised diastase activity decreasing in mānuka honeys 1 and 2 and clover honey (control and spiked) samples stored at 27 °C, over ~200 days.

The mānuka honey samples contain a range of phenolic compounds that clover honey either does not have or has in much lower concentrations (Table 1.7). When clover honey is spiked with just MGO, the decrease in diastase activity is not as large as when the clover matrix is spiked with 3PLA+MGO (Figure 4.7). This suggests there is a requirement for a proton for MGO-induced modification reactions (3PLA can donate protons due its antioxidant properties discussed earlier in section 4.4). Furthermore, a similar trend is observed when clover honey has DHA or 3PLA+DHA added (diastase activity in 3PLA+DHA spiked honey decreased more than for the DHA spiked honey as seen in Figure 4.7). In this case, the 3PLA may also be contributing to converting the DHA dimer to two DHA monomers (which requires a proton), the monomers then convert to MGO, as suggested by Grainger *et al.*[166]

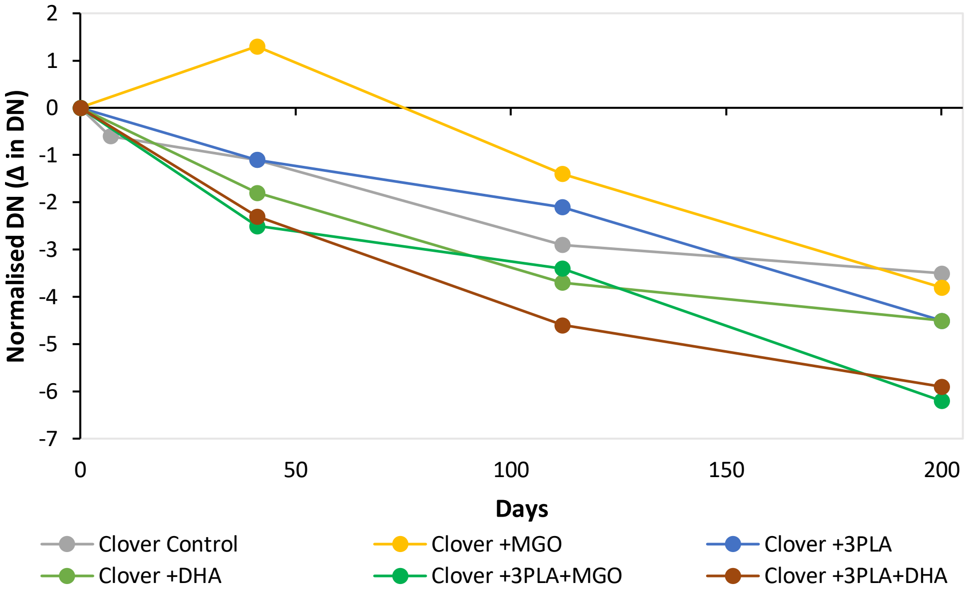


Figure 4.7. Normalised diastase activity decreasing in clover control and spiked honeys stored at 27 °C, over 200 days.

Enzyme assays consisting of a full characterisation of the enzyme catalytic capacity and binding affinity to starch were conducted to deconvolute the effects of honey matrices on diastase activity. Over the course of 200 days, changes in the $V_{\max}$ and K_M values were observed for both mānuka honeys and clover honey spiked with 3PLA+DHA (Figure 4.8), as determined by Michaelis-Menten fitting (Appendix Figure 8.1). Since $V_{\max}$ is the maximum catalysed reaction velocity at complete substrate saturation and reflects the enzyme's catalytic capacity, it is influenced by enzyme concentration (the folded active portion, exclusive of any denaturation), catalytic efficiency of the enzyme (which may be altered by surface level modifications) and any inhibitory effects.

For both mānuka honeys the $V_{\max}$ is observed to decrease over 200 days indicating the enzyme is either denaturing or being inhibited over time leading to a loss of diastase activity. This is consistent with the measured decrease in DN number in honey over time (Figure 4.6) and previous studies[4]. Clover honey spiked with MGO displayed an initial increase in $V_{\max}$ from day 7-43 and then a steady decline following day 43 compared to clover honey spiked with 3PLA+DHA which showed an initial increase in $V_{\max}$ between days 7-112 and then a decrease following day 112. The delay in the decrease for the 3PLA+DHA sample is expected due to the requirement to convert DHA to MGO (Figure 4.3) and consolidates the findings that MGO is a key factor in the decreasing $V_{\max}$.

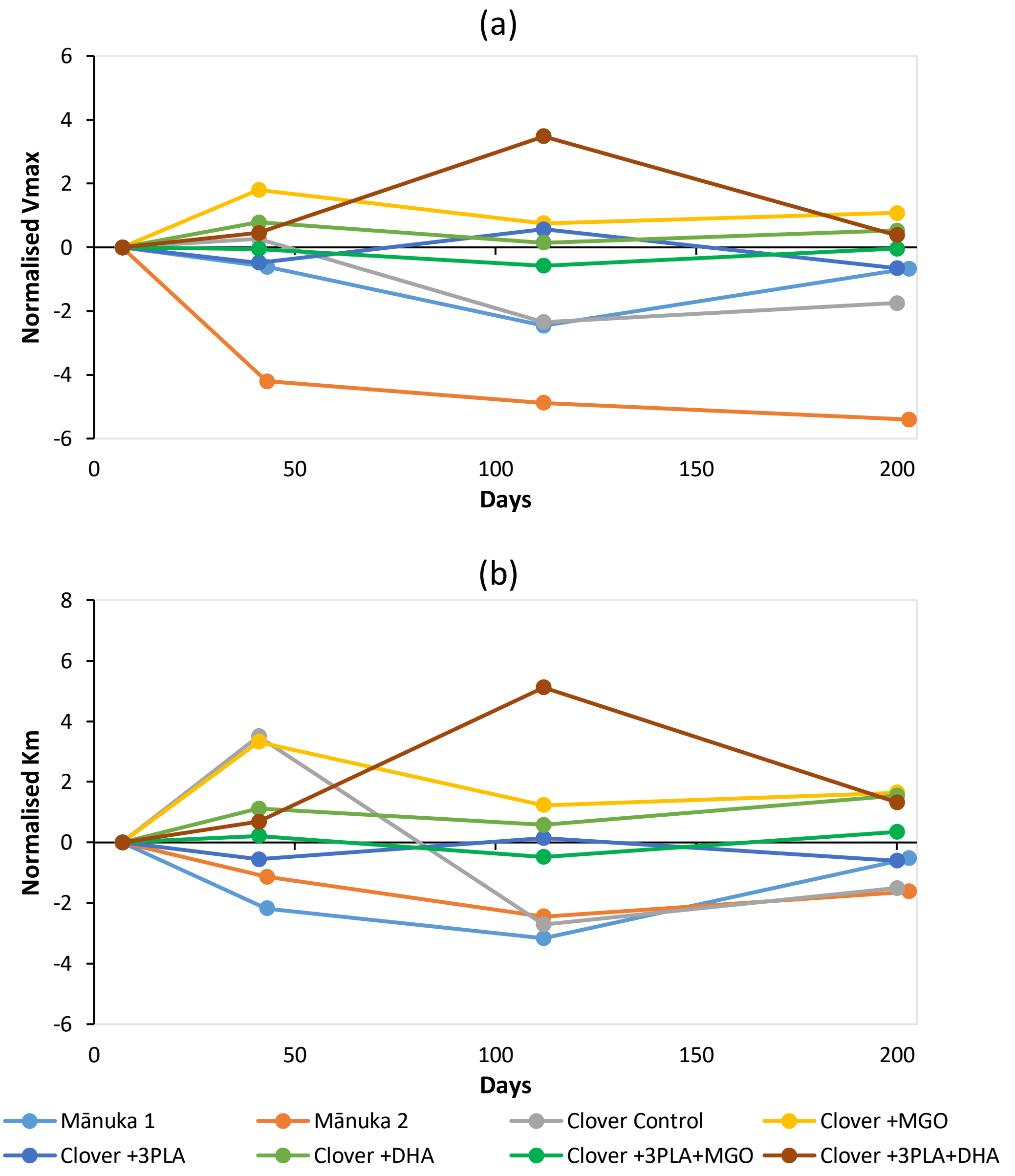


Figure 4.8. Normalised changes in $V_{\max}$ (a) and K_{M} (b) of diastase in various honeys stored at 27 °C.

Given that $V_{\max}$ (and DN) values can decrease both by denaturation and/or inhibitory modifications to the enzyme structure (honey dependent), $V_{\max}$ is not a useful parameter for deconvoluting these two effects. An enzyme binding constant (K_{M}) is independent of enzyme concentration, and thus this can be used to infer the surface level modifications suspected to occur in mānuka honey. Any measured changes in K_M indicate changes in enzyme-substrate interaction from the interference of surface level enzyme modifications. Since these modifications can also affect rates, if K_M changes are detected, it is an indication that V_{max} decreases are not exclusively a denaturation effect and is especially relevant to the more complex matrix of mānuka honey.

For both mānuka honeys and clover honeys spiked with MGO and 3PLA+DHA, the K_M value had pronounced changes. The K_M is independent of the active enzyme concentration (unlike DN and V_{max}), hence provides valuable information for comparing the analysed honey samples to deconvolute enzyme denaturation from inhibitory modifications. The changes in K_M indicate that at least some of the changes in diastase activity are due to changes in enzyme substrate binding effects from matrix-protein interactions, independent of denaturation effects. The greater observed effects in the mānuka samples indicate a greater potential activity interference in mānuka honey testing (i.e. in diastase activity analysis by the Phadebas or Schade method).

Since the effects on enzyme kinetics (V_{max} and K_M) and diastase activity for clover honey spiked with 3PLA+DHA and MGO were similar to the mānuka honeys, this indicated that the compounds 3PLA, DHA and MGO (all found in high concentrations in mānuka honeys) were contributing to these effects. The more pronounced changes observed for the 3PLA+DHA spike than the 3PLA+MGO spike confirms that DHA modifications are also contributing to diastase activity changes (as well as MGO modifications after DHA converts to MGO). Since changes in V_{max} and K_M for 3PLA+DHA are also more pronounced than DHA spiked clover honey, this confirms the role of 3PLA in facilitating proton requiring DHA and MGO modification forming reactions.

Of the two mānuka honeys, the greatest effects on enzyme kinetics and diastase activity were observed for mānuka 2. These effects can be explained due to the chemical composition of the tested compounds (Table 4.3). Mānuka honey 2 had higher levels of MGO, DHA and 3PLA at the start of the time trial than mānuka 1. This means that diastase in mānuka honey 2 had more exposure to protein modifying compounds (DHA and MGO), and since diastase activity rapidly decreased (DN reduced from 17 to <2) along with decreases in V_{max} (by ~5-fold) and K_M (~1.5-fold) it suggests that activity loss is associated with substrate binding affects (not purely due to denaturation). Since the changes in V_{max} and K_M are more pronounced for mānuka honey 2 (as compared to mānuka 1) and mānuka 1 contained lower MGO, DHA and 3PLA levels, the substrate binding is less affected in mānuka honey 1 resulting in a smaller decrease in diastase activity (DN reduced from 20 to 8.5).

Table 4.3. Summarised chemical composition (MGO and DHA concentrations, mg/kg), diastase activity (DN) and kinetics (V_{max} and K_M) of the time trial honeys (mānuka, clover control and clover spiked honeys).*

Honey	Time point	MGO (mg/kg)	DHA (mg/kg)	3PLA (mg/kg)	DN	V_{max}	K_M
Mānuka 1	Initial	107	895	650	20.0	3.54	3.57
	End	240	549	650	8.5	2.87	3.04
Mānuka 2	Initial	154	1971	2200	17.5	6.68	4.67
	End	440	1330	2200	<2.0	1.28	3.05
Clover control	Initial	2	55	<20	18.0	4.17	4.91
	End	2	100	<20	16.5	2.42	3.40
Clover +MGO	Initial	241	27	<20	18.0	1.21	0.79
	End	157	87	<20	14.2	2.29	2.43
Clover +3PLA	Initial	1	45	1700	18.8	1.67	1.19
	End	2	102	1400	14.3	1.01	0.58
Clover +DHA	Initial	1	941	<20	20.0	1.72	1.01
	End	102	607	<20	15.5	2.25	2.56
Clover +3PLA+MGO	Initial	253	52	1700	18.9	2.24	1.59
	End	175	82	1400	12.7	2.20	1.94
Clover +3PLA+DHA	Initial	3	877	1500	19.9	1.56	1.08
	End	86	522	1300	14.0	1.94	2.40

*Initial time point refers to day 0 for all parameters except V_{max} and K_M which were assessed for day 7 samples. End time point refers to day 203 for mānuka honeys and day 200 for all clover honeys (control and spikes).

When comparing MGO spiked clover honey to mānuka 1, both contained similar MGO concentrations at the start of the time trial (241 mg/mL for MGO spiked and 106 mg/mL for mānuka 1 honey). However, a smaller decrease in diastase activity (DN 18 to 14.2) and a delayed decline in V_{max} and K_M (after day 43, Figure 4.8) is observed for the spiked sample. These differences can be attributed to MGO spiked clover honey not containing other key compounds that are found in mānuka 1 (such as 3PLA and DHA). Comparatively, clover honey spiked with 3PLA+DHA displayed a pronounced decline in $V_{\max}$ and K_{M} following day 112 and this delayed decrease in $V_{\max}$ and K_{M} is related to the time it takes for DHA to convert to MGO. Grainger suggested H^+ is required to dissociate the DHA dimer in the honey matrix into two monomers so that DHA is available to convert to MGO, which could be provided by 3PLA.[166] This would explain the lack of $V_{\max}$ and K_{M} changes in the DHA spiked clover honey over time, as well as the smaller decrease in diastase activity (23% for DHA spiked and 30% for 3PLA+DHA spiked clover honey). Honey is a highly saturated solution (contains a low moisture content) in which most of the protons are bound within sugar molecules, hence 3PLA is able to provide protons (due to its antioxidant abilities, discussed in 4.4) required for DHA dimer dissociation and MGO modification reactions. Even though both DHA and 3PLA+DHA convert to MGO, this conversion likely occurs faster in the 3PLA+DHA sample and 3PLA facilitates further reactions of MGO as well as other side reactions of DHA that involve modifying diastase. DHA is known to react with amino acids such as lysine, arginine, histidine and cysteine and is used as the active ingredient in sun-tanning products due its ability to modify amino acids through the Maillard reaction.[310, 311, 337] DHA is likely modifying the amino acid residues on diastase causing conformational or substrate binding affinity changes (therefore, changing $V_{\max}$ and K_{M}) which decreases the diastase activity. Hence, diastase activity, $V_{\max}$ and K_{M} decreases are more obvious for the 3PLA+DHA sample as compared to DHA spike alone.

The trend in $V_{\max}$ and K_{M} for the 3PLA+MGO spiked clover honey is less apparent and this likely highlights the role of DHA in modifying diastase. Since the 3PLA+MGO spiked sample does not contain DHA, only MGO modifications (as well as other generic sugar modifications) can occur on diastase. Whereas, in the 3PLA+DHA spiked sample, DHA and MGO are both present and can both contribute to modifications on diastase (enhanced by the presence of 3PLA proton donor).

From the earlier observations, DHA and MGO appear to be important contributors (in the presence of 3PLA) to the rapid decline in diastase activity highly likely due to rapid inhibition-causing modification formation on diastase through reversible and irreversible reactions. The modifications have the potential to inhibit diastase activity as this is a common phenomenon described in literature for other proteins but can apply to diastase as it contains the same reactive amino acids (lysine, arginine, cysteine and histidine). MGO modifications [such as MG-H1, carboxyethyl-lysine (CEL)] have been shown to affect protein binding interactions. Even minor modification changes on proteins can have adverse effects on proteins. For example, CEL is an MGO modification on lysine and it completely inhibits amyloid fibril formation when it joins onto lysine at position 15 of α-synuclein.[338] The accumulation of such modifications (i.e. Maillard reaction products) can have devastating effects on proteins and have been intensively studied for their involvement in diabetes-mellitus and some age-related diseases.[338, 339] The role of phenolic compounds as catalysts or hydrogen providers, the exact modifications that form on the protein sequence and the occurrence of these in honey is less studied. It is important to study the modification effects on *A. mellifera* diastase specifically for understanding diastase activity changes in honey (as independent to just denaturation effects). This is because upon doing a BLASTp sequence alignment between *A. mellifera* α-amylase (UniProt accession no. Q8N0N7) and the most studied α-amylase for modification effects, porcine pancreatic *A. mellifera* α-amylase (UniProt accession no. P00690), a low percentage identity of ~50% was found even though the query coverage was 98% (i.e. 98% sequences matched but only ~50% was identical and hence there are likely to be structural differences even if the basic enzymatic mechanisms are the same[4]). These structural differences can cause the mechanisms of inhibition to differ. Hence, identifying the ways in which diastase is modified due to MGO, DHA and 3PLA is important to understanding how diastase activity depletes quicker in mānuka honey compared to other honeys.

Across the range of time trial samples, a strong positive correlation between V_{max} and K_{m} was found (Figure 4.9). That is, decreases in V_{max} are correlated with stronger substrate affinity. This strong correlation is consistent with the hypothesis that compounds in mānuka honey cause surface modifications to the protein which effect both substrate binding and potential enzymatic rates. If denaturation were the only effect lowering enzyme rates in these honey samples, K_M would not change across the range of V_{max} values. Convolution of measured enzymatic rates from substrate binding affects means that measuring diastase activity rates in honey without considering substrate binding effects, misses relevant information and hence is a misguided approach to determining honey quality. The standardised diastase activity testing methods used in the honey industry only consider the reduced diastase activity as a denaturation effect from exposure of honey to harsh storage conditions and does not account for the enzyme modification effects that are occurring. In this, reduced enzyme activity from modifications is mistakenly attributed to enzyme denaturation. These modification effects are enhanced for mānuka honeys as seen by the large changes in K_M for both time trial mānuka honeys and clover honey spiked with 3PLA+DHA (key mānuka compounds, Figure 4.8) enhancing the confounding effects in mānuka testing for export quality.

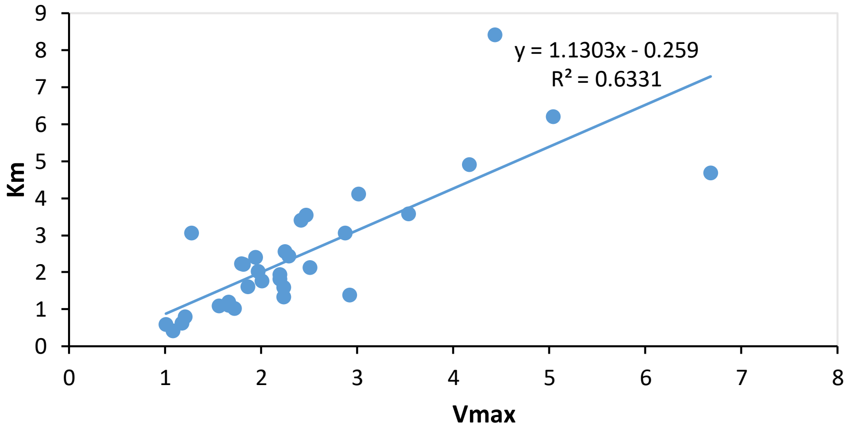


Figure 4.9. Correlation between the K_m and V_{max} of aged honeys (mānuka honeys 1 and 2, clover control and spiked honeys) at 27 °C for all four time points.

In conclusion, changes in K_M were observed for time trial mānuka honeys and a clover honey spiked with 3PLA+DHA (key mānuka honey compounds). Since K_M is independent of active enzyme concentration, changes to this indicate there are effects other than denaturation affecting diastase activity in honey over time and this may be confounding the measurement of enzyme rates too. These other effects are speculated to be inhibition effects. The inhibitions effects were investigated in Chapter 5 to identify what these modifications may be, where they are occurring and how they relate to the decreasing diastase activity.

5 Protein Modifications Occurring in Honey

The current honey quality test for diastase activity relies on enzymatic activity to assess the quality of the honey. However, mānuka honey is a complex matrix which contains many compounds that can interact with enzymes. Chapter 4 determined that MGO, DHA and 3PLA affect the activity of diastase. This chapter explores how compounds present in mānuka honey (DHA, MGO and 3PLA) interact with enzymes, modifying the protein structure in ways which may affect activity, to determine if enzymatic testing is a suitable method for assessing the quality of mānuka honey.

5.1 Changes in size of diastase over time in honey

SDS-PAGE gels were used to visualise proteins in honey that had been stored for various lengths of time. Protein identification is theoretically possible from molecular weight based on the SDS-PAGE gel alone, however, various honey proteins have similar molecular weights (e.g. *A. mellifera* α-glucosidase has a weight of 65.6 kDa and α-amylase has a weight of 56 kDa which can be difficult to discern from a SDS-PAGE gel), and this can be further complicated by surface level modifications altering the molecular weight. For this project, the honey protein bands were identified via LC-MS/MS (see section 2.8 for methodology and sections 5.2-5.3 for results).

Initially, fresh clover and mānuka honeys were separated on the SDS-PAGE gel to identify diastase and to determine LC-MS/MS information for the enzyme before storage or addition of compounds of interest. From the gel (Figure 5.1), five distinct bands were observed for both the clover and mānuka honey (labelled 1-5 in lane A of Figure 5.1), however, a sixth band (at ~85 kDa) was also observed for mānuka honey and was further investigated. The distinct bands (1-6) were analysed by LC-MS/MS to identify the presence of proteins by homology to the known protein sequences from the UniProt database (accession numbers the same as in Table 1.2).

The exact process for preparing gel bands and the process of LC-MS/MS analysis is detailed in section 2.8.2.

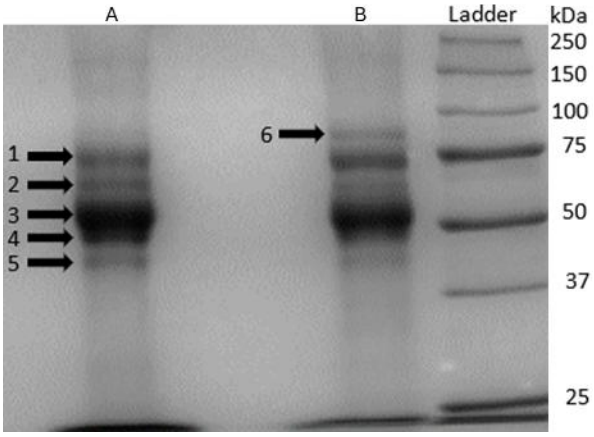


Figure 5.1. A 10% SDS-PAGE gel with 32% (w/v) diluted honeys run in lanes 1 and 2 and Precision Plus Protein^{TM} standard ladder (labelled). Lane A (left) contains a clover honey and lane B (right) contains a mānuka honey. The gel bands excised for LC-MS/MS protein identification are labelled 1-6.

Diastase was present in bands 3 and 4 (Table 5.1) but had low %coverage in both bands (~57 and 17% coverage for bands 3 and 4 respectively) likely due to the low abundance of diastase in honey compared to other proteins. The %coverage depends on the abundance of the protein, how much the peptides are modified and whether certain peptides are very hydrophobic (i.e. interacts strongly with the column in LC-MS/MS) or hydrophilic (elutes off the LC-MS/MS column quickly). A higher %coverage indicates the protein is more abundant in the sample, and/or more peptides and modifications were detected. All six of the bands were from fresh honeys and very few modifications were detected. Modifications which were detected were common protein modifications and are discussed later. Comparative to diastase, MRJP1 (an abundant honey protein) had ~84 and 67% coverage for bands 3 and 4 respectively (Table 5.1). Due to the high coverage, MRJP1 was also investigated which allowed more modifications to be assessed with greater accuracy due to the improved data coverage. This works towards the overall aim of assessing modifications to diastase on honey as the same basic principles apply (same modifications can affect the same amino acids on all proteins).

Table 5.1. Most abundant proteins in bands 1-6 (Figure 5.1) and the instances of α-amylase (bands 3 and 4). There are two entries in UniProt for α-amylase (Q9U8X5 and Q8N0N7).

Band	Protein (UniProt accession no.)	Average mass (kDa)	%Coverage
1	α-glucosidase (Q25BT6)	65.6	76.72
2	MRJP1 (C6K481)	48.9	70.37
3	MRJP1 (C6K481)	48.9	84.03
	α-amylase (Q9U8X5, Q8N0N7)	55.9, 56.0	59.43, 57.61
4	MRJP2 (C6K482)	51.1	86.73
	α-amylase (Q9U8X5)	55.9	16.84
5	MRJP3 (D3Y5T0)	61.6	56.8
6	α-glucosidase (Q25BT6)	65.6	48.5

Following the protein identification, samples from the storage time trial of mānuka, clover control and clover spiked honeys were run on SDS-PAGE gels to observe changes in the diastase protein size over time (Figure 5.2). The samples from the start of the time trial (i.e. day 0 samples) were compared to the samples from the end of the time trial (i.e. day 203 for mānuka honey samples and day 200 for clover control and spiked honey samples).

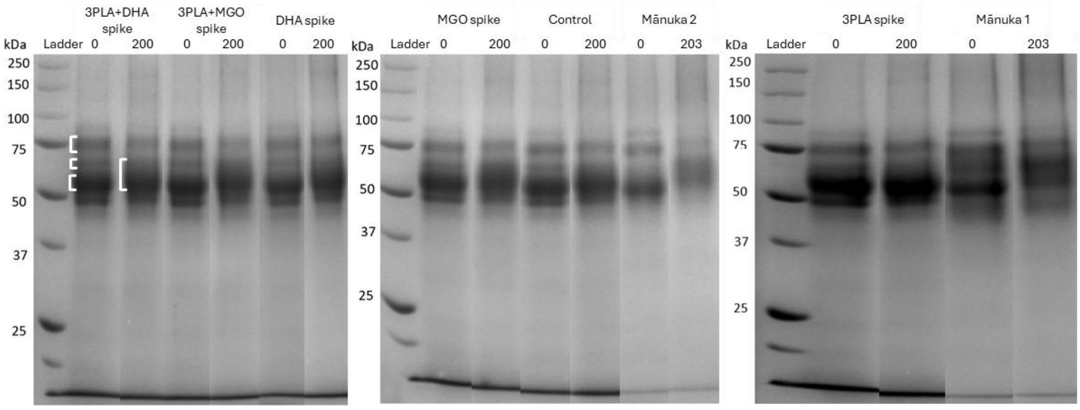


Figure 5.2. The 10% SDS-PAGE gel of time trial honeys. Day 0 and ~200 samples of each mānuka honey (1 and 2), clover control and clover spiked samples. The distinct banding observed for all day 0 samples as compared to the smeared band for end point samples (i.e. day 200 or 203) is illustrated with the example of the 3PLA+DHA spike (using the white brackets).

The day 0 samples for each clover spike treatment were compared to the day 0 sample of clover control and identical bands were observed (Figure 5.2). The largest band was observed at ~56 kDa (the size for *A. mellifera* α-amylase) for all day 0 clover honey samples. This confirmed that the diastase protein was appearing at the correct size (i.e. was not modified) for all day 0 clover control and spike honeys, hence the clover control day 0 sample could be used to represent all the honey treatments for day 0 for further analysis with LC-MS/M later. The day 0 of mānuka honey 1 showed a more intense and compact band at ~56 kDa than mānuka honey 2. This indicates that at day 0 mānuka honey 2 may already have modifications on diastase which cause diastase to have a bigger and more variable mass and run slower on the gel, thus, appearing higher with a smearing effect on the gel. Smearing is expected to occur, especially over time, because the addition of modifications to proteins is random in terms of which modifications are added, where they occur and how many occur, resulting in variations in protein mass. The smearing effect is illustrated by the comparison of the day 0 (which shows distinct banding) to the day 200 (shows a smeared band) for the 3PLA+DHA sample in Figure 5.3.

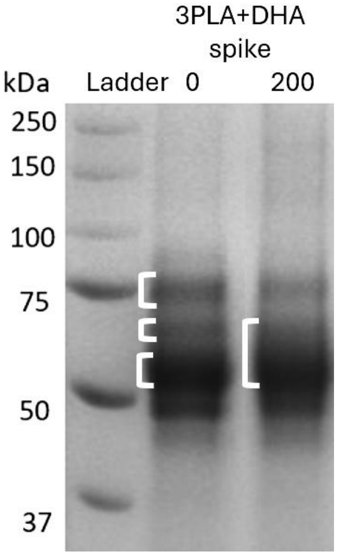


Figure 5.3. The distinct banding observed for all day 0 samples as compared to the smeared band for the end point samples (i.e. day 200 or 203) is illustrated with the example of the 3PLA+DHA spike (using the white brackets) on a 10% SDS-PAGE gel. This is an expansion of Figure 5.2.

All time trial end-point samples (i.e. day 203 for both mānuka honey samples and day 200 for clover control and spike samples) showed a smeared band higher on the gel between 50-75 kDa compared to the corresponding day 0 samples (Figure 5.2). Comparatively, the day 0 samples had more distinct banding at ~56 kDa, ~70 kDa and ~75 kDa. This indicates that the protein has been modified to varying degrees resulting in a smearing effect on the gel. A smearing effect is observed for the clover control as well, indicating that over time the protein is naturally modified, however the intensity of the smear is greater for 3PLA+MGO spiked, and MGO spiked samples (Figure 5.2). These observations compliment past literature which showed that the protein bands of MRJP1 (major royal jelly proteins) increased in molecular weight due to MGO-related modifications forming on the protein.[340] Another study observed the decrease in the intensity of bands as well as a smearing effect after testing honeys from a storage trial and related this to the formation of phenolic compound (i.e. quinone) modifications on proteins which they predicted was causing the proteins with modifications to be too big to enter the gel and to be less soluble.[341] The study was able to quantify phenolics from honey samples using size-exclusion chromatography which suggested the formation for polyphenolic modifications on the proteins. The formation of such modifications on proteins can affect enzymatic activity.[342]

Enzymatic activity is heavily dependent on the 3D structure of the enzyme including modifications at the protein surface.[343] These surface modifications involve the addition of a compound or a part of compound to the protein structure.[9] The degree and manner by which the enzyme activity is influenced depends on whether the modifications occur in the active site (results in conformational changes in the active site, directly affecting substrate binding) or on other parts of the enzyme (i.e. allosteric effects which cause conformational changes on the protein elsewhere from the active site and indirectly affect substrate binding and catalysis).[343] This modification-activity relationship is a well-established effect in biology, and is a mechanism extensively used by cells for the targeted modification of enzyme activity as part of metabolic regulation.[342]

Surface modifications can form either by cleaving the peptide backbone or by undergoing reactions with side chains of amino acid residues and becoming covalently incorporated into the protein structure.[9] Through incorporation, these modifications can affect the enzyme kinetics (by acting as activators[344] or inhibitors[9]). The most common biological surface modifications include acetylation (addition of CH_3CO to serine and lysine), glycosylation (*N*- linked glycans attach to asparagine and *O*- linked attach to serine and threonine residues, and can be permanent), phosphorylation (reversible, addition of a phosphate group to typically serine, threonine and tyrosine residues), ubiquitination (ubiquitin is added to lysine), methylation (CH_3 group added to lysine), nitration and acylation.[9]

Non-enzymatic glycosylations (i.e. the Maillard reaction) are likely the most prevalent modifications in honey. The reactive amine parts of lysine and arginine residues (in proteins) react with carbohydrates to form Schiff bases that rapidly rearrange (due to instability) into Amadori products.[9] These Amadori products can transform into advanced glycation end products (AGEs) which are permanently bound surface modifications.[345] Over time, as the honey matures these modifications are more likely to occur and the accumulation of these AGEs on honey proteins are predicted to be impacting enzymatic activity (including diastase activity). When the number and spread of sites (Cys, Lys, Arg and His) available for modifications (Figure 5.4) on diastase are considered with all of the different modifications from MGO alone (Figure 5.5) it presents the opportunity for complex alteration patterns to be occurring on diastase in honey with the potential for significant effects on diastase activity levels. These effects are likely to be occurring on all proteins in honey.

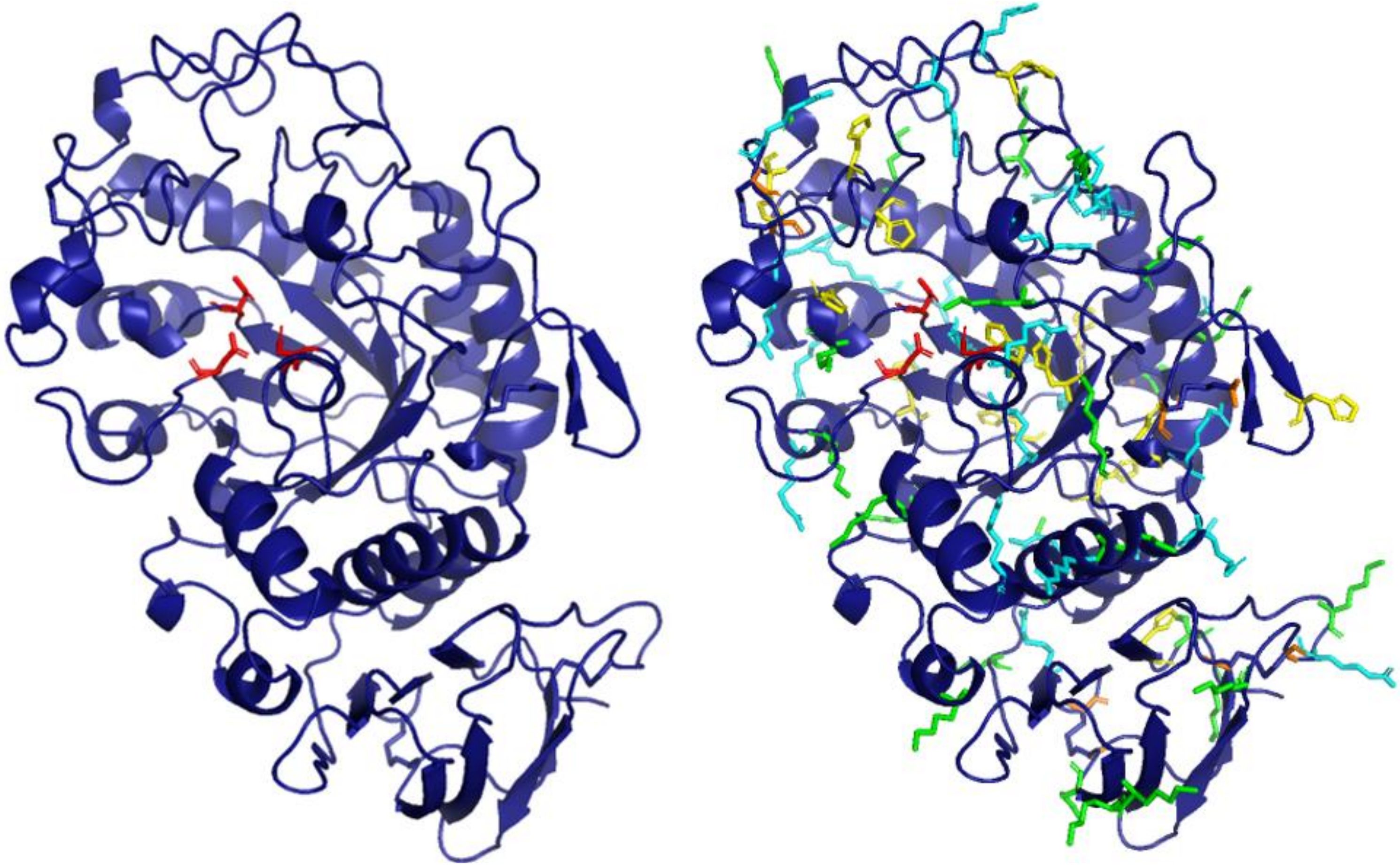


Figure 5.4. The *A. mellifera* diastase with active site residues highlighted red in stick format (left) and same structure (right) with the His (yellow), Cys (orange), Lys (green) and Arg (cyan) residues in stick format. These highlighted surface residues present potential modification sites.

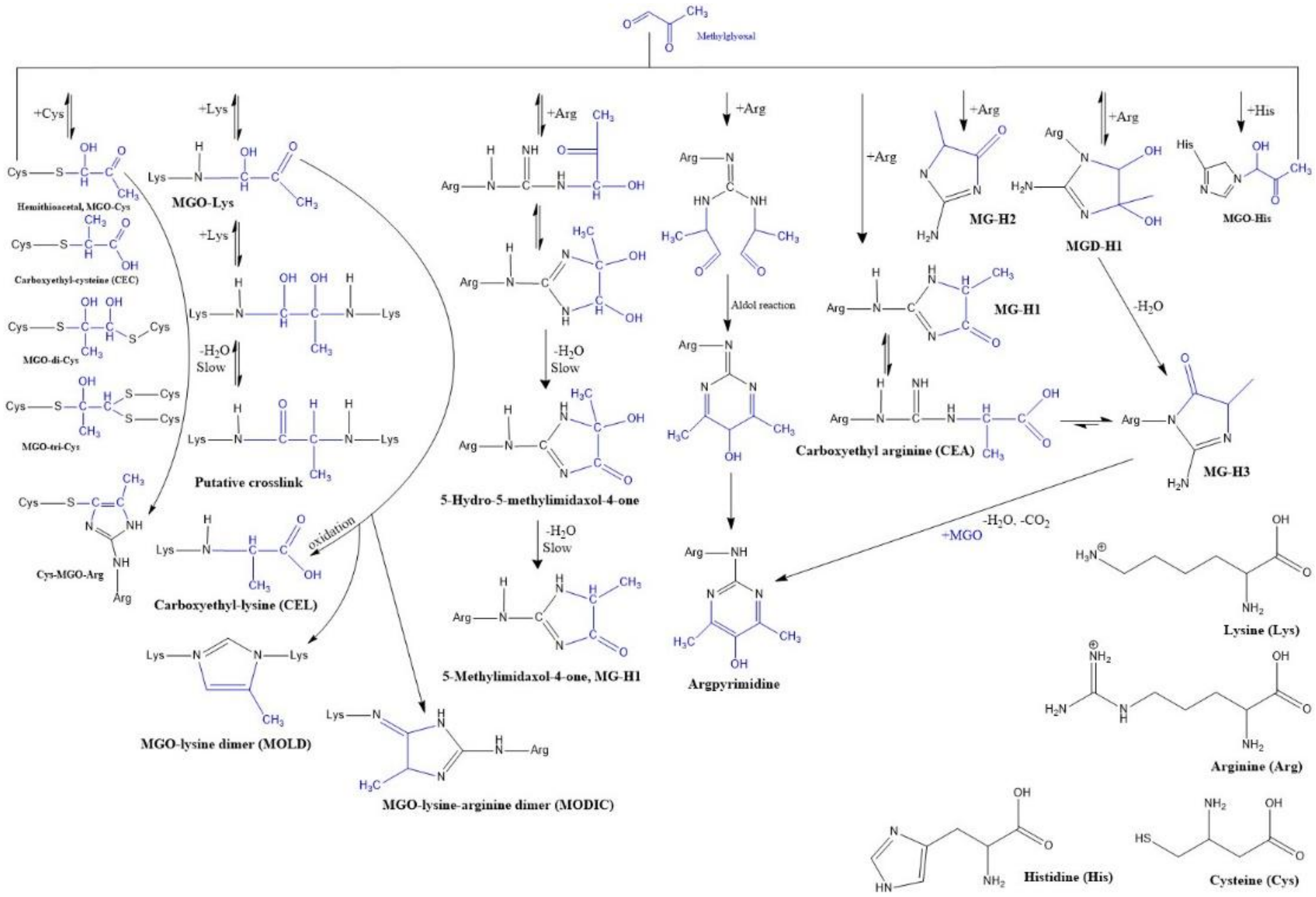


Figure 5.5. Examples of advanced glycation end products (AGEs) formed from MGO reactions with amino acid residues (cysteine, lysine, arginine and histidine) mentioned in literature.[119, 166, 298-307, 346-352]

Previously, protein modification in mānuka honey has been investigated by comparison of non-mānuka honey to mānuka honey or non-mānuka honey spiked with MGO. Majtan *et al* (2012) showed that the protein band for MRJP1 appeared higher on a SDS-PAGE gel (i.e. had higher mass) for honey that had increasing concentrations of MGO added.[340] They also showed that MGO significantly reduced antibacterial activity of the honey protein defensin-1.[340] Another study stored mānuka (of varying MGO spiked concentrations) at 37 °C for 10 weeks and observed an increase in MGO related modifications (specifically MG-H1 and CEL, discussed below in section 5.2) over time.[303] Hellwig *et al* (2017) showed MGO-related modifications were strongly correlated to MGO concentrations in honey after observing modifications of mānuka honey proteins compared to non-mānuka honey proteins. No study thus far, has investigated the role of other individual compounds (3PLA and DHA) also found at high concentration in mānuka honey or potential synergistic effects (between 3PLA+MGO and 3PLA+DHA) on modification formation over time in honey and how this relates to enzymatic activity (specifically diastase activity). In this study, LC-MS/MS was used (from an external laboratory) to test for modifications on proteins in honey samples from the time trial and this was related to back to diastase activity and enzyme kinetics.

5.2 LC-MS/MS modification databank formation

To test for the possible modifications on honey proteins, first a databank (with which a mass spectrum search could be conducted) was made of the most probable modifications that could occur from the compounds known to be present in mānuka honey. This was comprised of four common modifications including carbamidomethylation (+57.02 m/z, iodoacetamide was used in sample preparation as an alkylating agent which modifies Cys residues to prevent disulfide bond formation and improve LC-MS/MS results;[353] this is especially important for *A. mellifera* diastase which contains five disulfide bonds), deamidation (+0.98 m/z, removal of an amide group from asparagine and glutamine[354]), oxidation (+15.99 m/z, methionine residues are readily oxidised[355]) and phosphorylation

(+79.97 m/z, addition of phosphate to serine, threonine and tyrosine[9]). The carbamidomethylation modification was set as a fixed modification while the deamidation, oxidation and phosphorylation modifications were set as variable. For a fixed modification, the LC-MS/MS software only searches for the modified peptide and assumes it is present on every occurrence of the set amino acid. By comparison, variable modifications search for the modified and non-modified peptide sequence (this adds complexity but is required to account for different modification sites and distributions which is more realistic).[356, 357] The N-fructosyl lysine modification was added as another variable modification which is commonly found in honey and is a result of the reaction between glucose and maltose with the side-chain amine group of lysine.[5] In addition to these common modifications, modifications specifically formed due to the reaction with MGO, caffeic acid (as an example of a phenolic compound modification) or DHA were added to the search (Table 5.2). Surface modifications that involved modification to more than one amino acid residue were not assessed as this introduced complexities to testing and data processing. Examples of surface modifications with more than one amino acid involved in the modification include MGO-di-Cys[119], MGO-tri-Cys[119], putative crosslinking[166, 298], Cys-MGO-Arg (MICA)[300, 346, 348], MGO-lysine dimer (MOLD)[119, 299, 301, 304, 305], MGO-lysine-arginine dimer (MODIC)[119, 299, 346], and adduct[347]. Although this is a good library (Table 5.2) of modifications, it is not exhaustive given the complexity of all the possible interactions and hence, known and predicted MGO modifications were the main focus of this study.

Table 5.2. Literature and proposed surface modifications resulting from reactions of MGO, DHA and phenolic compounds with amino acid residues (Cys, Arg, Lys and His).

Name of modification (acronyms/other names)	Modifying chemical	Amino acid residues affected	Monoisotopic Mass Shift (Da)	Formula of Modification Group
Hemithioacetal (MGO-Cys/ Hemimercaptal)[119, 166, 298-300, 347]	MGO	Cys	72.02113	$C_3H_5O_2$
Cys-diMGO[119, 166, 298-300, 332, 347]	MGO x2	Cys	144.0423	$C_6H_9O_4$
Cys-triMGO[119, 166, 298-300, 332, 347]	MGO x3	Cys	216.0634	$C_9H_{13}O_6$
Cys-4MGO[119, 166, 298-300, 332, 347]	MGO x4	Cys	288.0845	$C_{12}H_{17}O_8$

Name of modification (acronyms/other names)	**Modifying chemical**	**Amino acid residues affected**	**Monoisotopic Mass Shift (Da)**	**Formula of Modification Group**
Cys-5MGO[119, 166, 298-300, 332, 347]	MGO x5	Cys	360.1057	$C_{15}H_{21}O_{10}$
Cys-6MGO[119, 166, 298-300, 332, 347]	MGO x6	Cys	432.1268	$C_{18}H_{25}O_{12}$
Cys-7MGO[119, 166, 298-300, 332, 347]	MGO x7	Cys	504.1479	$C_{21}H_{29}O_{14}$
Carboxyethyl-cysteine (CEC)[119, 299]	MGO	Cys	72.0211	$C_3H_5O_2$
Cys-triMGO aromatic[119, 299, 332]	MGO x3	Cys	200.0685	$C_9H_{13}O_5$
MGO-Lys[119, 166, 298, 301, 349]	MGO	Lys	72.0211	$C_3H_5O_2$
Lys-diMGO[119, 166, 298-300, 332, 347]	MGO x2	Lys	144.0423	$C_6H_9O_4$
Lys-triMGO[119, 166, 298-300, 332, 347]	MGO x3	Lys	216.0634	$C_9H_{13}O_6$
Lys-4MGO[119, 166, 298-300, 332, 347]	MGO x4	Lys	288.0845	$C_{12}H_{17}O_8$
Lys-5MGO[119, 166, 298-300, 332, 347]	MGO x5	Lys	360.1057	$C_{15}H_{21}O_{10}$
Lys-6MGO[119, 166, 298-300, 332, 347]	MGO x6	Lys	432.1268	$C_{18}H_{25}O_{12}$
Lys-7MGO[119, 166, 298-300, 332, 347]	MGO x7	Lys	504.1479	$C_{21}H_{29}O_{14}$
Carboxyethyl-lysine (CEL)[119, 299, 301-305, 347]	MGO	Lys	72.0211	$C_3H_5O_2$
Lys-triMGO aromatic[119, 299, 301-305, 347]	MGO x3	Lys	200.0685	$C_9H_{13}O_5$
5-Hydro-5-methylimidazol-4-one[166]	MGO	Arg	70.0055	$C_3H_4O_2$
Arg-diMGO[119, 166, 298-300, 332, 347]	MGO x2	Arg	142.0266	$C_6H_8O_4$
Arg-triMGO[119, 166, 298-300, 332, 347]	MGO x3	Arg	214.0477	$C_9H_{12}O_6$
Arg-4MGO[119, 166, 298-300, 332, 347]	MGO x4	Arg	286.0689	$C_{12}H_{16}O_8$
5-Methylimidazol-4-one (MGO Imidazolone, MG-H/ Hydroimidazolone isomer 1, MG-H1/ Nδ-(5-methyl-4-oxo-5-hydroimidazo-linone-2-yl)-l-ornithine)[119, 166, 306, 350]	MGO	Arg	53.0027	C_3H_4O
Arg-diMGO 2[119, 166, 298-300, 306, 332, 347, 350]	MGO x2	Arg	125.0239	$C_6H_8O_3$

Name of modification (acronyms/other names)	**Modifying chemical**	**Amino acid residues affected**	**Monoisotopic Mass Shift (Da)**	**Formula of Modification Group**
Arg-triMGO 2[119, 166, 298-300, 306, 332, 347, 350]	MGO x3	Arg	197.0450	$C_9H_{12}O_5$
Arg-4MGO 2[119, 166, 298-300, 306, 332, 347, 350]	MGO x4	Arg	269.0661	$C_{12}H_{16}O_7$
Argpyrimidine[119, 304, 306, 307, 351]	MGO	Arg	79.0184	C_5H_7O
Hydroimidazolone isomer 2 (MG-H2/ 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid)[299, 306, 307]	MGO	Arg	54.0106	C_3H_4O
Arg-diMGO 3[119, 166, 298-300, 306, 307, 332, 347]	MGO x2	Arg	126.0317	$C_6H_8O_3$
Arg-triMGO 3[119, 166, 298-300, 306, 307, 332, 347]	MGO x3	Arg	198.0528	$C_9H_{12}O_5$
Arg-4MGO 3[119, 166, 298-300, 306, 307, 332, 347]	MGO x4	Arg	270.0740	$C_{12}H_{16}O_7$
Carboxyethyl arginine (CEA)[299, 306, 347]	MGO	Arg	72.0211	$C_3H_5O_2$
Arg-triMGO aromatic[119, 166, 298-300, 306, 332, 347]	MGO x3	Arg	200.0685	$C_9H_{13}O_5$
MGD-H1[307, 347]	MGO	Arg	72.0211	$C_3H_6O_2$
Hydroimidazolone isomer 3 (MG-H3/ 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid)[299, 306, 307, 347, 352]	MGO	Arg	54.0106	C_3H_4O
Arg-diMGO 4[119, 166, 298-300, 306, 307, 332, 347, 352]	MGO x2	Arg	126.0317	$C_6H_8O_3$
Arg-triMGO 4[119, 166, 298-300, 306, 307, 332, 347, 352]	MGO x3	Arg	198.0528	$C_9H_{12}O_5$
Arg-4MGO 4[119, 166, 298-300, 306, 307, 332, 347, 352]	MGO x4	Arg	270.0740	$C_{12}H_{16}O_7$

Name of modification (acronyms/other names)	**Modifying chemical**	**Amino acid residues affected**	**Monoisotopic Mass Shift (Da)**	**Formula of Modification Group**
MGO-His[119]	MGO	His	72.0211	$C_3H_5O_2$
His-diMGO[119, 166, 298-300, 332, 347]	MGO x2	His	144.0423	$C_6H_9O_4$
His-triMGO[119, 166, 298-300, 332, 347]	MGO x3	His	216.0634	$C_9H_{13}O_6$
His-4MGO[119, 166, 298-300, 332, 347]	MGO x4	His	288.0845	$C_{12}H_{17}O_8$
2-S-Cysteinyl caffeic acid (2-CCA)[358, 359]	Caffeic acid	Cys	178.0266	$C_9H_7O_4$
Lys-DHA[310]	DHA	Lys	108.0211	$C_6H_5O_2$

Most of the MGO-induced modifications were derived from literature, however, some of the MGO-induced modifications in Table 5.2 were hypothesized structures based on the tendency of MGO to participate in self-condensation reactions to form long chain AGEs in acidic conditions (honey is mildly acidic).[166, 332, 360] It was hypothesised that these long chains of MGO could be forming and then modifying proteins. Another possible hypothesis was that single MGO compounds could be modifying proteins and then undergoing self-condensation once bound to the protein.

Modifications on cysteine residues involve a nucleophilic attack of the thiol group (R-SH) in the cysteine on either the aldehyde group of MGO (to form hemithioacetal) or the ketone group of MGO (to form CEC or Cys-triMGO aromatic) with subsequent chemical reactions (dehydration or oxidation).[298] The formation of hemithioacetal is a reversible reaction whereas CEC is a stable modification product.[299, 300, 361] The structures of the proposed MGO-induced modifications on cysteine residues based on known modifications (aforementioned cysteine specific reactions and self-condensation reactions) are depicted in Figure 5.6.

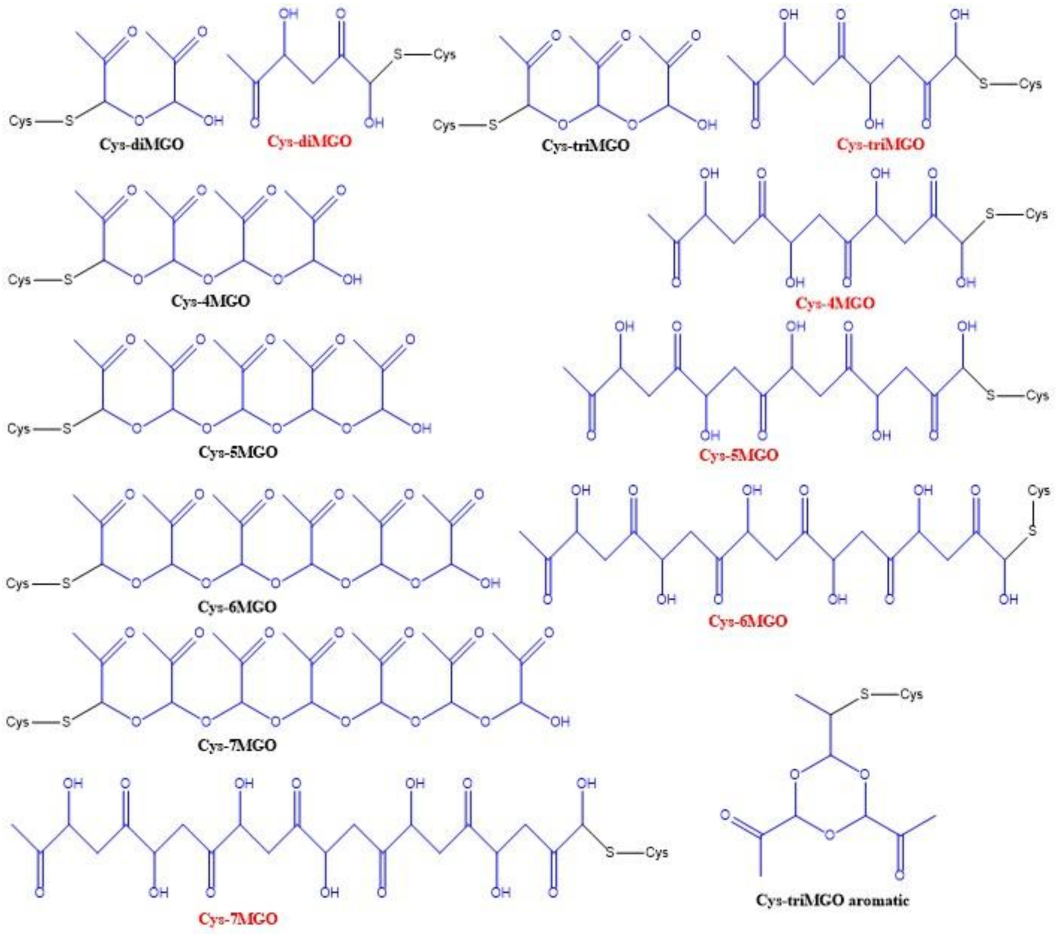


Figure 5.6. The proposed structures for MGO-chain modifications on cysteine, based on known modifications (such as hemithioacetal or CEC). In blue is the modification, black and red text is used to differentiate different isomeric forms that give the same mass shift.

Modifications on lysine residues involve a nucleophilic attack of the terminal amine group of lysine on the aldehyde group of MGO (to form MGO-Lys, an unstable intermediate and is a reversible reaction[361]) or the ketone group of MGO (to form CEL which is a stable product) followed by subsequent dehydration and/or oxidation to get the final structure.[301, 347, 361] The structures of the proposed MGO-induced modifications on lysine residues based on known modifications (e.g. MGO-Lys and CEL modifications with known self-condensation reactions) are depicted in Figure 5.7.

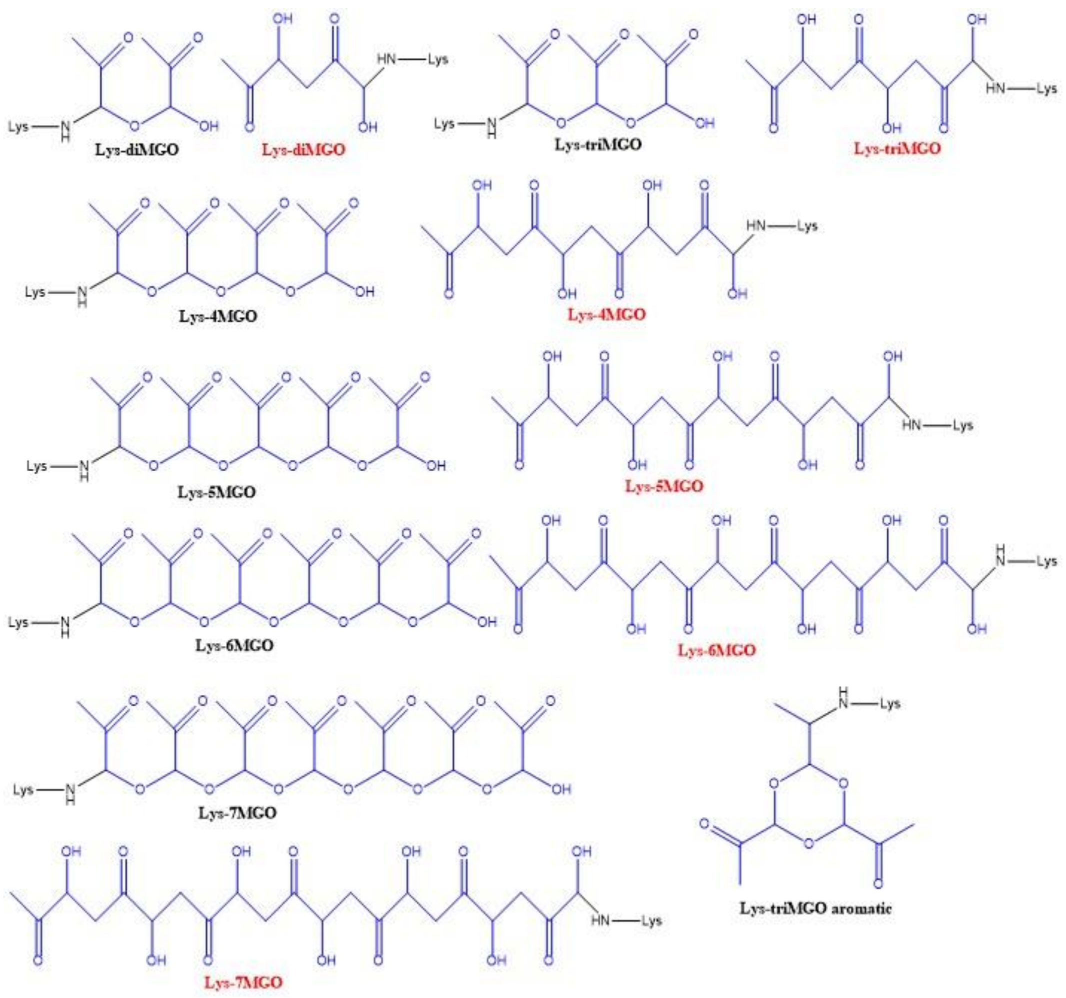


Figure 5.7. The proposed structures for MGO-chain modifications on lysine, based on known modifications (such as MGO-Lys and CEL). In blue is the modification, black and red text is used to differentiate different isomeric forms that give the same mass shift.

Modifications on arginine residues involve the nucleophilic attack of one or more of the terminal amine groups on the aldehyde and or ketone group of MGO with subsequent chemical reactions (e.g. dehydration and/or oxidation).[119, 166, 299, 306, 307, 347, 351, 352] Most of the MGO-induced modifications on arginine residues (including argpyrimidine, CEA, MG-H1, MG-H2 and MG-H3) are stable products.[361] The structures of the proposed MGO-induced modifications on arginine residues based on known modifications (e.g. CEA and MG-H1 modifications with known self-condensation reactions) are depicted in Figure 5.8. The surface of diastase is covered by lysine and arginine residues (green and cyan highlighted structures in Figure 5.4) and hence this is potentially a highly important set of modifications.

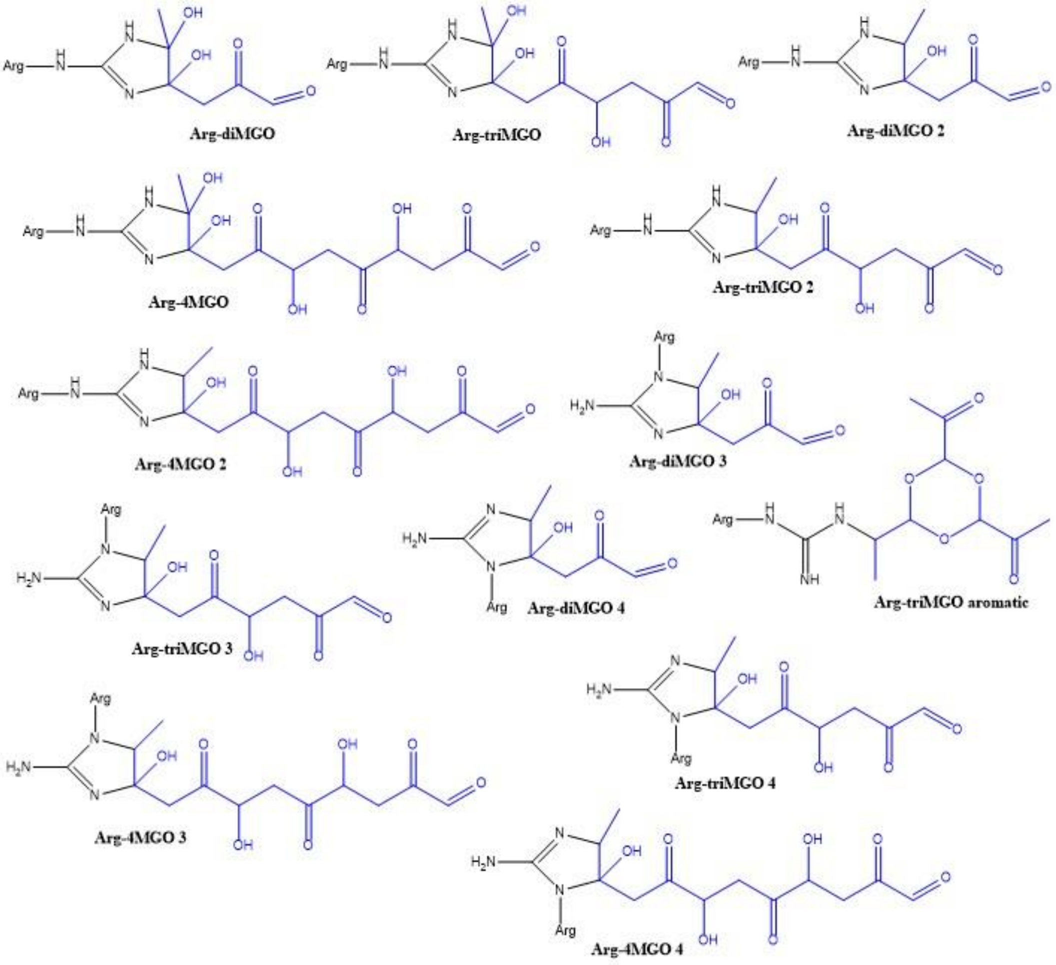


Figure 5.8. The proposed structures for MGO-chain modifications on arginine, based on known modifications (such as CEA and MG-H1). The blue part of the structure is the modification and the black part is arginine.

DHA is an important compound to investigate as it is unique to mānuka honey and an important factor in the rapid loss of diastase activity in mānuka honey (as discussed in section 4.5). DHA is commonly discussed in literature as the main agent used in sun-tanning products due to its ability to partake in the Maillard reaction to form melanoidins. However, only Sun *et al* (2022) gave exact structures of DHA melanoidins on arginine, histidine and lysine. Of these, DHA-Lys (Figure 5.9) was investigated in this study because DHA-Lys is formed from the reaction of DHA with the side chain amine group of lysine (which is more plausible in nature) instead of the amine group which is actually involved in the polypeptide chain of the protein (which is the case for the melanoidin of arginine and histidine).[310] Hence, DHA-Lys seemed the realistic modification to be occurring for a tertiary protein structure.

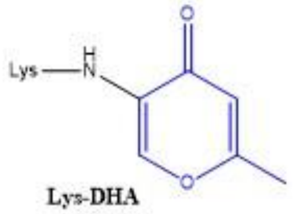


Figure 5.9. The structure of the DHA-Lys modification (proposed mechanism of formation from the reaction of DHA to lysine is given by Sun *et al*).[310] In blue is the DHA modification and in black is the lysine residue.

Histidine is another amino acid that can react with methylglyoxal. Hence, the masses of structures of MGO and various chain lengths of MGO bound to histidine (based on self-condensation, Figure 5.10) were calculated. It was predicted that the aldehyde group of MGO undergoes a nucleophilic attack from the amine group in the side chain of histidine (similar to the formation of MGO-Lys), forming the His-MGO structure.

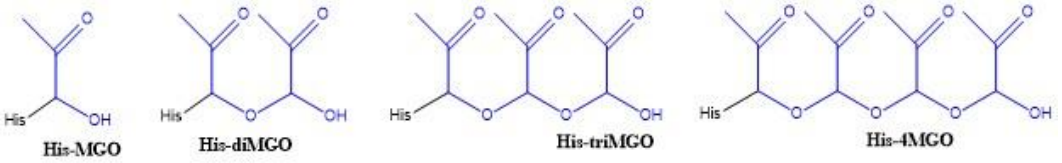


Figure 5.10. The proposed structures for MGO-chain modifications on histidine, based on known modifications (such as MGO-Lys). In blue is the modification.

Although there is literature that states that phenolic compounds form irreversible modifications on proteins through covalent linkage and the formation of reactive quinones,[362] only one structure was found in literature depicting caffeic acid (phenolic compound) attached to a protein residue (cysteine). The condensed name used in this thesis for the 2-S-cysteinyl-caffeic acid modification is 2-CCA[358, 359] (Figure 5.11). Hence, this was investigated in this study. There were no structures in literature showing 3PLA bound to protein residues and due to time-constraints predicted structures of 3PLA modifications could not be made or investigated.

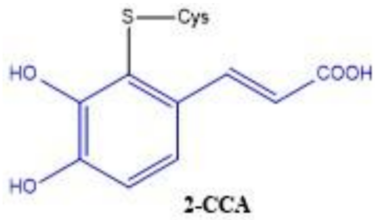


Figure 5.11. The structure of the 2-caffeic acid (2-CCA) modification on cysteine,[358, 359] with the modification shown in blue.

In addition to modification, 3PLA and other phenolics could also be providing protons for acid-catalysed modification reactions which involve other compounds (e.g. MGO). Hence the 3PLA spiked samples were investigated using LC-MS/MS to identify whether MGO, DHA or 2-CCA modifications were enhanced in these samples.

Another study discussed the role of phenolic compounds as trapping agents for the highly reactive MGO by forming adducts through nucleophilic addition reactions.[119] This provides a potential pathway for the interaction of phenolic compounds and MGO in mānuka honey. It was predicted that MGO already bound to protein residues could be reacting with phenolic compounds (e.g. quercetin, rutin and caffeic acid) to form complex AGEs with free aldehyde or ketone groups on the MGO.[119] The predicted structures of these AGEs are shown in Figure 5.12 and were based off literature MGO-phenolic compound complexes.[119] 3PLA and other phenolic compounds (such as those found at high concentrations in mānuka honey, section 1.4.10) could also be forming modifications on proteins however, there are no structures or mechanisms of formation reported in literature for phenolic compounds bound directly to amino acids. Cysteine was used as an example, but the same chemistry would work for arginine and lysine (where instead of the thiol group, the amino group on the side chains of arginine and lysine would do a nucleophilic attack on the ketone group of MGO). Due to the complexities of the structures, the possibility of various isomers and time-constraints these predicted structures (Figure 5.12) were not investigated in this study but are a possible future research avenue.

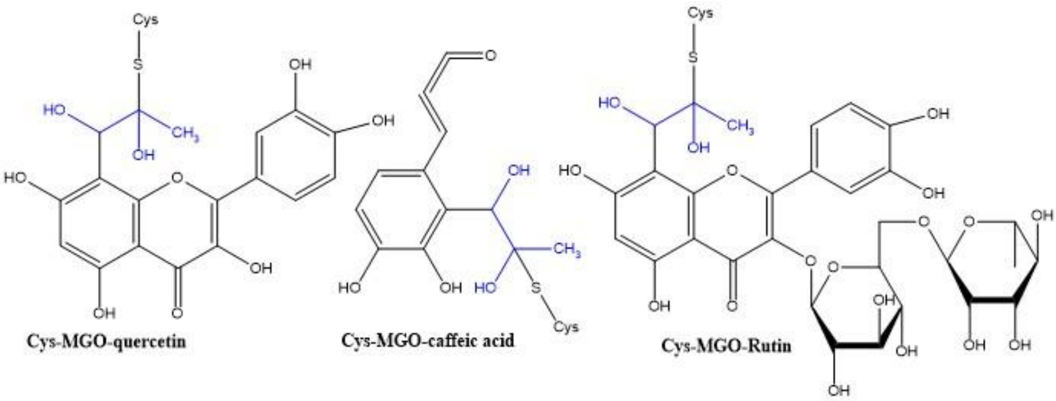


Figure 5.12. The potential structures of Cys-MGO-phenolic compound modifications. The modification from MGO is depicted in blue.

Once the databank was prepared (Table 5.2), samples were prepared for LC-MS/MS to match mass shifts to the databank (resulting in detected peptides) and identify modifications on honey proteins (including diastase) from the storage time trial samples (Figure 5.2).

5.3 LC-MS/MS analysis of modifications on honey proteins

The extracted bands from the SDS-PAGE gel for day 0 samples for mānuka 1 and 2 and clover control and end-point samples (Figure 5.2) for all honeys were analysed using the LC-MS/MS to observe modification changes on diastase and MRJP1 in the honeys. For the day 0 samples, the distinct band at ~56 kDa was excised and for the day 200/203 gel samples the smeared band 50-75 kDa was excised from the gel (Figure 5.2). Each band was individually prepared to remove the staining dye, solubilise the denatured protein from the gel into solution, digested using trypsin (digestive enzyme that cleaves highly specifically on the C-terminal side of arginine and lysine residues in proteins[363]) and analysed by LC-MS/MS. Resultant fragment masses (i.e. change in m/z) were analysed to match mass shifts to the databank (Table 5.2) and identify modifications on diastase and MRJP1 isolated from the storage trial studies.

The resulting mass shifts were observed by comparing the mass spectrum data from the experiment to honey bee protein sequences from UniProt (Table 1.2) and using the modification data from the databank (Table 5.2). This generated results in the format of %coverage, number of peptides detected, number of each modification detected (based on the databank) and −10lgP.

The number of peptides detected depends on whether the protein was modified, and if the specified modification is accounted for in the databank. If no modifications are present, the number of peptides detected should reflect the trypsin-cleavage sites on the proteins (lysine and arginine cleavage sites shown in Figure 5.13). If the protein was modified, the number of peptides assigned are dependent on the present modifications being accounted for in the databank so mass shifts can be accounted for.

The −10lgP value is the significance score calculated as −10 logarithm of a *p*-value. A higher −10lpP value indicated higher statistical confidence in identifications (e.g. peptide to spectrum matches, or peptides to proteins, etc) and suggests they are less likely due to random chance. All results in this study had −10lgP values >150 indicating good confidence in the identifications.

5.3.1 Modification changes on diastase in honey over time

The LC-MS/MS analysis of the end-point samples (day 200/203) showed differences in the numbers and types of modifications identified. It was predicted that the mānuka honey samples and clover honeys spiked with MGO, DHA or 3PLA or a combination of these would have a higher number of modifications identified due to the modifications that could potentially form.

The %coverage for diastase (UniProt accession number Q8N0N7) ranged from 17-58% (lowest coverage was observed for the mānuka day 203 samples and clover spiked with 3PLA+MGO and 3PLA+DHA day 200 samples). The low %coverage of diastase made it more difficult to observe modification changes. The %coverage decreased from day 0 to the end-point samples (i.e. day 200/203); this indicated that there may be other modifications bound to peptides that were unaccounted for by the modifications entered in the databank. For example, mānuka honey 1 showed no unique honey-related modifications for day 0 or 203 but the number of peptides detected for day 203 (17 peptides) was much lower than the number of peptides detected for day 0 (28 peptides, Figure 5.13). Only oxidation (+15.99) and carbamidomethylation (+57.02) were detected; these are common modifications to most biological contexts and are not specific for honey or any type of honey. At day 203, peptides were not detected near the active site residues for mānuka honey 1 (Figure 5.13). This indicates that there may be modifications of unassigned weight (i.e. modifications not accounted for by the databank, Table 5.2) causing the peptides to no longer be assigned in the processing. Since this is near the active site residues of diastase (near 207D, and 309D, i.e. aspartic acid positions 207 and 309 shown in Figure 5.13) could be interfering with its activity (and hence this may be a reason for why there is a decline in diastase activity, Figure 4.6). Other than unaccounted modifications, another factor that can prevent peptides being assigned is if the cleavage sites are modified (by a known or unknown modification) and the modification interferes with the ability of trypsin to cleave the peptide sequence. The cleavage sites were identified (shown as red and orange triangles in Figure 5.13) and if any modification occurred on the end lysine (K) and arginine (R) residues of the peptide it would interfere with the cleavage of the peptide and may also cause non-specific cleavage (i.e. trypsin cleaves at amino acids other than lysine or arginine, not at the sites indicated by the red and orange triangles in Figure 5.13) as well. Non-specific cleavage was not observed for mānuka 1. These modification-induced peptide assignment changes were further investigated by comparing the changes between the various time trial honey samples (Figure 5.14).

The peptides that were detected at the start of the time trial which were not detected at the end of the time trial are given in Figure 5.14. These map modifications that are occurring within the time trial but are either not accounted for in the databank or are not detected due to interference from cleavage site interference. Certain peptides were detected in the clover control sample at day 0 but not at day 200 (highlighted grey in Figure 5.14) and hence modifications outside those accounted for in the databank, occur even in the relatively simple matrix of clover honey. The 3PLA and DHA spike samples showed no differences in peptide detection patterns compared to the clover control. Interestingly, both mānuka honeys (1 and 2) and all samples spiked with MGO (i.e. MGO and 3PLA+MGO spiked samples) showed unique modification patterns (Figure 5.14). Several of the modifications highlighted in this analysis were near the active site residues (active site residues shown with red triangles in Figure 5.14) which could play a key part in blocking the active site from the substrate (i.e. starch) and causing diastase activity loss. These peptides were identified on the AlphaFold model of *A. mellifera* α-amylase to determine their 3D arrangement (Figure 5.15). Roughly four unique sites of these peptide changes were identified in this analysis in aged honey (hinted by the arrows for mānuka honeys 1 and 2 and clover honey spiked with MGO, 3PLA+MGO and 3PLA+DHA in Figure 5.15). The peptide site (not specified by any arrow in Figure 5.15) on the left hand side just below the catalytic triad (highlighted red in Figure 5.15) is present in most of the samples (except mānuka honey 1 and the MGO spiked honey) suggesting this is a common modification to any honey. The 3PLA+DHA spiked sample and both mānuka honeys 1 and 2 are the only samples to observe a unique peptide change (hinted by an arrow in Figure 5.15) on the tip of diastase above the active site cleft and this suggests the presence of a DHA modification. Most of the unique peptide sites identified were in close proximity to the active site. Hence this shows that any modifications that occur in these regions are likely candidates for the observed changes in substrate binding affinity (K_M changes as observed in Figure 4.8) as they are in proximity to the substrate binding site. As well as this, unique modification sites across the entire diastase can affect substrate binding affinity due to allosteric effects (other unique sites shown on diastase on the other side of its active site cleft are in the Appendix on Figure 8.2).

Mānuka honey 2 experienced the largest diastase activity loss, hence the sites of the modifications not accounted for in the databank for this honey are of particular interest (indicated by orange bars in Figure 5.14). Most of these peptides are in locations away from the active site residues (as compared to the MGO spiked sample modification sites) and it is possible that these regions are closer to the active site when arranged in 3D but it is also important to note that allosteric modifications (i.e. modifications on regions outside of the active site) can influence substrate binding to the protein and enzyme activity by introducing slight protein conformation changes that alter the protein's affinity or accessibility to the substrate and catalytic efficiency.

A non-specific cleaved peptide change was observed for all honeys at the end of the signal peptide (Figure 5.14) which is a common occurrence in biological contexts.[364] Another non-specific cleaved peptide was observed for mānuka honey 1 and all clover samples (except the MGO spike) at the C-terminal end of glutamine (Glue, Q) at position 243 of the diastase sequence (Figure 5.14). This indicates that there is likely a modification occurring on the nearest arginine residue (or on another amino acid in close proximity) which prevents the cleavage at the arginine.

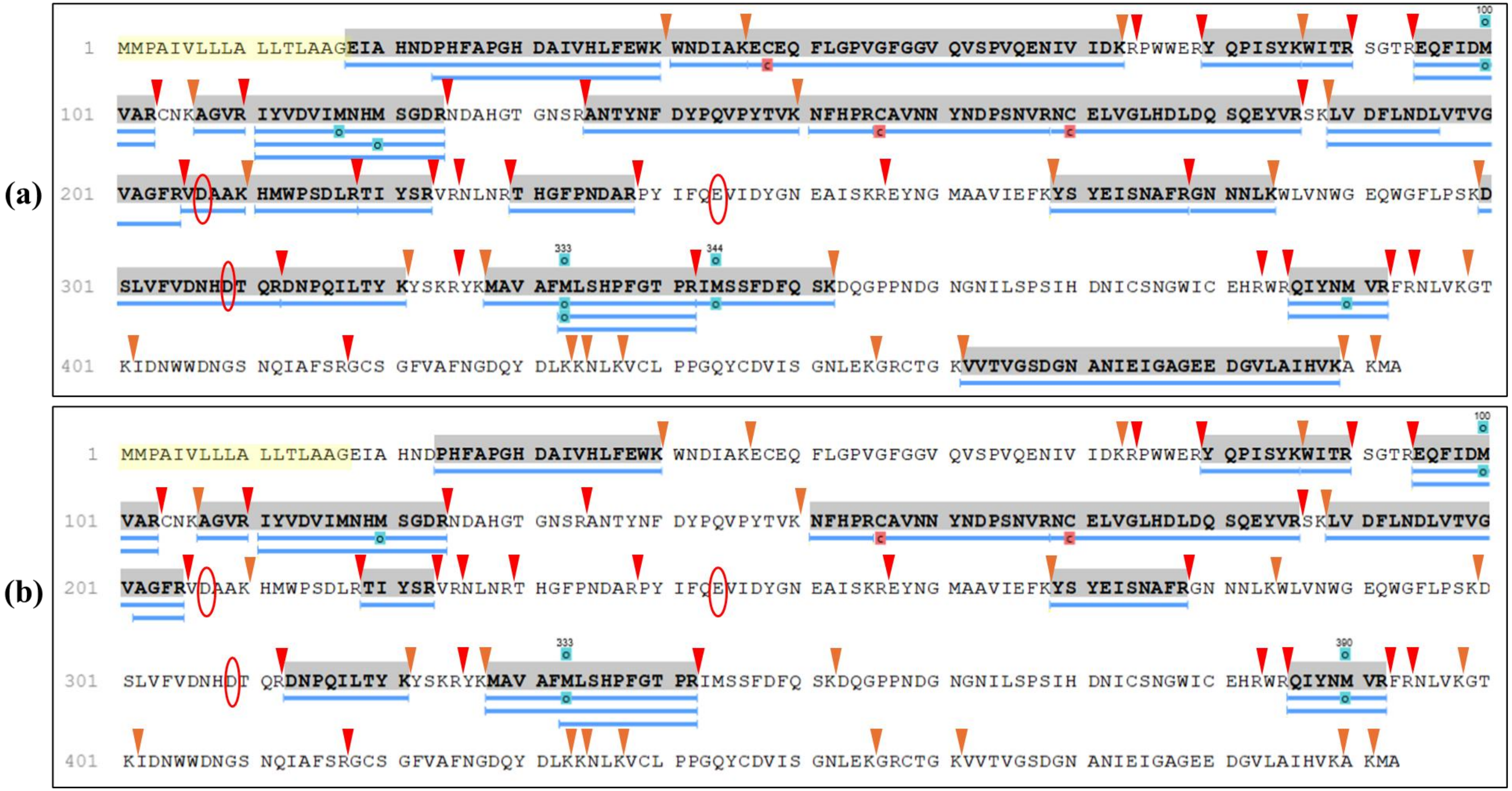


Figure 5.13. The coverage map of the amino acid sequence for diastase (UniProt accession number Q8N0N7), (a) mānuka 1 day 0 and (b) mānuka 1 day 203. Peptides detected by LC-MS/MS are highlight grey with blue underlines; detected modifications are shown as small coloured boxes with letters identifying oxidation (aqua) and carbamidomethylation (pink), active site residues are identified by red circles and the signal peptide is highlighted yellow. Trypsin cleavage sites are identified with triangles red for arginine C-terminal end and orange for lysine C-terminal end.

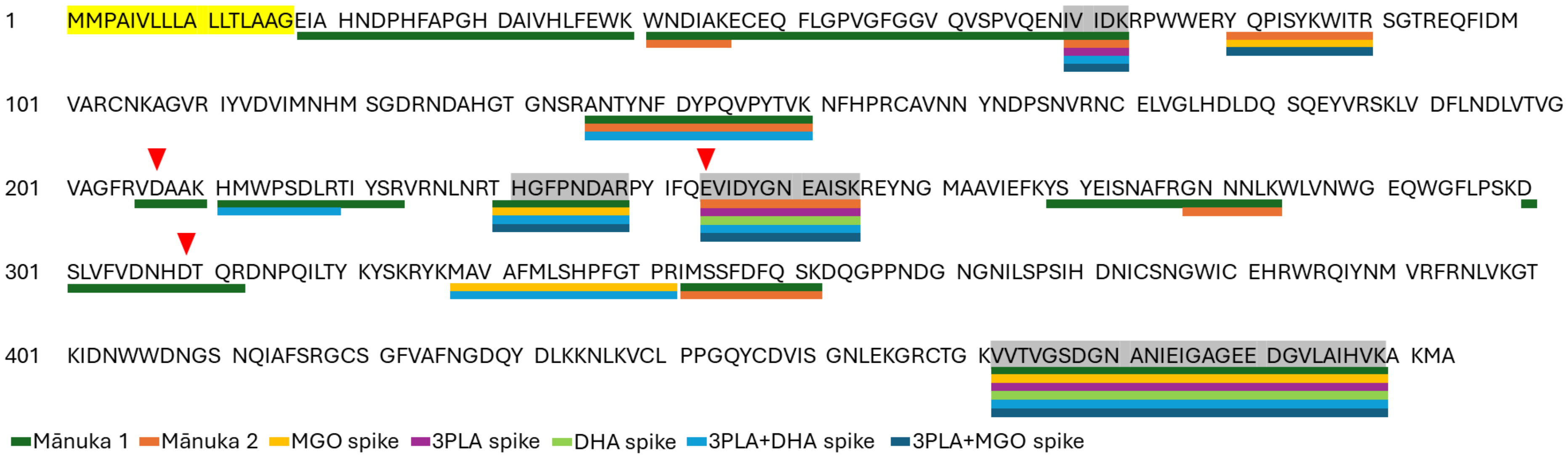


Figure 5.14. The diastase sequence (UniProt accession no Q8N0N7) peptides that were present for the day 0 samples but absent for day 200/203 time trial samples for all the honeys (mānuka 1 and 2, clover control and clover spiked honeys). These represent the peptides where modifications, not in the databank, occurred over the course of the time trial. The signal peptide is highlighted yellow, the active site residues are identified by the red downwards triangles, clover control peptides are highlighted grey while all other honey peptides were assigned a colour according to the legend.

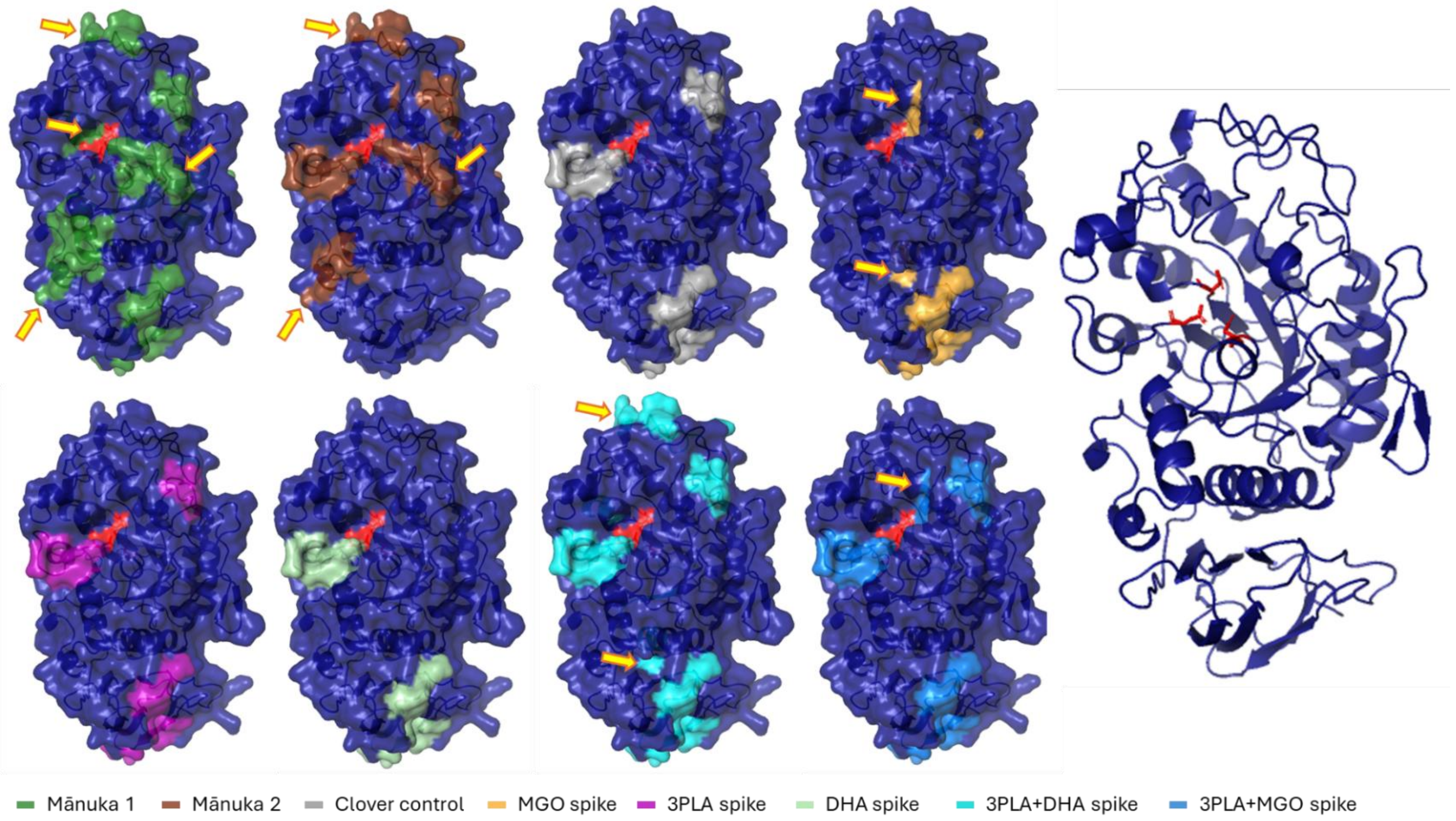


Figure 5.15. The diastase sequence peptides, identified on the surface of the AlphaFold model of *A. mellifera* α-amylase (UniProt accession no Q8N0N7 without the signal peptide), that were present for the day 0 samples but absent for end point samples for all honeys (mānuka 1 and 2, clover control and clover spiked honeys). The active site residues are coloured red, while peptide change for each sample was recorded by a colour legend. The skeletal structure of diastase is shown on the right without the surface or any peptide changes. The arrows indicate unique peptide changes not observed for the clover control.

Given the above analysis showing that modifications not accounted for in the databank occurring on diastase, the number of detected peptides was analysed as a proxy for total modification number. When the LC-MS/MS data for diastase was compared to the enzyme activity data (Chapter 4 Time Trial Results and Discussion), a positive correlation was observed between the total number of detected peptides (for diastase) and the diastase activity DN number (Figure 5.16). This indicates that the decrease in diastase activity is correlated to the formation of surface modifications on the proteins which result in the detection of fewer peptides based on the current modification databank (when analysed using LC-MS/MS). For this, correlations were assessed across end point samples only, as these are assumed to have had sufficient time to produce observable modifications and have equivalent denaturation effects (if any).

Day 0 results were then compared to this trend as an indirect means of assessing denaturation over the time trial. If denaturation had a significant effect over the time trial, the day 0 samples (points in orange in Figure 5.16) would sit higher on the y-axis (as denaturation in the end point samples has an additional negative effect on activity) and would deviate from the main trendline indicating the extent of denaturation occurring. The day 0 points are low in modifications and high in DN value and sit within the observed modification-activity trend. This indicates that the DN value of the day 0 samples is well accounted for by the extent of protein modifications. Thus, confirming that more complex effects are occurring to diastase than simple denaturation over time in honey and that denaturation is not a significant contributor to the activity effects. These effects are not considered in standard diastase testing. As such, these tests may assign decreases in diastase activity in mānuka honey to denaturation due to heat or aging, when effects are instead due to surface level modifications effecting rates due to changes in enzyme-substrate interactions and catalytic capacity. This confirms that the low V_{max} over the time trial is not exclusively due to denaturation and that inhibition effects are taking place.

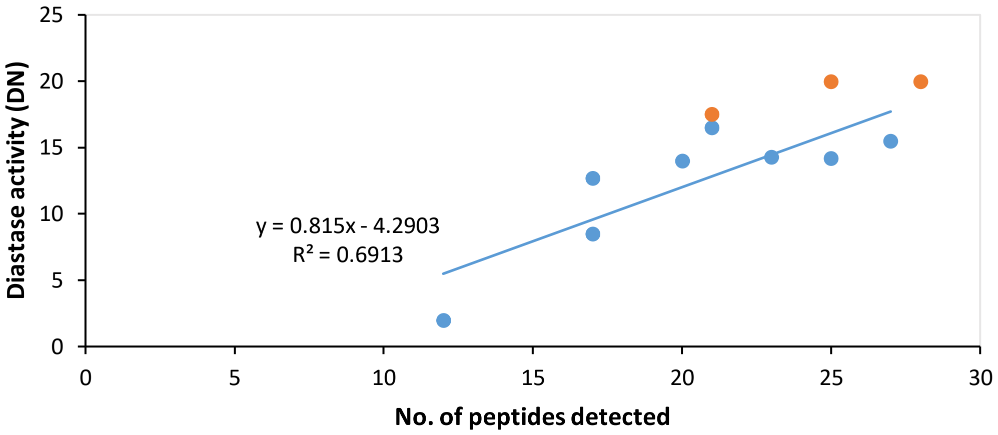


Figure 5.16. The correlation between the diastase activity (expressed DN as evaluated using the Phadebas method) and the number of peptides detected for diastase using LC-MS/MS for end point time trial honeys (blue). A higher number of peptides detected correlates to lower modification levels due to the incomplete nature of the current modification library. Day 0 samples (orange) follow the trend of the aged samples which indicates that denaturation is not a significant contributor to activity loss.

5.3.2 MRJP1 modification changes in honey as a case study

To observe the widest range of modifications possible to proteins in honey, MRJP1 (UniProt accession O18330) was studied due to its higher coverage (>80% for all the samples at day 0 and 200/203). The highest coverage indicates that this was the most abundant protein in the honey samples. If diastase had been more abundant, it is likely that similar observations would have been made since both proteins contain cysteine, lysine, histidine and arginine residues and are both exposed to the same conditions in the honey matrices, giving equal opportunity for surface modifications.

The modifications on MRJP1 from day 0 were compared to those at day 200/203 for all the time trial honeys in Figure 5.17. Mānuka honeys 1 and 2 had increased number of identified modifications at day 203 (from day 0), an increase of 46 and 81% respectively. This indicates mānuka honey 2 has more modifications occurring on proteins. A high number of modifications is also likely to be occurring to diastase which helps to explain why mānuka honey 2 has a greater decrease in diastase activity (DN decreased from 17.5 to <2 for mānuka honey 2 compared to a decrease from 20 to 8.5 for mānuka honey 1). Mānuka honey 1 had 65 MGO-induced modifications at the end of the storage trial, whereas, mānuka honey 2 had 86. Also, interestingly the total number of common modifications (i.e. oxidation, deamidation, phosphorylation and carbamidomethylation) remained relatively stable for mānuka honey 1 whereas the number increased by 17% for mānuka honey 2. This further correlates with the rapid decline in diastase activity in mānuka 2 and indicates that certain compounds (such as 3PLA which was found at a much higher concentration in mānuka 2 than in mānuka 1) could be acting as catalysts for these common modifications.

The number of modifications for the clover control honey remained relatively stable (only 1% increase in the number of modifications was observed at day 200 from day 0). Whereas, the spiked samples, particularly the MGO and DHA spiked samples, had 226 and 133% increases in the total number of modifications detected compared to the day 0 clover control. This was closely followed by the 3PLA+MGO, 3PLA and 3PLA+DHA spiked samples which experienced an increase in the total number of detected modifications by 80, 79 and 44%. This confirms that MGO is a key modification factor in honey and hence results in more modifications in honey containing high concentrations of MGO. However, it needs to be noted that fewer modifications were observed for the 3PLA+DHA sample because only one DHA-dependent modification (DHA-Lys) was entered into the databank. Additionally, the changes in $V_{\max}$ and K_{M} were more obvious for 3PLA+DHA compared to 3PLA+MGO and MGO spiked samples (Figure 4.8) which indicates that DHA binding effects, not accounted for in the modification databank, play a significant role in activity decreases. Hence, other DHA related modifications could be investigated in future studies.

Although these modification changes were studied on MRJP1, similar changes are expected for diastase because the same reactive amino acids (lysine, arginine, cysteine and histidine) are also present in diastase (Figure 5.4). The proposed MGO modifications composed of more than one MGO compound (Table 5.2) were not detected in any of the samples for any protein and this may be because any proteins with these modifications ran much slower on the gel and fell outside of the region of the gel that excised (50-75 kDa) for LC-MS/MS analysis. The region above 75 kDa was smeared and more intense for all the end point samples as compared to the day 0 samples (Figure 5.2) and could be the focus in future studies.

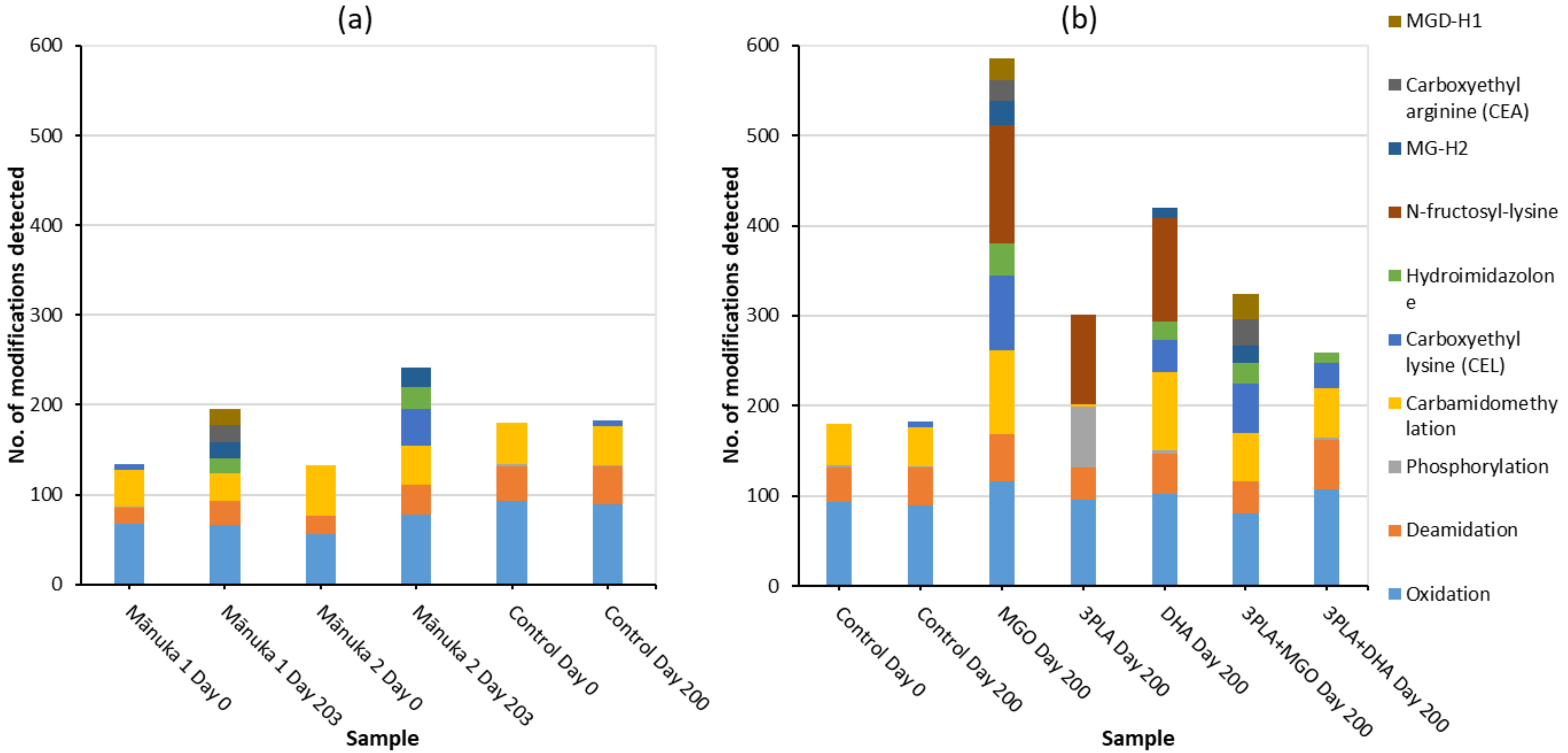


Figure 5.17. The modifications detected on MRJP1 from the various time trial honey samples. (a) Comparison of the modifications detected between the day 0 and day 200/203 samples of mānuka honeys to the clover control. (b) Comparison of the modifications detected between day 0 and day 200 of the clover control and spiked (MGO, 3PLA, DHA, 3PLA+MGO and 3PLA+DHA spikes) samples. Note that the number of modifications only reflects those in the databank and hence DHA-specific modifications are not accounted for.

6 Conclusion and Future work

6.1 Conclusion

New Zealand’s multi-million dollar apiculture industry exports more than 10,000 tonnes of honey annually to various countries. Some of these countries (such as those in the European Union and Canada) set a minimum level for diastase activity (DN >8) as a honey quality parameter. Mānuka honey fails to meet diastase activity more often than any other NZ floral type which is detrimental to NZ’s apiculture industry. It was hypothesized that bioactive compounds found at high concentrations (MGO, DHA and 3PLA) modify diastase and the accumulation of these modifications cause the rapid loss of diastase activity in mānuka honey. This research aimed to investigate the extent, diversity and location of modifications on diastase (focusing on MGO modifications in comparison to MRJP1) and from this, determine whether honey industry standards overlook protein modification as a factor in the loss of diastase activity and variability between different types of honey.

The enzyme kinetics analysis method was developed based on the IHC Schade method and validated (Chapter 3) to explore the changes in key enzyme kinetics parameters ($V_{\max}$ and K_M) and the statistically significant agreement of parameters across validation assays (at 95% confidence intervals) allowed Michaelis-Menten fitting to the resulting data.

The chemical composition of two mānuka honeys, clover control and spiked clover samples was tracked over ~200 days of incubation at 27 °C. Clover honey was used as the matrix to isolate the effects of MGO, 3PLA, DHA, 3PLA+MGO and 3PLA+DHA on the inherent diastase activity. This was required as *A. mellifera* α-amylase is not commercially available and clover honey contains few bioactive compounds. Mānuka honeys 1 and 2 contained low and high concentrations (respectively) of MGO, DHA and 3PLA. Mānuka honey 2 had the largest decrease in diastase activity (DN 17.5 to <2) and a decrease in $V_{\max}$ (maximum catalysed reaction velocity at complete substrate saturation) and K_M (substrate binding constant). The decrease in $V_{\max}$ (and DN) indicate denaturation and/or inhibitory modifications to the diastase structure (honey dependent). The K_M is independent of enzyme concentration, and therefore, measured changes in the K_M indicate surface level modifications occurring on diastase in mānuka honey. These modifications can effect enzyme activity, suggesting diastase activity loss is not exclusively due to denaturation (due to high temperature or extended storage time). Of the clover honeys, 3PLA+DHA spiked honey showed a rapid loss of diastase activity and the most pronounced changes to $V_{\max}$ and K_M, strongly suggesting that DHA and MGO were both involved in forming modifications on diastase and that this was enhanced by 3PLA (acting as a proton donor to accelerate the reactions). In comparison to smaller changes observed for individually spiked DHA and 3PLA clover honey. A strong positive correlation in $V_{\max}$ and K_m for a range of time trial samples confirmed surface modifications were an aspect involved in the loss of diastase activity. Hence, it is incorrect to assume loss in diastase activity in honey (according to current honey industry standard tests) is exclusively due to denaturation caused by heat treatment or storage longevity.

Surface modification identification was determined by separating protein content of the time trial honeys on SDS-PAGE gels, carrying out a trypsin digest and investigating modification changes to the resulting peptides. Results showed more peptides that were not assigned over time for both mānuka honeys (1 and 2) and MGO and 3PLA+DHA spiked honeys. The peptides that were not assigned were mapped on the AlphaFold model of *A. mellifera* α-amylase and several were identified to be in proximity to the active site pocket. The most abundant honey protein, MRJP1, was used to accurately identify modification changes on proteins over time as a model for the changes to diastase. Both mānuka honeys showed increased number of modifications (due to the formation of MGO modifications) over time, while the number remained relatively constant for the clover control. All spiked samples experienced an increase in the total number of modifications, while MGO, DHA and 3PLA+MGO spiked honeys showed the greatest increase in the number of modifications. The number of modifications for 3PLA+DHA spiked honey was predicted to be higher as DHA modifications were not available in the library. These trends in modifications were expected for diastase due to the presence of similar reactive amino acids.

In conclusion, MGO, DHA and 3PLA were found to have significant roles in forming modifications on proteins (diastase and MRJP1) in honey. Enzyme kinetic parameters revealed that denaturation is not the exclusive effect causing loss of diastase activity and that surface modifications on diastase may be contributing to the rapid loss of diastase activity observed for mānuka honey. This evidence indicates that the current honey industry standard diastase assay methods (IHC Schade and Phadebas) which assume total loss of diastase activity is due to denaturation from extended storage times and high temperature is incorrect and that a different approach to determining excessive heat treatment is required.

6.2 Recommendations for future research

Throughout this research, several research avenues could not be explored due to time constraints, however, they have the potential to form future research projects and expand on the findings from this work.

Expansion of the protein modification library:

- The protein modification identification method used a databank of literature and predicted modifications of predominantly methylglyoxal. However, during this research DHA and phenolic compounds were found to be key contributing factors to the loss of diastase activity from the enzyme kinetics results. Hence, future work should expand the databank with a focus on DHA and phenolic compound modifications. It is expected that this will increase the instances where modifications on peptides are assigned for the time trial samples.
- Larger gel slices should be excised above 75 kDa to be analysed by LC-MS/MS to determine if larger sized modifications were being missed because they ran slower on the SDS-PAGE gel.

Isolation of 3PLA, MGO and DHA and their effects on diastase:

- This study used a predicted AlphaFold model of *A. mellifera* α-amylase to identify regions of interest based on LC-MS/MS results. However, future work should aim to recombinantly express, purify and crystallize

A. mellifera α-amylase to obtain a crystal structure (as the predicted model and actual protein can differ in their 3D arrangement based on factors not accounted for by literature and databases). Currently, there is no solved crystal structures of *A. mellifera* α-amylase.

- This current work involved analysing the native diastase from clover honey and spiking clover honey with the compounds of interest (MGO, 3PLA, DHA, 3PLA+MGO and 3PLA+DHA). However, to isolate and confirm that MGO, DHA , 3PLA and their synergistic effects are causing the diastase activity loss, purified *A. mellifera* diastase (recombinantly produced) should be spiked into an artificial honey matrix with the same compounds. This would account for contribution from other compounds in clover honey that are currently unaccounted.

Expansion of compounds of interest:

- The compounds of interest for this study were based on previous findings[4], however, mānuka honey contains high levels of a diverse range of phenolic compounds. Future work should track the changes in the concentrations of these phenolic compounds in various honeys over time, if losses are seen, those compounds should be spiked into blank honey matrices (i.e. clover or artificial honey) to explore their potential role in forming irreversible modifications on diastase.

7 References

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8 Appendix

Table 8.1. The standard abbreviations (3-letter and 1-letter codes) given to all 20 amino acids.

Amino acid name	3-letter code	1-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asp	N
Cysteine	Cys	C
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Table 8.2. The concentrations of MGO, DHA, HMF, 3PLA, 2MAP, 2MBA and 4HPLA (mg/kg) in the mānuka honeys for the time trial experiment (provided by the supplier).

Honey	MGO	DHA	HMF	3PLA	2MAP	2MBA	4HPLA
Mānuka 1	107	1318	1.0	340	10.1	6.1	4.4
Mānuka 2	156	2700	1.9	1180	39.0	10.9	8.8

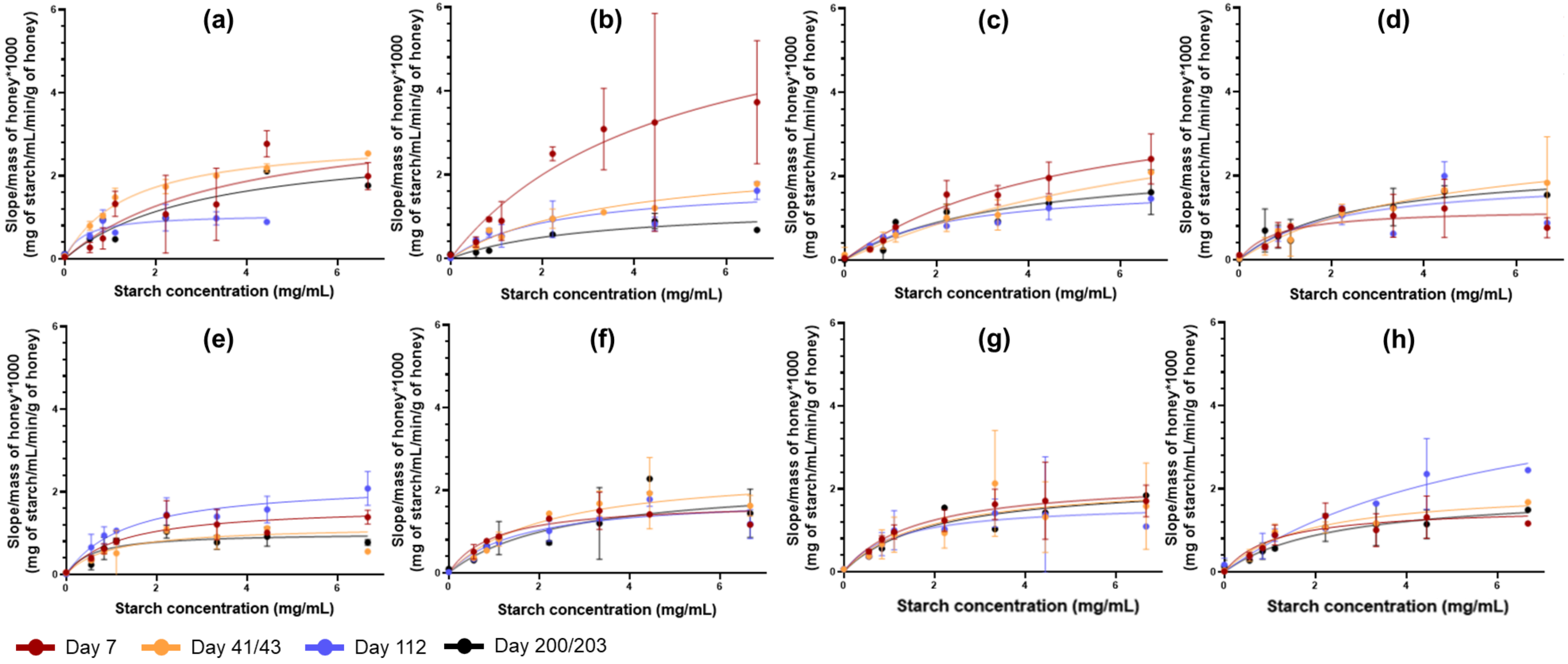

Figure 8.1. The Michaelis-Menten fitting of time trial honey data for 4 different time points (days, 7, 41/43, 112 and 200/203). Days 43 and 203 for the mānuka honeys and days 41 and 200 for the clover control and spiked honeys. (a) Mānuka honey 1, (b) Mānuka honey 2, (c) clover control, (d) clover +MGO, (e) clover +3PLA, (f) clover +DHA, (g) clover +3PLA+MGO and (h) clover +3PLA+DHA honey.

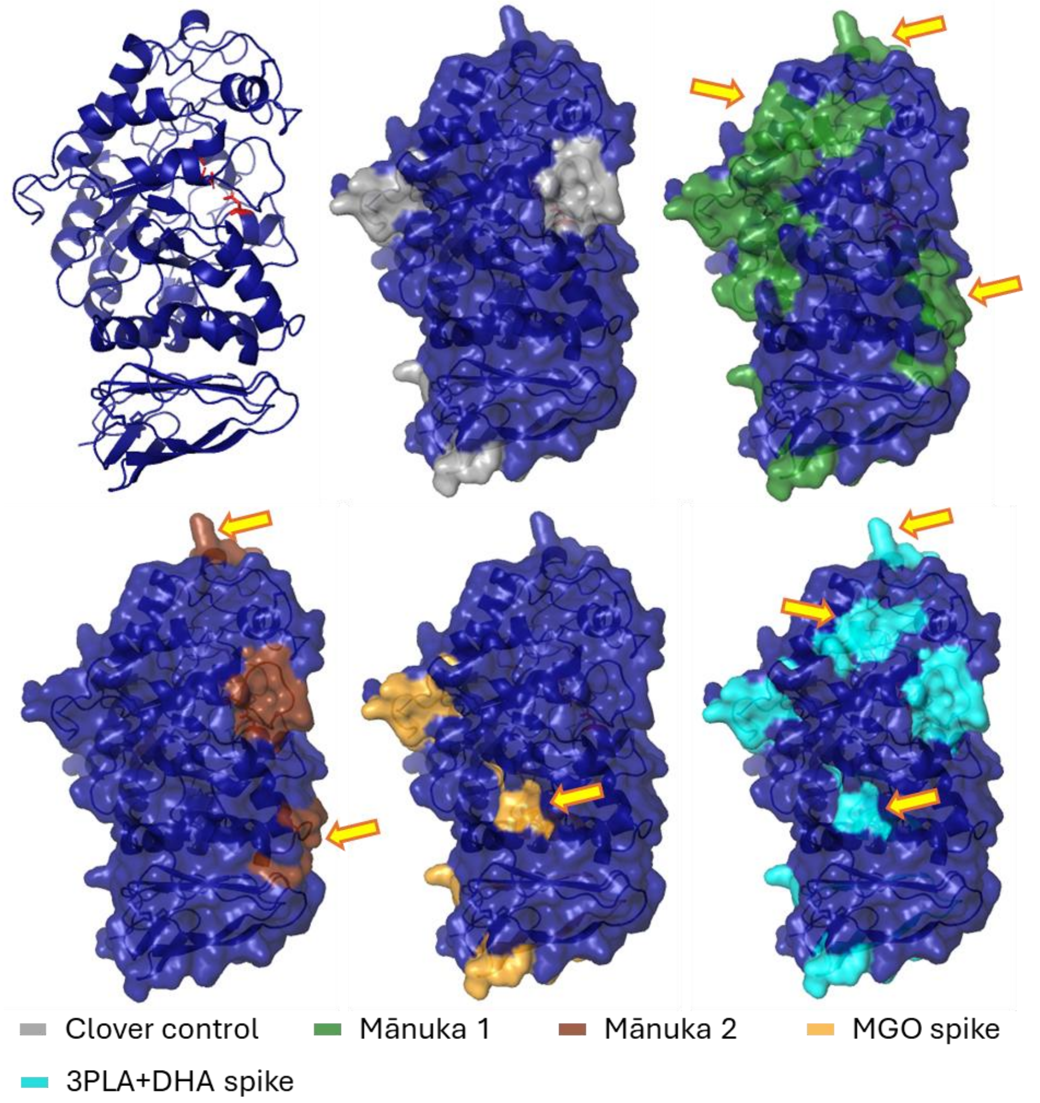

Figure 8.2. The unique diastase sequence peptides, identified on the surface of the AlphaFold model of *A. mellifera* α-amylase (UniProt accession no Q8N0N7 without the signal peptide) rotated away from the active site cleft, that were present for the day 0 samples but absent for end point samples of mānuka honeys 1 and 2, clover control and clover spiked with MGO and 3PLA+DHA. The active site residues are coloured red, while peptide change for each sample was recorded by a colour legend. The skeletal structure of diastase is shown on the top left without the surface or any peptide changes.