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Optimising honey production processes and supply chain management for uni-floral honeys

29 June 2023

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Version 1.0

First published as BICWA Industry Quality Standard – Certified Western Australian Uni-Floral and Multi-Floral Honeys.

Written by Kenneth Dods, ChemCentre, under license from BICWA, ABN 68 609 634 059 as part of the project outcomes for the CRC Honey-Bee products.

The project manager Kenneth Dods wishes to acknowledge the assistance of the following.

Co-authors, Dr Tristan Campbell, Dr Lynne Milne, Dr Robert Manning, Dr David Chandler, Dr Chris May, Dr Kathryn Linge and their associated project teams to this report.

Dr Rob Manning, Field research coordinator
Rob Davis, Technical Officer, WA Herbarium, WA Department of Biodiversity Conservation and Attractions (DBCA)
Dr David Chandler –DNA and phylogenetic leader and his team from Australian Genomic Research Facility (AGRF)
Dr Lynne Milne,: Forensic Palynologist
Steven Davis (year 1), Colin Fleay, (years 2 and 3), Mat Welsh (Year 3), Dr Tristan Campbell (years 4 and 5 and onward). Role: BICWA and WA Farmers Federation (WAFF) WA Honey Research and Development Group (WAHRDG) Committee Chair. Michael Bellman (Treasurer), Kerry Fewster (Secretary), Kim Cheeseman, Ben Pan, Eduard Planken, Simon Green.

Final edit has been provided by Dr John Moursounidis.

1. Background and description

Rapid development of export markets for the WA uni-floral honey industry has challenged the continuity of product supply, particularly for Jarrah honey. Jarrah is a predominantly biannual crop with a lower supply volume in its 'off' season but can deliver abundant supplies in the subsequent season.
Export market requirements demand that the WA honey industry manages the supply chain using methods such as optimal use of localities, hive placements and bee nutrition to maximise

harvest, and with optimal storage conditions to retain honey characteristics.
Modified management practice and optimum conditions are required for appropriate storage of honey when supply is more abundant, especially as the industry moves to establish secondary product development in the nutritional supplement, health food and medicinal space.

2. Storage and supply management

The current supply chain in Western Australia was examined, and research was conducted to better inform the industry of constraints or areas requiring management control in the supply chain. The effects of key variations on Jarrah and Marri uni-floral honeys were assessed in the following areas:

1. Critical control points in the uni-floral honey supply chain.
2. Factors influencing honey quality in the short term.
   a. pH and atmosphere exposure
   b. Temperature
   c. Metal ion content.

   These aspects were investigated as short- term indicative investigative trials that provided guidance to the content and scope of the longer-term storage trial.
3. Long term exposure trial that confirmed trends noted in the short-term trials to be conclusively demonstrated as factors requiring attention and control for honey storage.
4. An investigative trial to determine chemical composition indicators that sterilisation temperatures had been applied to a honey.

Figure 1 Typical honey processing facility as established at Davis Apiaries, purchased and now operated by Honey for Life.

3. Control points

Every food industry has a series of steps that occur during harvest, manufacture and final packaging that are characteristic of those industries normal practices. During the project, five large scale production facilities were reviewed. Interviews were conducted to determine the understanding of normal beekeeper and production practice. Interviews were conducted with:

1. Steven Davis, builder of Davis Apiaries, which later became Honey for Life owned by Shane McLinden

2. Ben Pan, who established and built Australian Natural Botanical (ANB)
3. Simon and Sarah Green, Great Southern Honey
4. Kim Cheeseman, Buzz from the Bees
5. Michael Bellman, Hive and Wellness (formally Capilano).

From these interviews both the beekeeper and the processor and packer supply chains were assessed.

Each step in each supply chain was then assessed for:

1. The variables that could potentially occur with each step.
2. What measurements were possible.
3. Could the measurement result in an improvement of control in the process?

From this an industry supply chain description with critical control points was established as documented in Figure *2* and Figure 3.

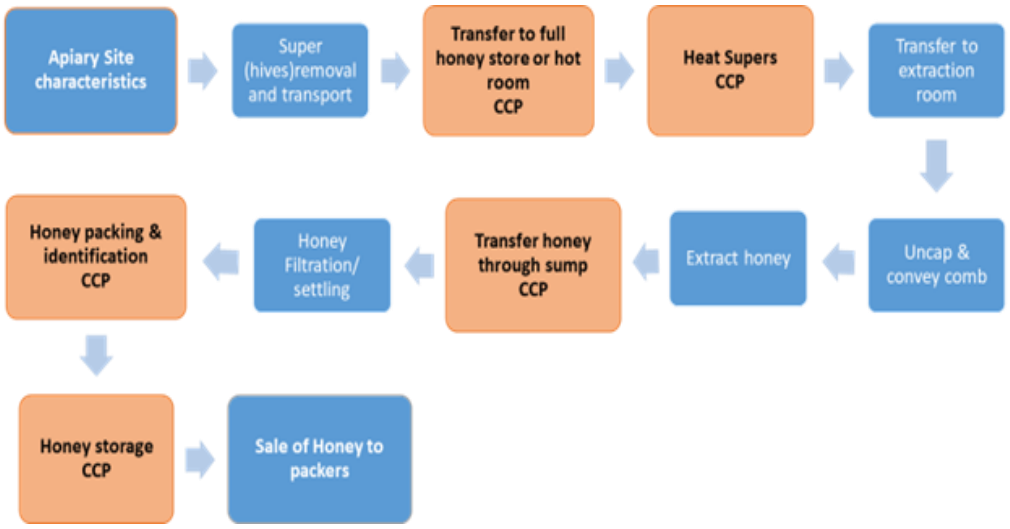

Figure 2 Beekeeper supply chain critical control points (CCP) are highlighted where a CCP protocol is required.

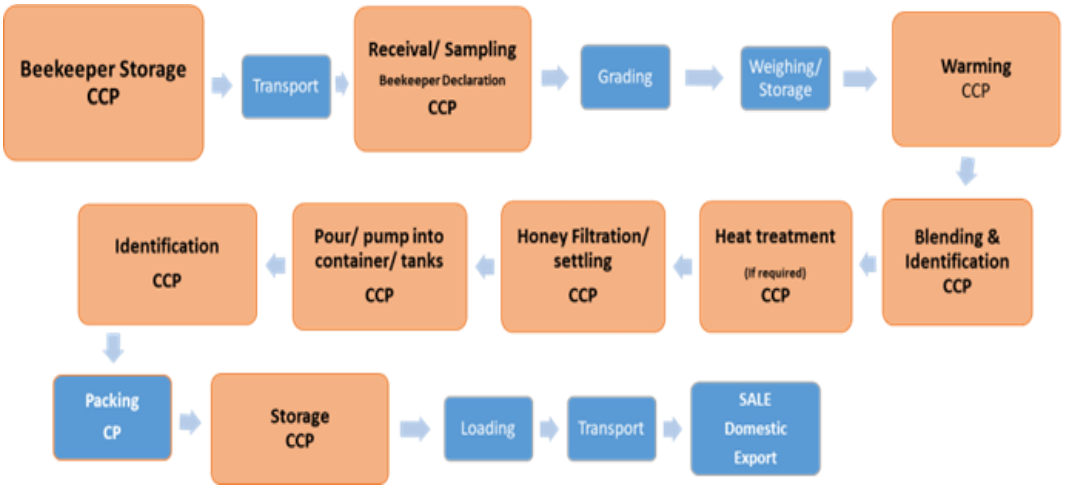

Figure 3 Honey packer supply chain critical control points (CCP) are highlighted where a CCP protocol is required.

4. Short term storage trial – pH change and atmosphere exposure

4.1. Introduction

A short-term storage trial was initiated to investigate the effect of pH and atmosphere on several honey parameters over time. Mallee (as an internal standard) and Marri honey were examined, representing high antimicrobial activity (Marri) and low antimicrobial activity (Mallee) varieties, with Mallee honey commonly used in the laboratory as a control sample with well-defined physico-chemical parameters.

4.2. Methods

Mallee honey and Marri honey samples were diluted to approximately 20% in distilled water by weight and adjusted to pH 8.0 using 1 M NaOH. Each honey variety was then titrated slowly to pH 2.0 using 0.01 mL additions of 5% gluconic acid, with one minute of gentle stirring between each addition.

Samples of diluted honey (20% in water) were prepared in glass jars wrapped in aluminium foil to protect against light. The pH of the dilute honey solutions was adjusted to either pH 10.0 or pH 2.7 from their natural level of pH 4 using 1 M NaOH or 25% gluconic acid respectively. Measurement was then taken at time zero for all three pH conditions. Where an inert atmosphere was required, nitrogen gas was blown into the top of the jar for 1 minute prior to sealing. Sample jars were stored at 22 °C for under various exposure conditions for up to three days. Once exposure was completed, samples were stored at -20 °C until analysis. A summary of the samples, honey varieties and exposure conditions is detailed in Table 1.

Table 1 List of samples and exposure conditions.

Sample Number	Honey Variety	Exposure Conditions	Exposure Duration (h)	Sample Number	Honey Variety	Exposure Conditions	Exposure Duration (h)
001	Mallee	N_2 - Control 1	0	025	Marri	N_2 - Control 1	0
002	Mallee	N_2 - Control 2	0	026	Marri	N_2 - Control 2	0
003	Mallee	N_2 - Control 3	0	027	Marri	N_2 - Control 3	0
004	Mallee	N_2 – pH 2.7	24	028	Marri	N_2 – pH 2.7	24
005	Mallee	N_2 – pH 2.7	48	029	Marri	N_2 – pH 2.7	48
006	Mallee	N_2 – pH 2.7	72	030	Marri	N_2 – pH 2.7	72
007	Mallee	N_2 – pH 4.0	24	031	Marri	N_2 – pH 4.0	24
008	Mallee	N_2 – pH 4.0	48	032	Marri	N_2 – pH 4.0	48
009	Mallee	N_2 – pH 4.0	72	033	Marri	N_2 – pH 4.0	72
010	Mallee	N_2 – pH 10.0	24	034	Marri	N_2 – pH 10.0	24
011	Mallee	N_2 – pH 10.0	48	035	Marri	N_2 – pH 10.0	48
012	Mallee	N_2 – pH 10.0	72	036	Marri	N_2 – pH 10.0	72
013	Mallee	Air - Control 1	0	037	Marri	Air - Control 1	0
014	Mallee	Air - Control 2	0	038	Marri	Air - Control 2	0
015	Mallee	Air - Control 3	0	039	Marri	Air - Control 3	0
016	Mallee	Air – pH 2.7	24	040	Marri	Air – pH 2.7	24
017	Mallee	Air – pH 2.7	48	041	Marri	Air – pH 2.7	48
018	Mallee	Air – pH 2.7	72	042	Marri	Air – pH 2.7	72
019	Mallee	Air – pH 4.0	24	043	Marri	Air – pH 4.0	24
020	Mallee	Air – pH 4.0	48	044	Marri	Air – pH 4.0	48
021	Mallee	Air – pH 4.0	72	045	Marri	Air – pH 4.0	72
022	Mallee	Air – pH 10.0	24	046	Marri	Air – pH 10.0	24
023	Mallee	Air – pH 10.0	48	047	Marri	Air – pH 10.0	48
024	Mallee	Air – pH 10.0	72	048	Marri	Air – pH 10.0	72

Following completion of exposure, all honey samples were returned to room temperature and analysed in accordance with the methods listed in Table 2 .

Table 2 List of analyses and methods performed on honey samples.

Analyte	Method Code	Method Description
EC	ORG404	Electrical Conductivity of honey
pH	ORG403	pH/Free Acidity in honey
Free Acidity	ORG403	pH/Free Acidity in honey
H_2O_2	ORG171	Determination of Hydrogen Peroxide by Spectrophotometric Method
FRAP	ORG423	Antioxidant Activity (Ferric Reducing Antioxidant Potential - FRAP)
Total Phenolics	ORG426	Total polyphenolic analysis in honey

4.3. Results and discussion

A brief discussion regarding each of the parameters is included below. Where results are presented graphically, error bars represent the experimentally determined measurement uncertainty of the analytical method.

4.3.1. Titration curves

Titration curves were produced for both varieties of honey to determine if natural buffering points within the honey could be identified. Marri and the Mallee honeys were run sequentially using an auto-titrator. During the time delay between pH adjustment and commencement of titration curve measurements, the pH of Mallee honey was observed to have decreased from 8.0 to approximately 7.0 by the time measurements commenced. Titration curves for Marri (Figure 4) and Mallee (Figure 5) are detailed below. The curves are truncated to facilitate examination of the region between pH 8.0 and pH 3.0 where natural buffering points were most likely to occur.

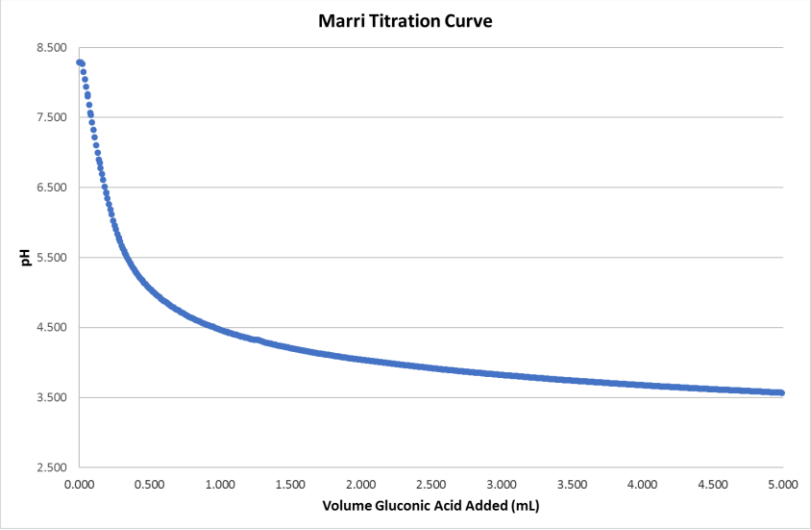

Figure 4 Titration curve for Marri honey with addition of gluconic acid.

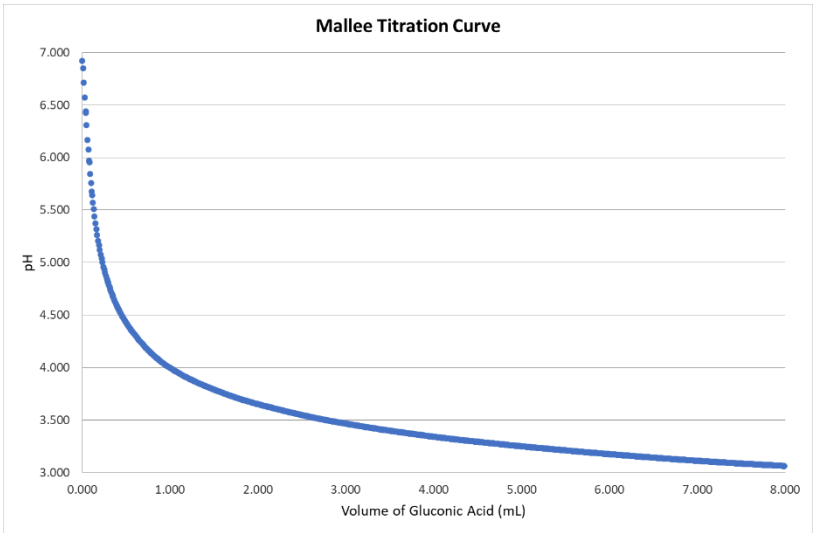

Figure 5 Titration curve for Mallee honey with addition of gluconic acid.

No natural buffering points could be identified, so pH values of 2.7 and 10.0 were selected to observe the effect of extreme pH changes on the various honey parameters in further experiments. A brief discussion regarding each of the parameters is included below:

4.3.2. Electrical conductivity

Electrical conductivity of both pH adjusted honeys (2.7 and 10.0) was observed to be higher than natural pH honey (4.0), after 24 hours regardless of honey variety or exposure to air.
The immediate increased conductivity is due to the addition of NaOH and gluconic acid for the pH adjusted honeys, with these compounds dissociating in the aqueous solution. Despite the short exposure time, there was an indication that electrical conductivity would increase over time in the pH 10.0 honey.
These trends in electrical conductivity are illustrated in Figure 6.

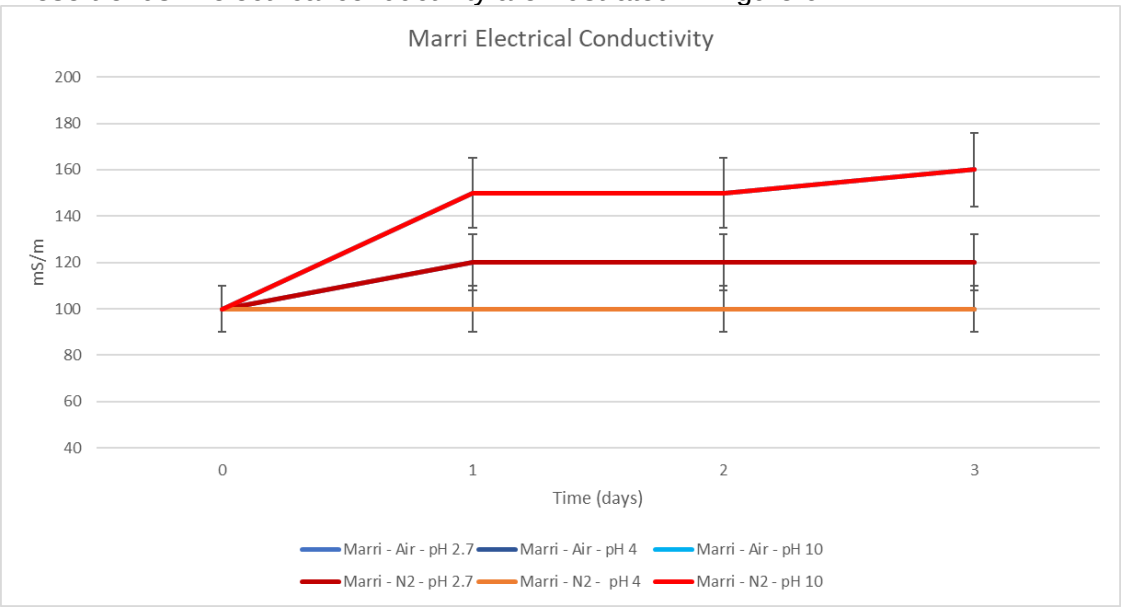

Figure 6 Electrical conductivity of Marri honey.

4.3.3. Free acidity

Free acidity of both varieties of honey were observed to be inversely proportional to their pH values. Honey adjusted to pH 2.7 exhibited high free acidity values that were stable through

the 72 hours of exposure. Honey maintained at pH 4.0 exhibited a similarly stable free acidity, though the free acidity was observed to be higher in an air atmosphere.
Honey adjusted to pH 10.0 exhibited a low free acidity value, though as the pH decreased naturally during exposure, the free acidity increased over time. Free acidity was observed to be higher in samples stored in an air atmosphere compared to those in an inert atmosphere. Consequently, it appears that the production of organic acids in honey occurs at a slower rate when the honey is not exposed to oxygen.
The trends in free acidity are illustrated in Figure 7.

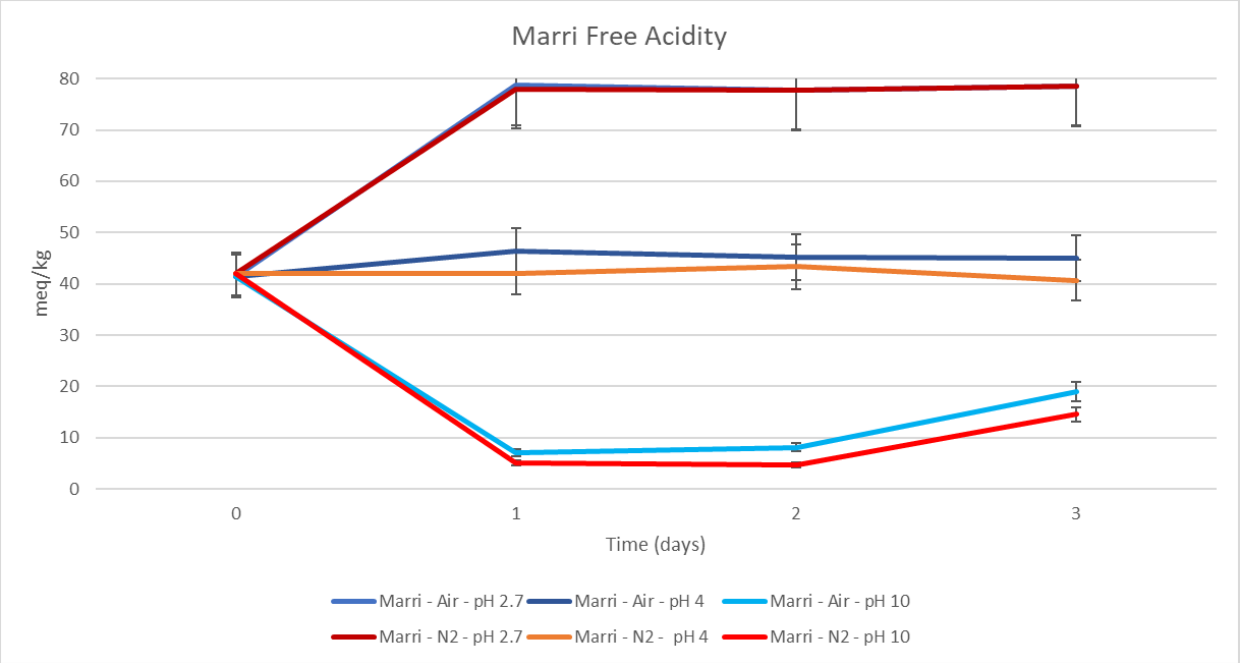

Figure 7 Free acidity of Marri honey.

4.3.4. Hydrogen peroxide

Mallee and Marri honey varieties were observed to respond differently with regards to hydrogen peroxide content during the exposure period. The hydrogen peroxide content of Mallee honey exhibited minimal variation across the exposure period with values ranging between 1.6 to 1.9 mg/kg across all conditions and no clear trend with regards to exposure atmosphere or pH value.
Conversely, Marri honey stored in air exhibited a significantly higher hydrogen peroxide content compared with Marri honey stored in an inert atmosphere (7.2 and 5.4 mg/kg respectively).
These results are consistent with the requirement of oxygen to facilitate the oxidation of glucose to produce hydrogen peroxide. Marri honey stored at pH 4.0 exhibited slightly decreasing hydrogen peroxide content over the exposure period, though samples in both atmospheres remained within 20% of control values.
Both sets of pH adjusted Marri honey samples exhibited very low hydrogen peroxide content, consistent with decreased glucose oxidase enzyme activity. Trends in the hydrogen peroxide content of Marri honey are illustrated in Figure 8.

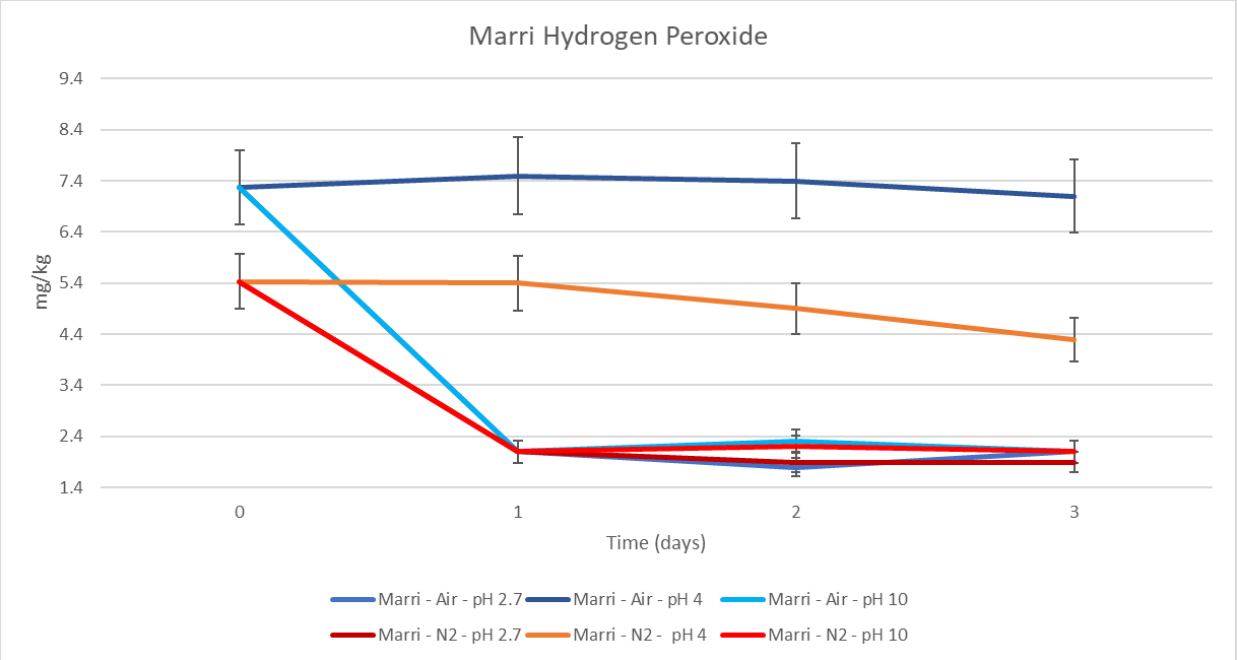

Figure 8 Hydrogen peroxide content of Marri honey.

4.3.5. Total phenolics

Total phenolic content of Mallee and Marri honey was observed to be relatively stable over the 72-hour exposure period for samples stored at pH 4.0. In both varieties, total phenolic content was slightly elevated in honey samples stored at modified pH conditions (pH 2.7 and 10) with pH 10 samples exhibiting a higher total phenolic content overall.

When comparing the two exposure atmospheres, Mallee honey exhibited higher total phenolic content in control samples stored in inert conditions, while Marri honey exhibited higher total phenolic content when exposed to air.

Total phenolic content in Mallee honey is detailed in Figure 9.

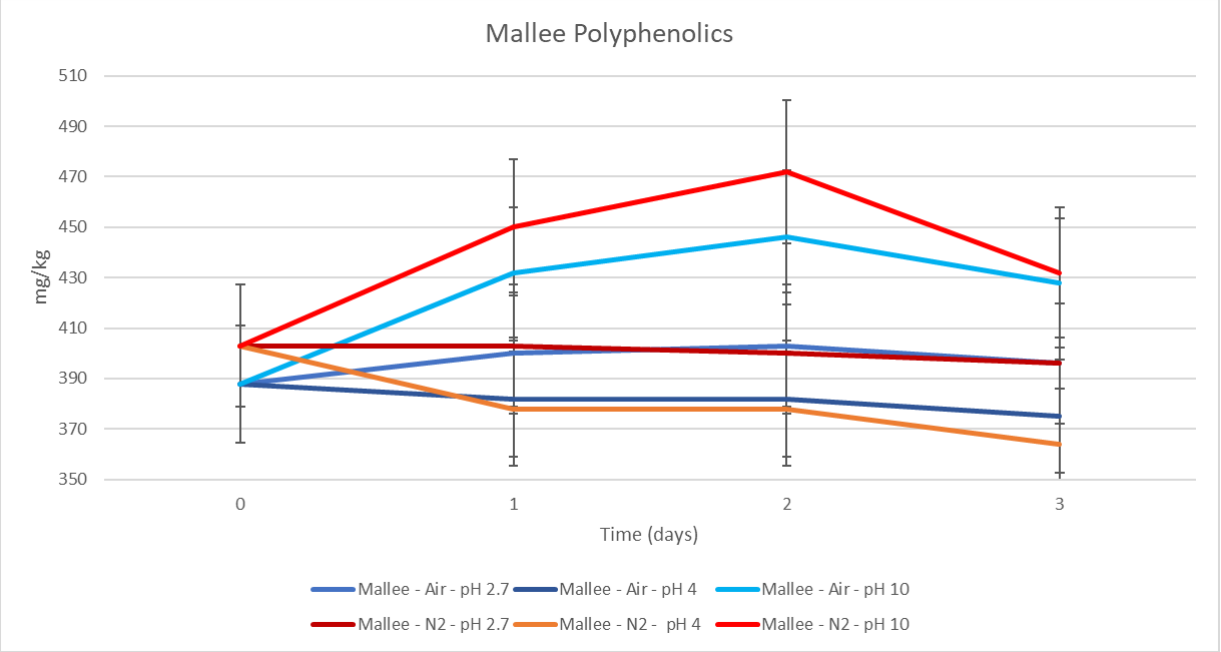

Figure 9 Total phenolic content of Mallee honey.

4.3.6. FRAP

The antioxidant activity of both honey varieties was observed to be higher in air than in an

inert atmosphere. Samples stored at pH 2.7 exhibited higher overall antioxidant activity.
When comparing samples stored at pH 4 and pH 10, Mallee honey exhibited slightly higher antioxidant activity in honey stored at normal pH levels while levels in Marri honey were generally similar between the two pH conditions.
Figure 10 illustrates the antioxidant activity observed in Mallee honey over the exposure period.

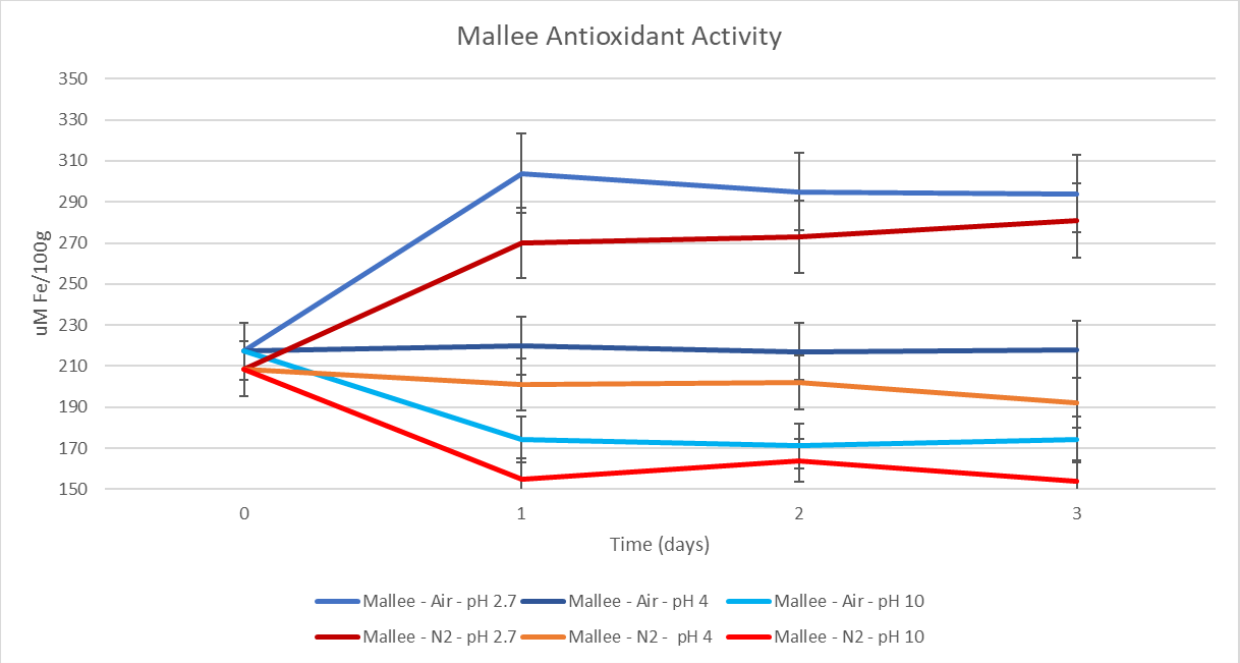

Figure 10 Antioxidant activity (FRAP) of Mallee honey.

4.4. Conclusion

A short-term storage trial was conducted to investigate the effect of pH and atmosphere on various parameters of honey.
Electrical conductivity results were affected by the addition and subsequent dissociation of NaOH and gluconic acid.
Free acidity and pH values were consistent and inversely proportional with each other. Despite the absence of any natural buffering points in titration curves, pH adjusted samples showed some evidence of buffering towards natural pH levels over time with samples stored in an inert atmosphere progressing at a slower rate.
The absence of natural buffering points in titration curves but the tendency towards returning to a pH near 4.0 warrants further investigation regarding the various organic acids present in honey and their natural ability to form a buffered system.
Hydrogen peroxide content in Marri honey samples were consistent with expectations regarding the oxidation of glucose, with higher concentrations present in samples exposed to oxygen and lower concentrations in honey samples stored in conditions outside the optimal pH range for glucose oxidase activity.
Total phenolic content was highest in samples stored at elevated pH levels suggesting that degradation to phenolic active derivatives is greater at elevated pH , while the inverse was true of antioxidant activity.

5. Short term storage trial – temperature

Figure 11 Measuring simple sugars by High Pressure Liquid Chromatography with Refractive index detection (HPLC-RI).

5.1. Introduction

A short-term storage trial was initiated to investigate the effect of temperature exposure over time on several honey parameters. Mallee and Marri honey were examined, representing high antimicrobial activity (Marri) and low antimicrobial activity (Mallee) varieties, with Mallee honey commonly used in the laboratory as a control sample with well-defined physico-chemical parameters.

5.2. Methods

Mallee (17S3064/001) and Marri (17S3064/002) honey samples were dispensed into a series of small glass hexagonal jars. Sample jars were grouped in accordance with intended exposure temperature and placed into laboratory incubator cabinets for known exposure periods up to three weeks.

Once exposure was completed, samples were stored at -20 °C until analysis.

A summary of the samples, honey varieties and exposure conditions are detailed in Table *3*.

Table 3 List of samples and exposure conditions.

Sample Number	Honey Variety	Exposure Temp. (°C)	Exposure Duration (weeks)	Sample Number	Honey Variety	Exposure Temp. (°C)	Exposure Duration (weeks)
001	Mallee	0	0	017	Marri	0	0
002	Mallee	19	1	018	Marri	19	1
003	Mallee	19	2	019	Marri	19	2
004	Mallee	19	3	020	Marri	19	3
005	Mallee	30	1	021	Marri	30	1
006	Mallee	30	2	022	Marri	30	2
007	Mallee	30	3	023	Marri	30	3
008	Mallee	40	1	024	Marri	40	1
009	Mallee	40	2	025	Marri	40	2
010	Mallee	40	3	026	Marri	40	3
011	Mallee	50	1	027	Marri	50	1
012	Mallee	50	2	028	Marri	50	2
013	Mallee	50	3	029	Marri	50	3
014	Mallee	70	1	030	Marri	70	1
015	Mallee	70	2	031	Marri	70	2
016	Mallee	70	3	032	Marri	70	3

Following completion of exposure, all honey samples were returned to room temperature and analysed in accordance with the methods listed in Table 4.

Table 4 List of analyses and methods performed on honey samples.

Analyte	Method Code	Method Description
Moisture	ORG401	Moisture in honey by refractometry
pH	ORG403	pH/Free Acidity in honey
Free Acidity	ORG403	pH/Free Acidity in honey
EC	ORG404	Electrical conductivity in honey
H_2O_2	ORG171	Determination of Hydrogen Peroxide by Spectrophotometric Method
FRAP	ORG423	Antioxidant Activity (FRAP)
Total Phenolics	ORG426	Total polyphenolic analysis in honey
HMF	ORG188	Carbonyl compounds in honey by LC-MS/MS
Total Sugars	ORG155F	Sugars in food and plant material using liquid chromatography

5.3. Results and discussion

A brief discussion regarding each of the parameters is included below. Where results are presented graphically, error bars represent the experimentally determined measurement uncertainty of the analytical method.

5.3.1. Moisture

Mallee honey exhibited minimal variation in moisture content across the various exposure conditions, with all results within 1% of the control sample, determined at 15.1% moisture.
Similarly, Marri honey exhibited minimal variation for the majority of exposure conditions, with all results within 1% of the control sample, determined at 17.5% moisture. Overall, no effect was observed on moisture content for these temperatures and exposure period.

5.3.2. pH

Both Mallee and Marri honey exhibited minimal variation in pH, with control samples for both varieties recorded as pH 4.1.
Minor elevation of pH was observed in both honey varieties after 1-2 weeks exposure at 70 °C. However, in both instances pH returned to 4.1 by the third week.
Consequently, it was concluded that any variation was likely associated with measurement uncertainty. The measurement uncertainty was established during method validation as ± 2.6% relative, rounded up to the nearest 0.1, equivalent to an absolute pH value of ± 0.2.

5.3.3. Free acidity

Free acidity in Mallee honey was determined to be approximately 17 meq/kg. No clear trends were observed for the majority of exposure conditions. However, at 70 °C exposure, samples did exhibit increasing free acidity over the course of three weeks.
Marri honey exhibited higher free acidity values overall (approximately 39 meq/kg), with a similar trend of increasing free acidity with increasing exposure time at 70 °C.
Marri and mallee free acidity change over time in shown in Figure 12.

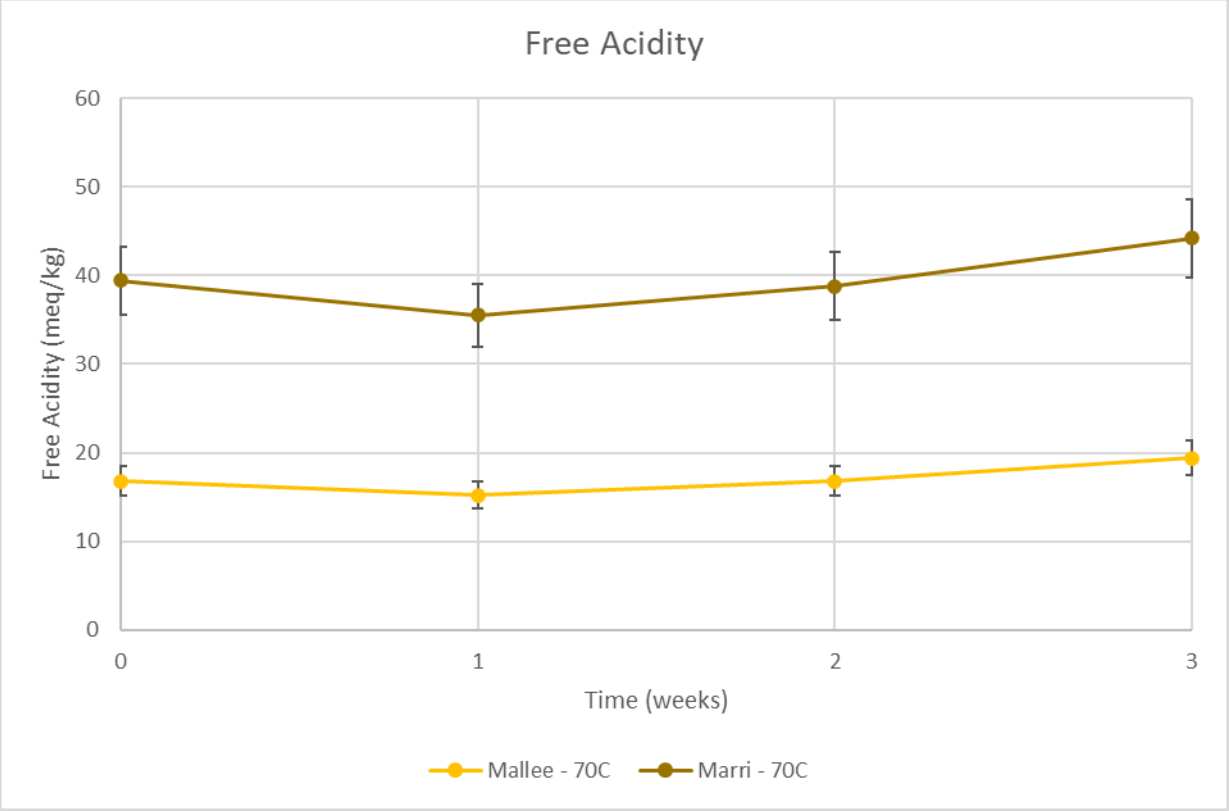

Figure 12 Free Acidity of honey following exposure at 70 °C.

5.3.4. Electrical conductivity

Electrical conductivity of Marri honey (approx. 100 mS/m) was observed to be higher than the electrical conductivity of Mallee honey (approx. 47 mS/m).

However, no variation was observed at the various exposure temperatures over time.

5.3.5. Hydrogen peroxide

Hydrogen peroxide values were observed to vary between 2–6 mg/kg in Marri honey across the various exposure times and conditions.

In general, hydrogen peroxide levels decreased with increasing exposure times at 19, 40 and 50 °C, while increasing hydrogen peroxide concentrations were determined with increasing exposure time at 70 °C.

Concentrations of hydrogen peroxide in Mallee honey were relatively lower (1 mg/kg) than in Marri honey. Minimal variation was observed in samples exposed to temperatures up to 50 °C.

However, increasing hydrogen peroxide concentrations over time were again observed in Mallee honey samples exposed to 70 °C conditions. These results are illustrated in Figure 13.

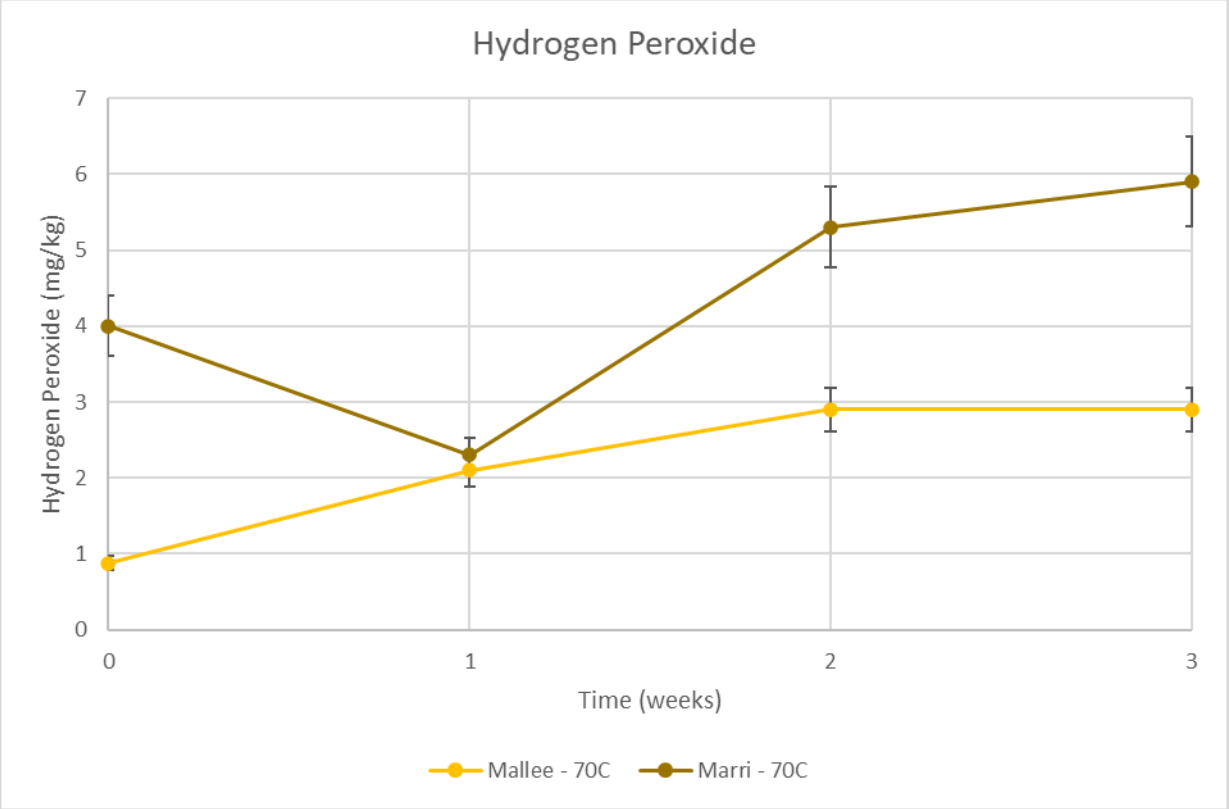

Figure 13 Hydrogen peroxide in honey following exposure at 70 °C.

5.3.6. Simple sugars

Total simple sugar content of Marri and Mallee honey samples were both determined to be approximately 79.8% in control samples.

No consistent trends with regards to simple sugar content were observed in either honey variety at storage temperatures up to 50 °C. However, at storage temperatures of 70 °C, a 3-7% absolute decrease in total simple sugar content was observed in both honeys.

5.3.7. HMF

HMF content of Marri and Mallee honey samples remained relatively stable over the three-week exposure period at storage temperatures up to and including 30 °C.

At storage temperatures of 40 °C and higher, HMF content increased over time, with the rate of increase proportional to the storage temperature (higher storage temperatures, faster rate of HMF production).

At storage temperatures of 70 °C, very high (> 350 mg/kg) levels of HMF were observed within one week of exposure. The increase in HMF content over time is illustrated in Figure 14.

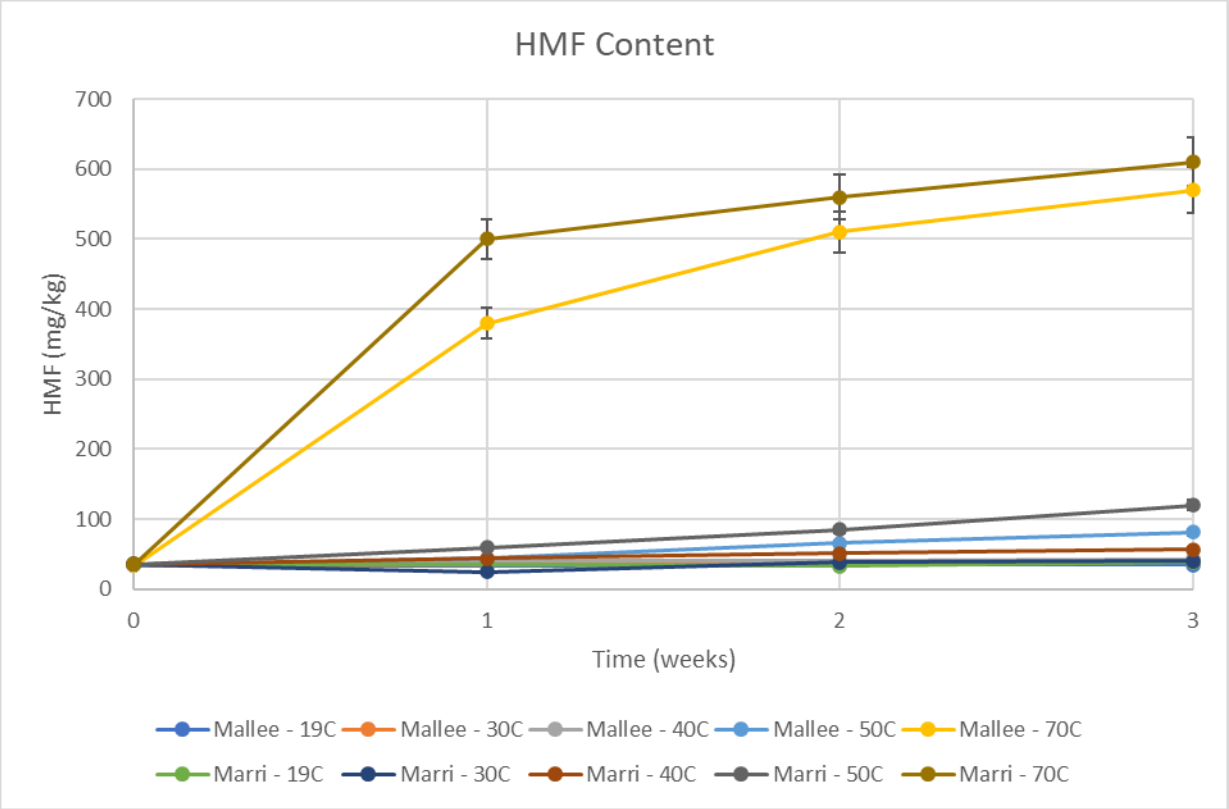

Figure 14 HMF content in honey following exposure to elevated temperatures.

5.3.8. FRAP

Using FRAP as a measure of antioxidant activity, the activity of Marri honey (approx. 500 µM Fe / 100 g) was higher than the activity of the Mallee honey (approx. 300 µM Fe / 100 g). No variation over exposure time was observed in honey samples stored at 19 °C.

However, a slight increase in activity over time was observed for both honey varieties stored at temperatures of 30, 40 and 50 °C. A significant increase (2-3x control values) in antioxidant activity was observed in samples stored at 70 °C. This increase in antioxidant activity is illustrated in Figure 15.

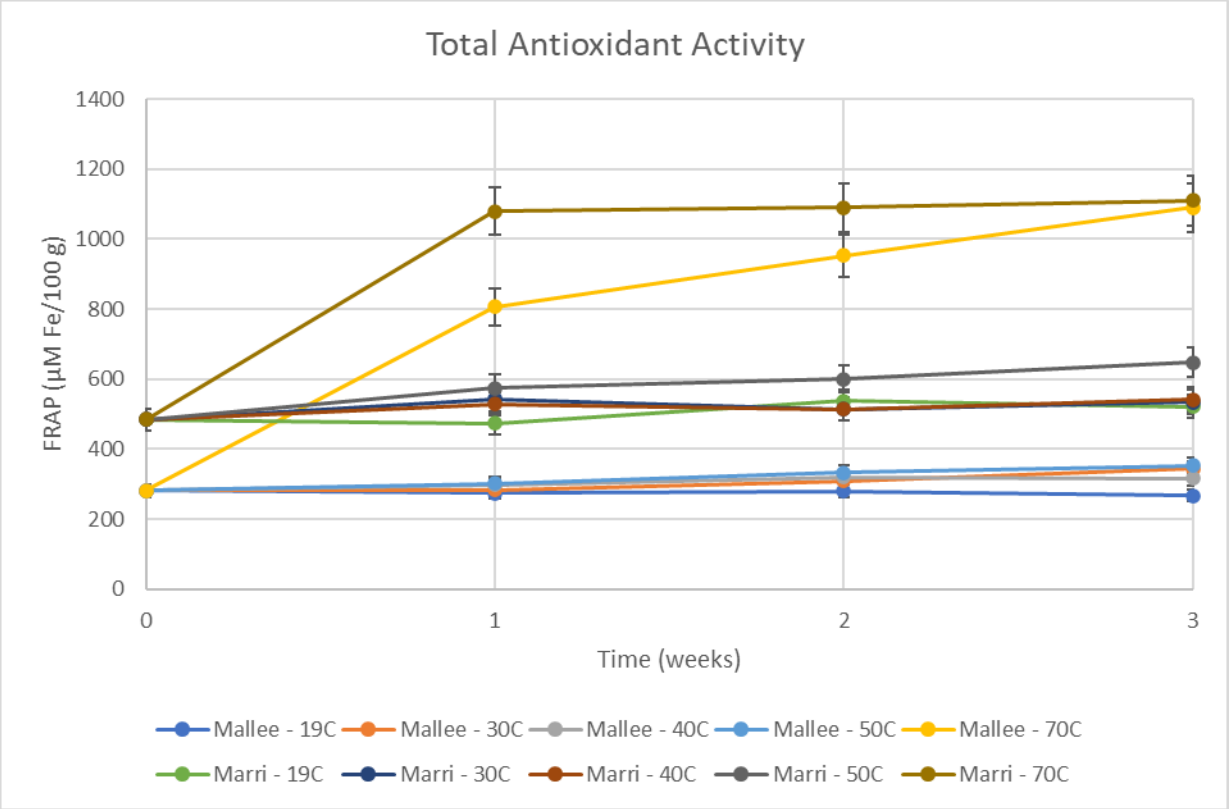

Figure 15 Antioxidant activity (FRAP) in honey following exposure to elevated temperatures.

5.3.9. Total phenolics

Total phenolic content of Marri honey was observed to be relatively stable over time at various storage conditions. A significant increase in total phenolics was observed in Week 3 samples for Mallee honey stored at 20 and 30 °C, though this was not observed in Marri honey, nor at temperatures of 40 and 50 °C.

However, at storage temperatures of 70 °C, an increase in total phenolic content was observed over time in both honey varieties. This increasing total phenolic content at 70°Cis illustrated in Figure 16.

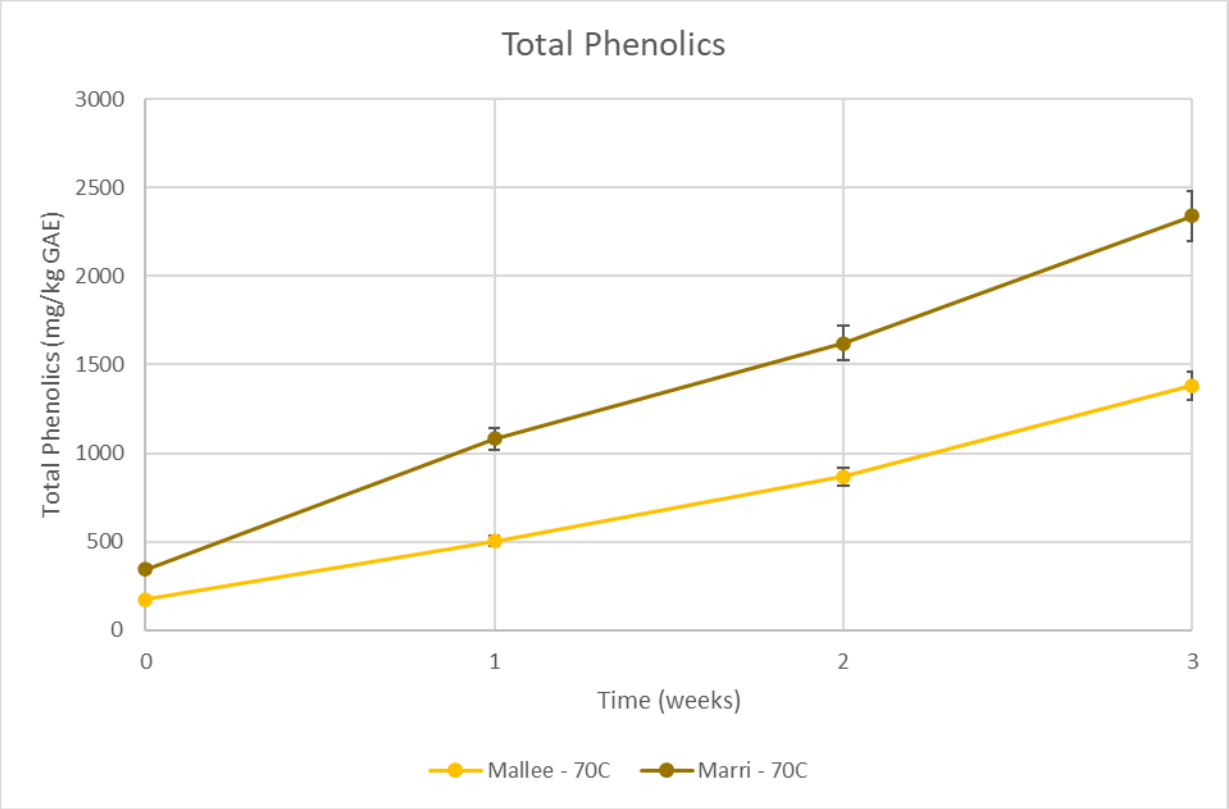

Figure 16 Total phenolic content in honey following storage at 70 °C.

5.4. Conclusion

A short-term storage trial investigating the effect of storage temperature on chemical characteristics of honey has been completed. At storage temperatures of 70 °C, total simple sugar content decreased while HMF, total phenolic content, hydrogen peroxide, free acidity and antioxidant activity all increased over time.

At lower temperatures, HMF content and antioxidant activity were observed to increase over time while other parameters remained relatively constant.

Due to the small number of samples and relatively short exposure period, further examination of these characteristics in a long-term storage trial are required before definitive conclusions regarding storage conditions can be reached.

However, preliminary results indicate that elevated storage temperatures induced a significant effect on several chemical characteristics of honey.

6. Short term storage trial – metal ion content

6.1. Introduction

A short-term storage trial was conducted to investigate the effect of added metal ion content on several honey parameters. Mallee and Marri honey were examined, representing high antimicrobial activity (Marri) and low antimicrobial activity (Mallee) varieties, with Mallee honey commonly used in the laboratory as a control sample with well-defined physico-chemical parameters.

The trial was commissioned based upon the results of a study by Nakamura & Ogura[1] in which the presence of certain metal ions (e.g., Cu^{2+}) resulted in inhibition of the glucose oxidase enzyme, while other metal ions (e.g., Zn^{2+}) increased glucose oxidase activity.

6.2. Methods

Mallee (17S3064/001) and Marri (17S3064/002) honey samples were accurately weighed into a series of small glass hexagonal jars and wrapped in aluminium foil.

Samples were spiked with appropriate amounts of $ZnCl_2$ or $CuCl_2.2H_2O$ to produce

concentrations of 1 mM Zn^{2+} or Cu^{2+} respectively. Sample jars were stored at 22 °C for known exposure periods up to three weeks.
Once exposure was completed, samples stored at -20 °C until analysis. A summary of the samples, honey varieties and exposure conditions are detailed in Table 5.

Table 5 List of samples and exposure conditions.

Sample Number	Honey Variety	Exposure Conditions	Exposure Duration (weeks)	Sample Number	Honey Variety	Exposure Conditions	Exposure Duration (weeks)
001	Mallee	Control	0	013	Marri	Control	0
002	Mallee	Control	0	014	Marri	Control	0
003	Mallee	Control	0	015	Marri	Control	0
004	Mallee	No Spike	1	016	Marri	No Spike	1
005	Mallee	No Spike	2	017	Marri	No Spike	2
006	Mallee	No Spike	3	018	Marri	No Spike	3
007	Mallee	1 mM Zn	1	019	Marri	1 mM Zn	1
008	Mallee	1 mM Zn	2	020	Marri	1 mM Zn	2
009	Mallee	1 mM Zn	3	021	Marri	1 mM Zn	3
010	Mallee	1 mM Cu	1	022	Marri	1 mM Cu	1
011	Mallee	1 mM Cu	2	023	Marri	1 mM Cu	2
012	Mallee	1 mM Cu	3	024	Marri	1 mM Cu	3

Following completion of exposure, all honey samples were returned to room temperature and analysed in accordance with the methods listed in Table 6.

Table 6 List of analyses and methods performed on honey samples.

Analyte	Method Code	Method Description
Moisture	ORG401	Moisture in honey by refractometry
pH	ORG403	pH/Free Acidity in honey
Free Acidity	ORG403	pH/Free Acidity in honey
H_2O_2	ORG171	Determination of Hydrogen Peroxide by Spectrophotometric Method
Total Phenolics	ORG426	Total polyphenolic analysis in honey

6.3. Results and discussion

A brief discussion regarding each of the parameters is included below. Where results are presented graphically, error bars represent the experimentally determined measurement uncertainty of the analytical method.

6.3.1. Moisture

Both Mallee and Marri honey exhibited minimal variation in moisture content across the various exposure conditions, with all results within ±0.2% of the control samples, determined at 15.6% moisture in Mallee and 17.8% in Marri. Overall, no effect was observed on moisture

content.

6.3.2. pH

Both Mallee and Marri honey exhibited no variation in pH, with control samples for both varieties recorded as approximately 4.0 across the various exposure conditions. Overall, no effect was observed on pH.

6.3.3. Free acidity

Both Mallee and Marri honey exhibited minor variation in free acidity during the storage period. Overall, a small increase in free acidity was observed in all Marri honey samples, with both Cu and Zn spiked samples exhibiting higher levels than the control (Figure 17A). Conversely, Mallee honey exhibited a slight decrease overall in free acidity, with Cu spiked samples exhibiting lower levels than the control (Figure 17B). However, all variations observed in free acidity fall within the uncertainty limits for the method and consequently no definitive effect in free acidity was observed over time in honey samples which had been spiked with metal ions.

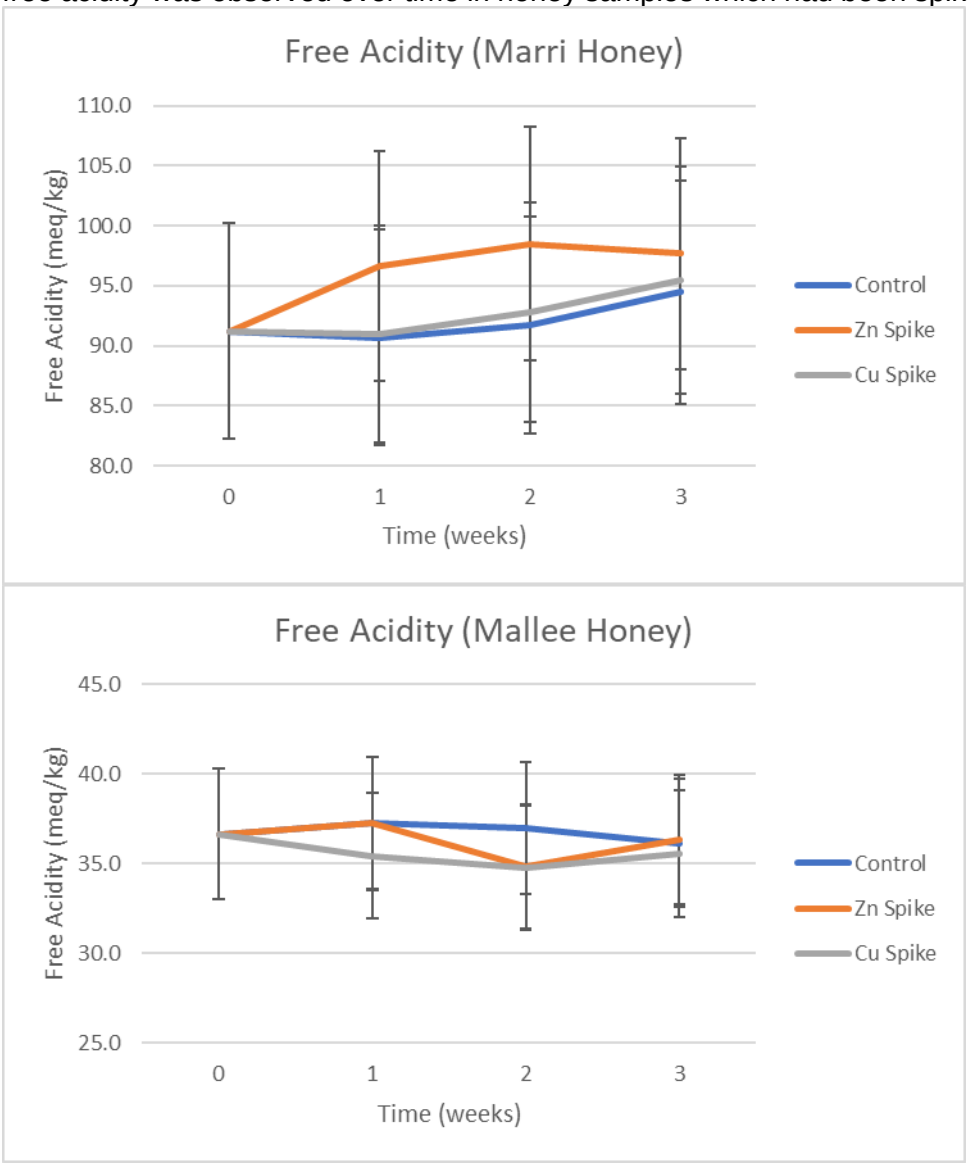

Figure 17A (top) and 17B (bottom): Free acidity of honey following spiking with metal ions.

6.3.4. Hydrogen peroxide

Both Mallee and Marri honey exhibited minor variation in hydrogen peroxide content during the storage period. Marri honey exhibited an overall increase in hydrogen peroxide content, with reduced production of hydrogen peroxide in samples spiked with metal ions (Figure 18A).

Hydrogen peroxide content in Mallee honey remained relatively stable during the exposure period, with both spiked samples generally exhibiting lower hydrogen peroxide content than the control (Figure 18B). Overall, the presence of spiked metal ions appears to have resulted in a minor decrease in hydrogen peroxide content in both honey varieties.

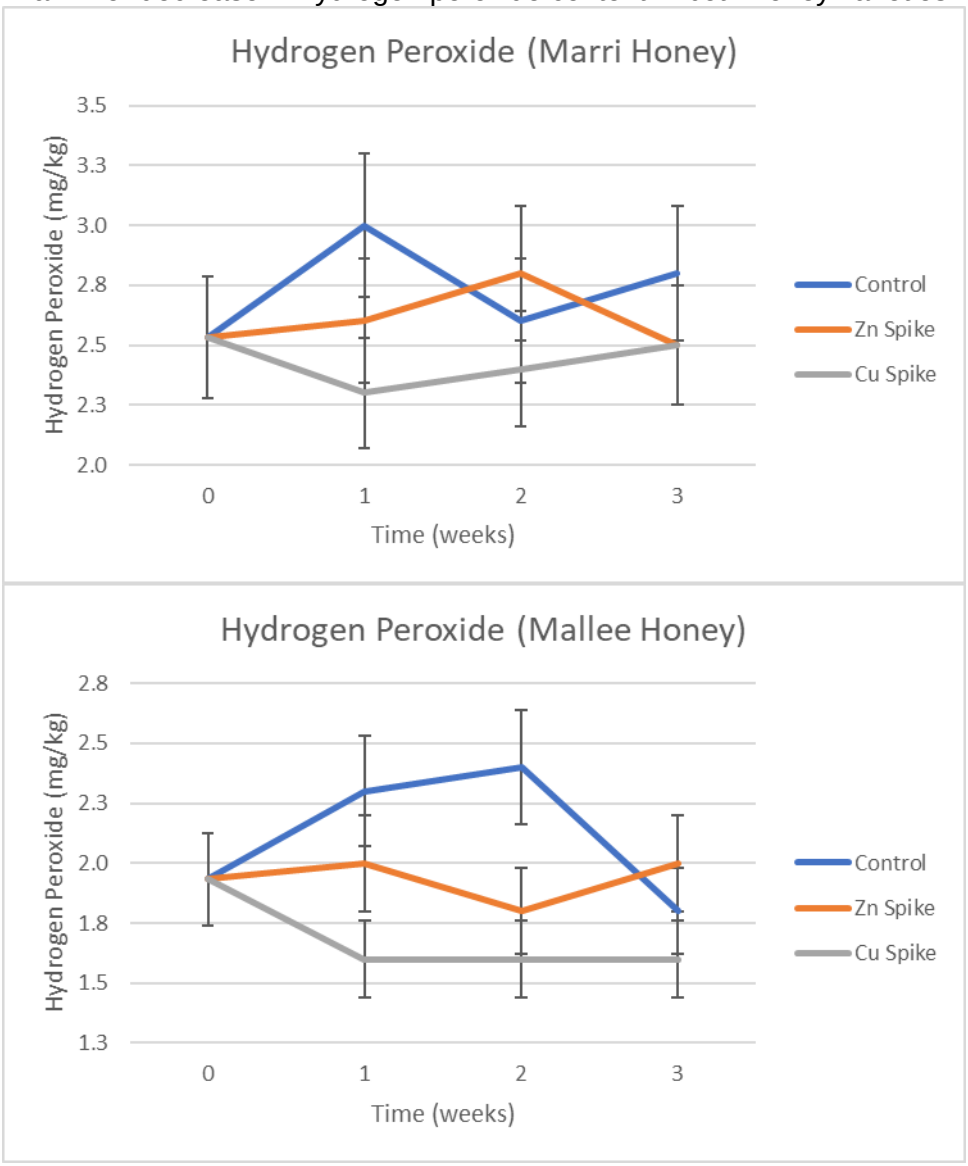

Figure 18A (top) and 18B (bottom): Hydrogen peroxide in honey following spiking with metal ions.

6.3.5. Total phenolics

Total phenolic content of Marri honey was observed to be relatively stable over time for the control and metal ion spiked samples (Figure 19A). The Mallee honey control exhibited a similar stability, though both metal ion spiked samples were determined to have a slightly lower phenolic content, with Cu spiked samples having the lowest phenolic content (Figure 19B). Overall, the effect of metal ions on phenolic content appears dependent upon the variety of honey, with Cu inhibiting phenolic production in Mallee honey but having no discernible effect on Marri honey.

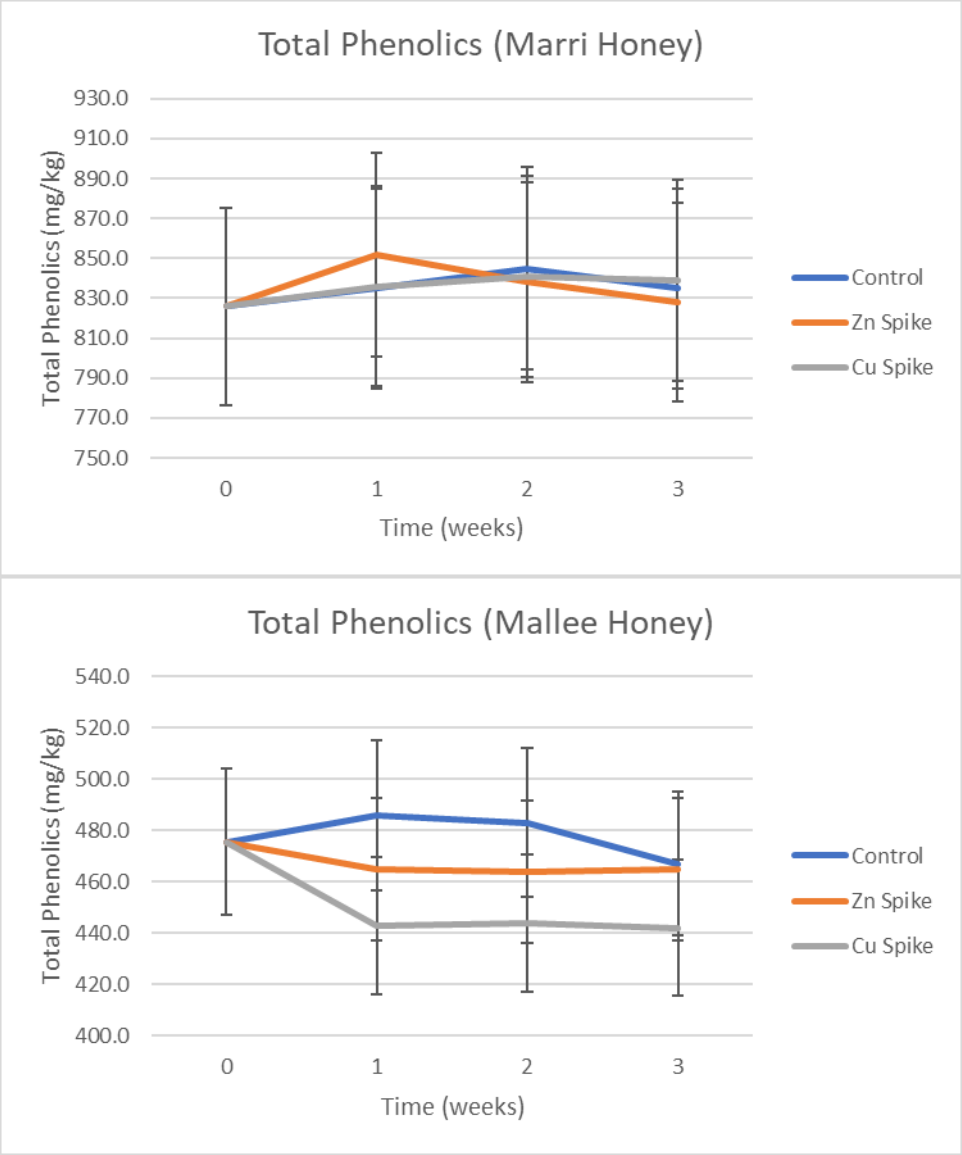

Figure 19A (top) and 19B (bottom): Total phenolic content in honey following spiking with metal ions.

6.4. Conclusion

A short-term storage trial investigating the effect of spiking honey with metal ions has been completed. Literature indicates that the presence of specific metal ions may either inhibit (e.g., Cu^{2+}) or enhance (e.g. Zn^{2+}) glucose oxidase activity[1].

In practice, Mallee and Marri honey spiked with Cu resulted in decreased hydrogen peroxide content, consistent with inhibited glucose oxidase activity.

However, increased activity was not observed when Zn was added. In addition to the effect on hydrogen peroxide content, Mallee honey spiked with Cu exhibited lower total phenolic content and lower free acidity than equivalent control honey.

No effect was observed on the total phenolic content of Marri honey, with free acidity in Marri honey increasing with the presence of the metal ions. Despite the small sample size, the presence of copper in honey does exhibit characteristics consistent with inhibition of the glucose oxidase enzyme.

Consequently, it is possible that the copper content of honey may have an effect on the hydrogen peroxide, and in turn the antibacterial properties of the honey. Further investigation, specifically with regards to copper and hydrogen peroxide content in honey, may assist in confirming these initial observations.

7. Long term storage trial 1

7.1. Introduction

A long-term storage trial was initiated to investigate the effect of temperature exposure over time on several honey parameters. Mallee and Marri honey were examined, representing high antimicrobial activity (Marri) and low antimicrobial activity (Mallee) varieties, with Mallee honey commonly used in the laboratory as a control sample with well-defined physico-chemical parameters. Honey was stored at temperatures ranging from 4 °C to 50 °C for up to nine months prior to the determination of eight compositional parameters.

7.2. Method

Mallee (17S3064/001) and Marri (17S3064/002) honey samples were dispensed into a series of small glass hexagonal jars. These honey varieties were selected to represent a high antimicrobial activity honey (Marri) and a low antimicrobial activity honey (Mallee).
Sample jars were grouped in accordance with intended exposure temperature and placed into laboratory ovens for known exposure periods up to nine months. Once exposure was completed, samples were stored at -20 °C until analysis.
Following completion of exposure, all honey samples were returned to room temperature and analysed in accordance with the methods listed in Table 7.

Table 7 List of analyses and methods performed on honey samples.

Analyte	Method Code	Method Description
Moisture	ORG401	Moisture in honey by refractometry
pH	ORG403	pH/Free Acidity in honey
Free Acidity	ORG403	pH/Free Acidity in honey
EC	ORG404	Electrical conductivity in honey
H_2O_2	ORG171	Determination of Hydrogen Peroxide by Spectrophotometric Method
FRAP	ORG423	Antioxidant Activity (FRAP)
Total Phenolics	ORG426	Total polyphenolic analysis in honey
HMF	ORG188F	Carbonyl compounds in honey by LC-MS/MS
Total Sugars	ORG155F	Sugars in food and plant material using liquid chromatography

Three samples of both Mallee and Marri honey were stored at each unique exposure condition. Electrical conductivity, hydrogen peroxide and pH analyses were performed on all three of these samples, while the remaining tests were performed on only one sample from each exposure condition.
For the tests that were performed on all three samples from each exposure condition, the average of the three results was calculated and used for the subsequent data analysis.

7.3. Results and discussion

A brief discussion regarding each of the parameters is included below. Where results are presented graphically, error bars represent the experimentally determined measurement uncertainty of the analytical method.

7.3.1. Moisture

Mallee and Marri honey both exhibited minimal variation in moisture content across the various exposure conditions. All results for Mallee honey were within 4% relative of the control sample result, which was determined at 15.5% moisture. Similarly, all results for Marri honey were

within 3% relative of the control sample result, which was determined at 18.0% moisture.
It was therefore concluded that the temperatures and storage times investigated during this trial had no significant effect on moisture content of either honey. These findings are detailed in Figure 20.

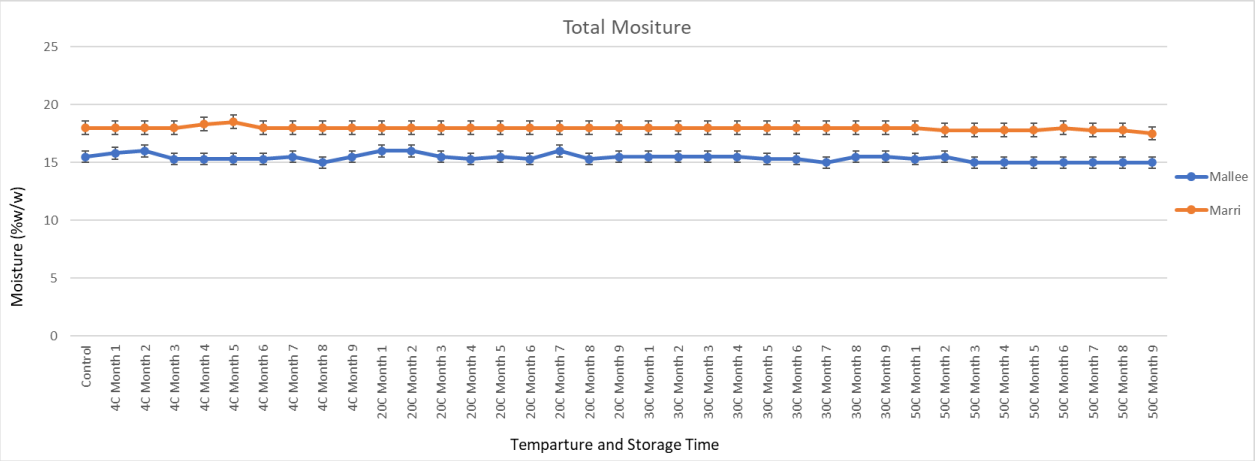

Figure 20 Moisture content in honey following exposure to various temperatures.

7.3.2. pH

Mallee and Marri honey both exhibited minimal variation in pH across the various exposure conditions, with these findings detailed in Figure 21.

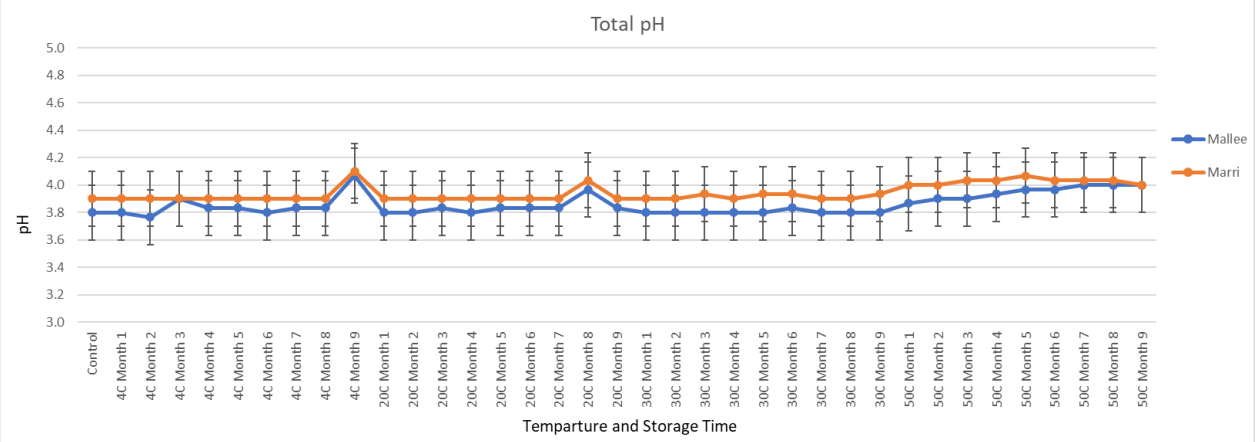

Figure 21 pH in honey following exposure to various temperatures.

Two features of the above graph warrant discussion. Firstly, two minor increases were observed at the data points associated with exposure for 4 °C for 9 months and for 20 °C for 8 months. As these increases were observed in both types of honey with no trends surrounding and were only small increases of approximately 5% relative, they are attributed to measurement uncertainty.
A small gradual increase in the pH of the Mallee honey stored at 50 °C was observed across the nine months of the trial. This increase was not observed in the Marri honey but represents a clear trend of decreasing acidity based upon the nine data points illustrated in Figure 22, with each data point representing the average of triplicate analyses.
Decreasing acidity may be the result of reduction of organic acids to alcohols, and potentially warrants further investigation once an organic acid screening tool becomes available. Consequently, honey pH may increase over time when exposed to elevated temperatures, depending upon honey variety and quantity of organic acids generated.

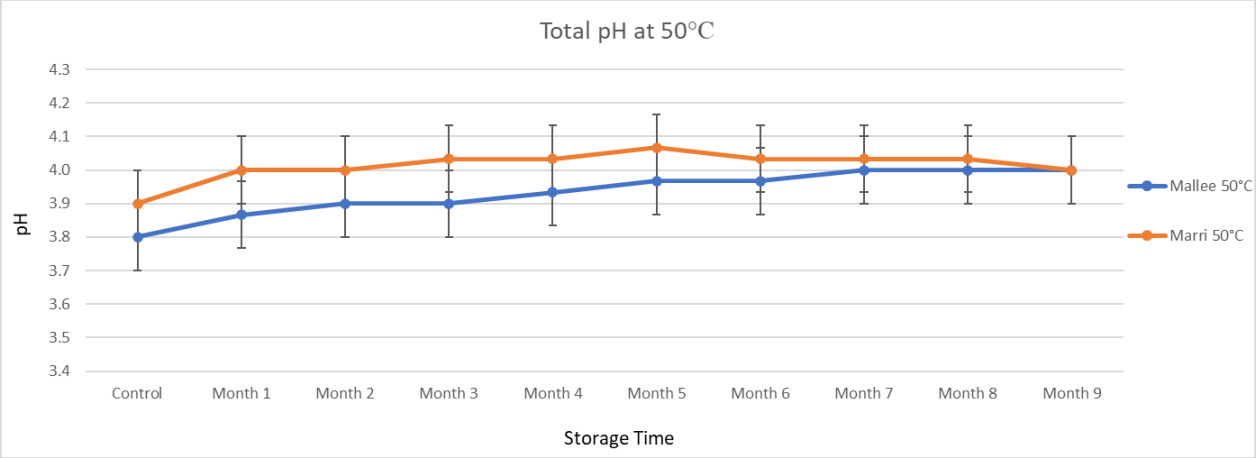

Figure 22 pH in honey following exposure to 50 °C conditions.

7.3.3. Free acidity

Minor variation was observed in the free acidity of Mallee honey across the various storage conditions, as detailed in Figure 23.

A 20% relative decrease in free acidity occurred in samples stored for nine months at 4 °C, correlating with the elevated pH value in those samples detailed previously.

Furthermore, a 14% relative decrease in free acidity was observed in Mallee honey samples stored at 50 °C, though this relative variation was reduced to 5% by the end of the trial (month 9) which would fall below measurement uncertainty of the method (10% relative).

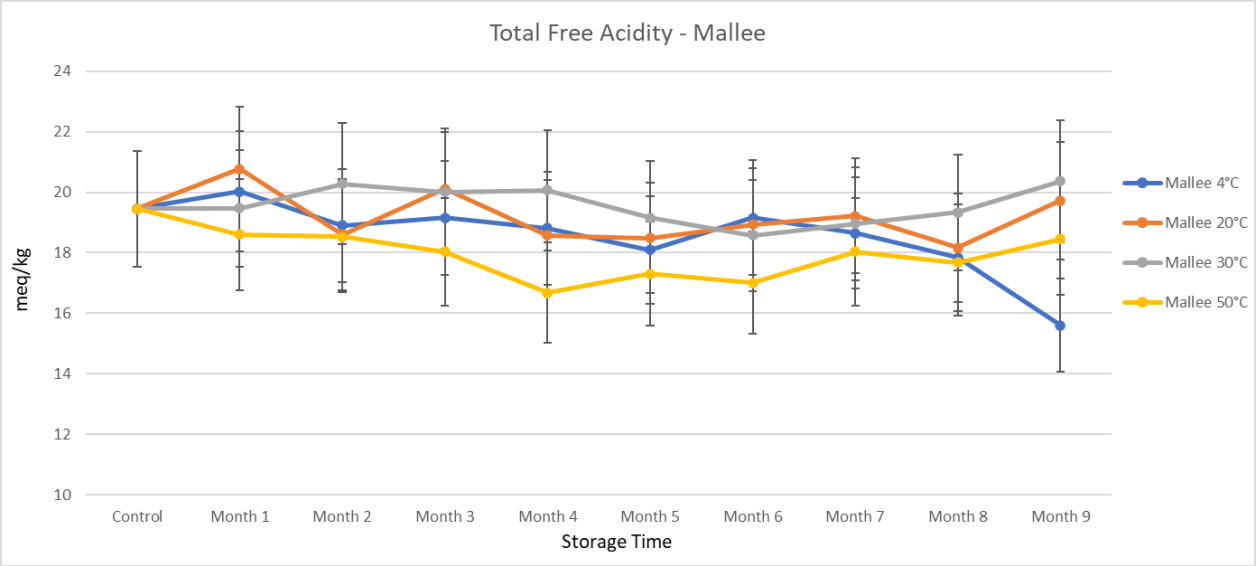

Figure 23 Free acidity in Mallee honey following exposure to various temperatures.

Similar results were observed for free acidity in Marri honey at temperatures of 4 °C, 20 °C and 30 °C. However, at 50 °C, the free acidity decreased approximately 19% compared to the control sample across the first five months of storage, before increasing to a value approximately 10% lower than the control value at the end of the nine months.

This finding is further detailed in Figure 24, though this trend was not reflected in the pH values detailed previously. Consequently, further examination of these samples may be warranted, with a particular focus on organic acids production, to better understand this phenomenon.

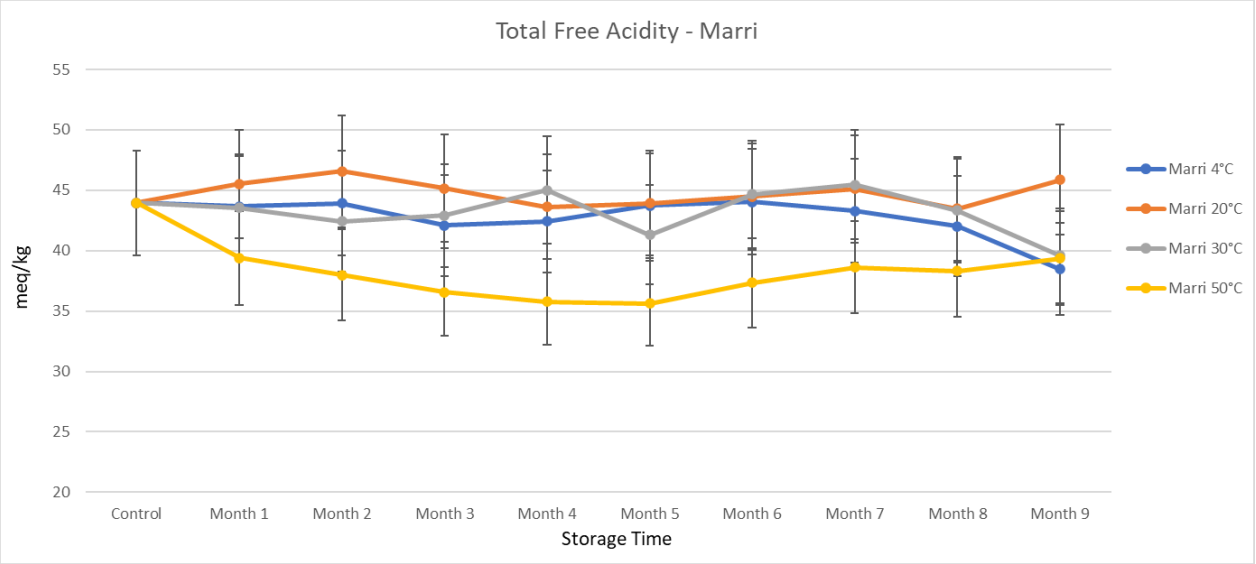

Figure 24 Free acidity in Marri honey following exposure to various temperatures.

7.3.4. Electrical conductivity

As detailed in Figure 25, the electrical conductivity of both Mallee and Marri honey did not exhibit any significant variation across the various storage conditions.

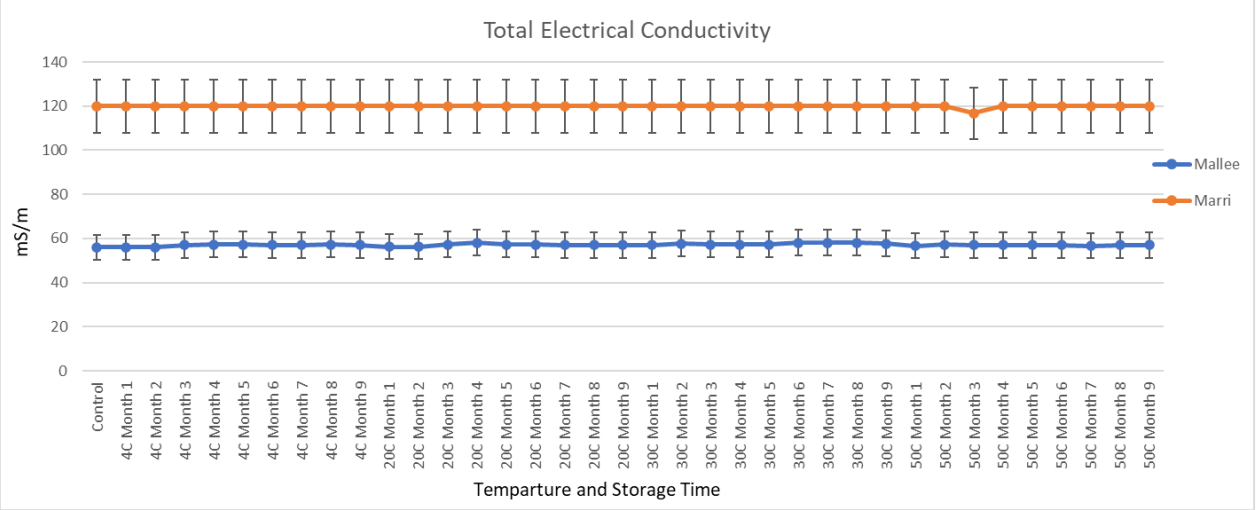

Figure 25 Electrical conductivity in honey following exposure to various temperatures.

7.3.5. Hydrogen peroxide

The hydrogen peroxide concentration varied significantly for the two different types of honey across the various storage conditions.

In Mallee honey, the hydrogen peroxide exhibited no significant variation across the nine months at temperatures of 4 °C, 20 °C and 30 °C. However, at 50 °C, the hydrogen peroxide increased steadily after one month until it was over double the original concentration by the end of nine months, as detailed in Figure 26.

Previous short-term studies (see Section 5) had indicated increasing hydrogen peroxide may occur at elevated temperatures (70 °C) over an intermediate time scale (3 weeks). These results support those initial findings and potentially provide a mechanism by which hydrogen peroxide concentration, and consequently, total activity values for low peroxide-based antimicrobial activity honeys may be increased.

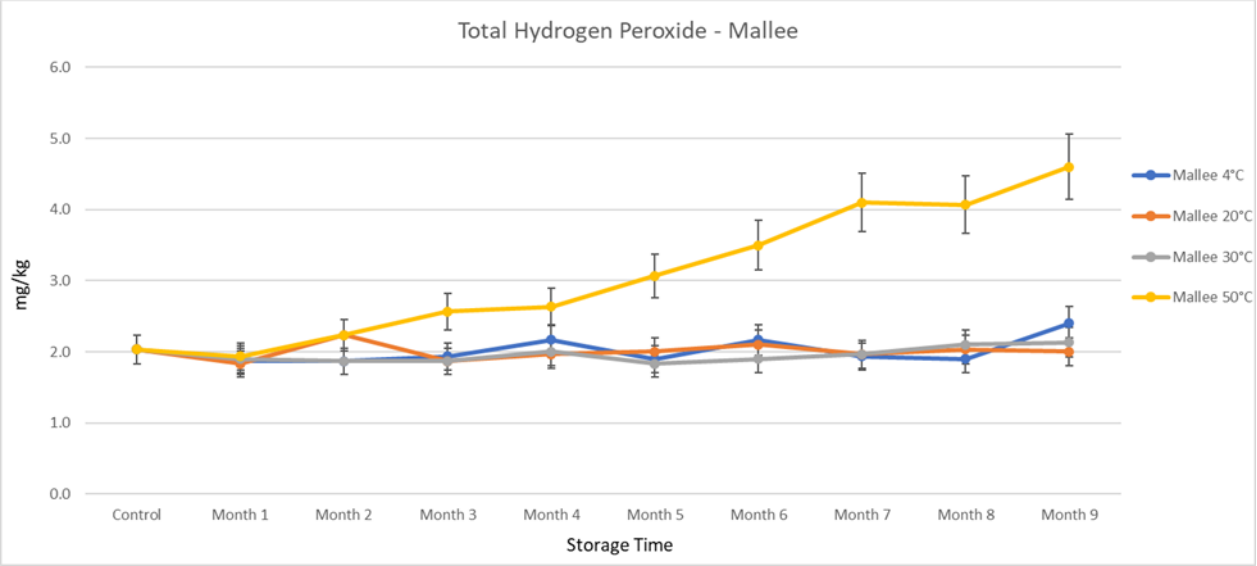

Figure 26 Hydrogen Peroxide in Mallee honey following exposure to various temperatures.

In Marri honey, as detailed in Figure 27, the hydrogen peroxide concentration was more variable across the various storage conditions than in Mallee honey.
This variation was most pronounced at 4 °C, where the hydrogen peroxide increased across the first four months, before decreasing for the next four months, before a large increase in the ninth month. This sharp increase in hydrogen peroxide concentration at month nine is supported by replicate analyses, however this is contrary to results detailed in the literature[2].
Literature studies[3] demonstrate a decrease in peroxide-based antimicrobial activity, and the associated hydrogen peroxide concentrations, in honey stored at 4 °C over time.
At 20 °C and 30 °C, hydrogen peroxide concentrations fluctuated, but were similar to the control sample after nine months.
At 50 °C, the hydrogen peroxide concentration decreased significantly over the first month and then steadily increased until the end of the ninth month, such that the final concentration was approximately 30% higher than the control sample. Consequently, it appears that elevated temperature conditions support increased hydrogen peroxide formation in honey over time.
This result is supported by the optimum temperature for glucose oxidase activity, the enzyme responsible for catalysing the oxidation of D-glucose creating hydrogen peroxide as a by-product. Literature indicates the optimal temperature for the enzyme is 40 °C, however temperatures up to 50 °C have similarly high activity[4]. Therefore, by optimising the temperature for glucose oxidase activity, hydrogen peroxide production and associated antimicrobial activity could potentially also be optimised.

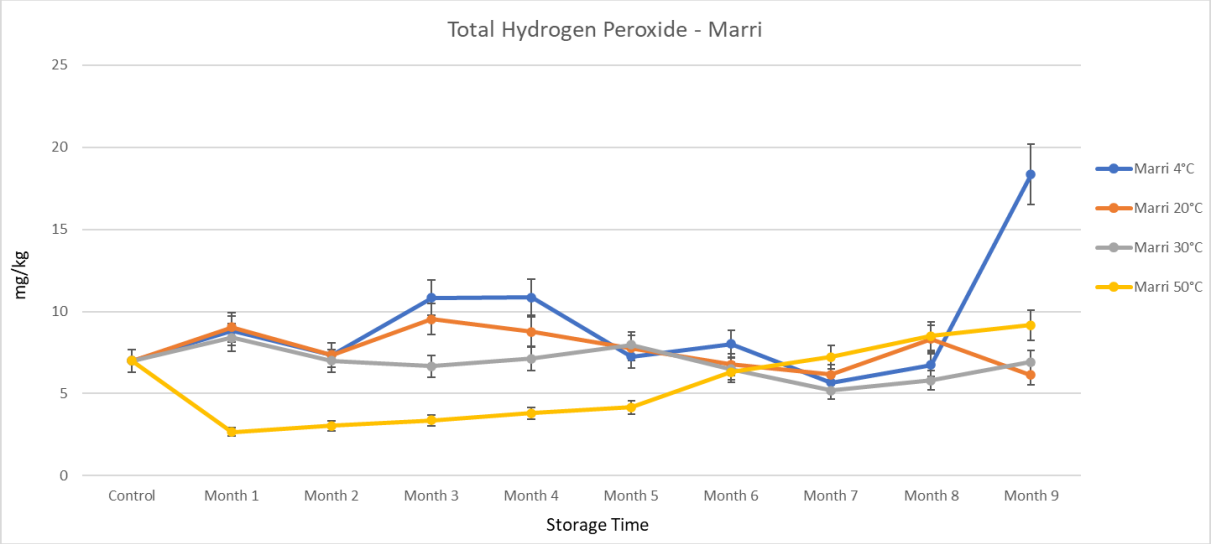

Figure 27 Hydrogen peroxide in Marri honey following exposure to various temperatures.

7.3.6. HMF

At 4 °C, the HMF content of both Mallee and Marri honey exhibited no significant variation over the nine months.

At 20 °C, the HMF content of Mallee and Marri honey increased steadily over the nine months, to values approximately 40% higher than the control sample for Mallee honey and 25% higher than the control sample for Marri honey. These findings are detailed in Figure 28.

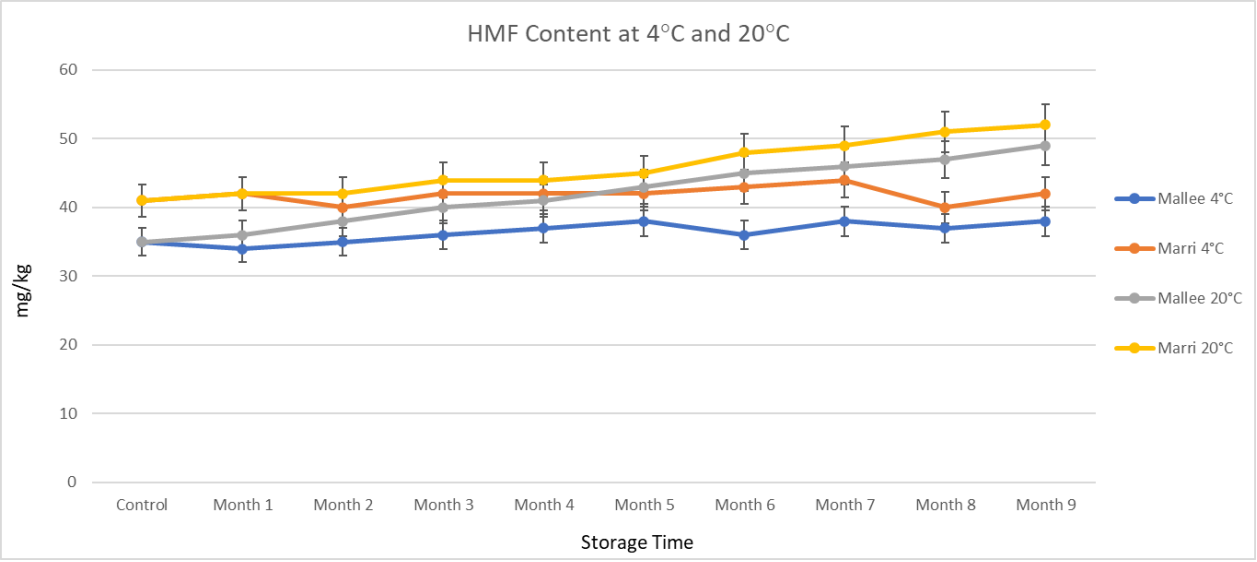

Figure 28 HMF content in honey following storage at 4 °C and 20 °C.

At 30 °C and 50 °C, the HMF content also increased over the nine months, but to a much greater extent than at 20 °C, with the rate of increase being proportional to the storage temperature. These findings are detailed in Figure 29 and Figure 30 to best illustrate the extent of the increases at the different temperatures.

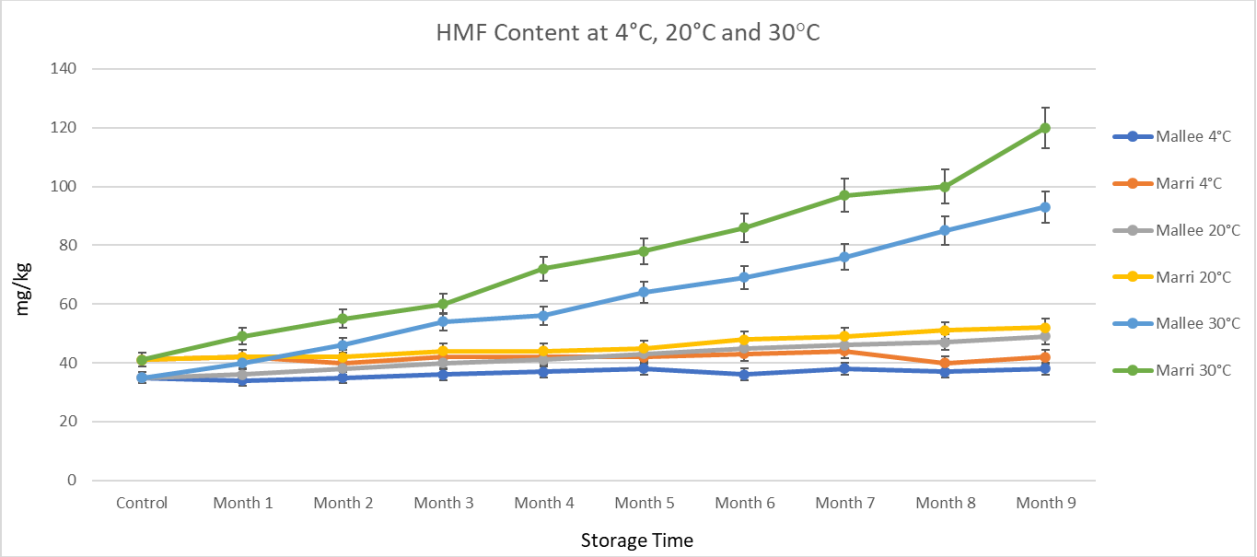

Figure 29 HMF content in honey following storage at 4 °C, 20 °C and 30 °C.

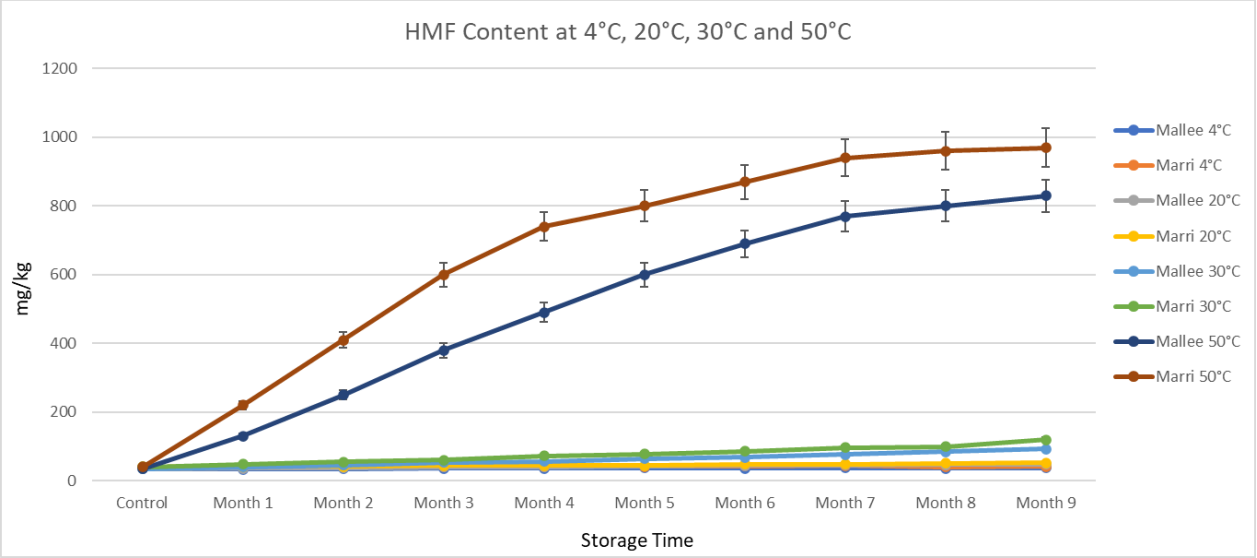

Figure 30 HMF content in honey following exposure to various temperatures.

Maximum HMF values are specified in the *Codex Alimentarius Honey* standard[5] of 40 mg/kg with an allowance of 80 mg/kg for honey originating from countries or regions with tropical ambient temperatures. Similarly, a 2014 submission from the Australian Honey-Bee Industry Council (AHBIC) proposed a maximum limit for Australian honey of 80 mg/kg. These limits should be considered when examining potential storage conditions.

After five months storage at 30 °C or one-month storage at 50 °C, Marri honey would exceed these limits. An equivalent exceedance would be reached for Mallee honey after eight months storage at 30 °C or one-month storage at 50 °C.

This indicates care in storage is critical to the maintenance of honey quality and the ability to market and sell honey internationally. Low temperature storage and handling are critical to HMF management in honeys.

7.3.7. Total phenolics

At 4 °C and 20 °C, the phenolic content of both Mallee and Marri honey exhibited no significant variation over nine months.

At 30 °C, the phenolic content of both Mallee and Marri honey steadily increased over nine

months, with an increase of approximately 10% for the Mallee honey and 25% for the Marri honey over the duration of the trial.
However, at 50 °C, the phenolic content of both honey varieties more than trebled over nine months. These findings are detailed in Figure 31 and Figure 32. These significant increases in total phenolics are likely attributable to the hydrolysis of bound phenolics over time at elevated temperatures in the acidic honey matrix, increasing their availability.

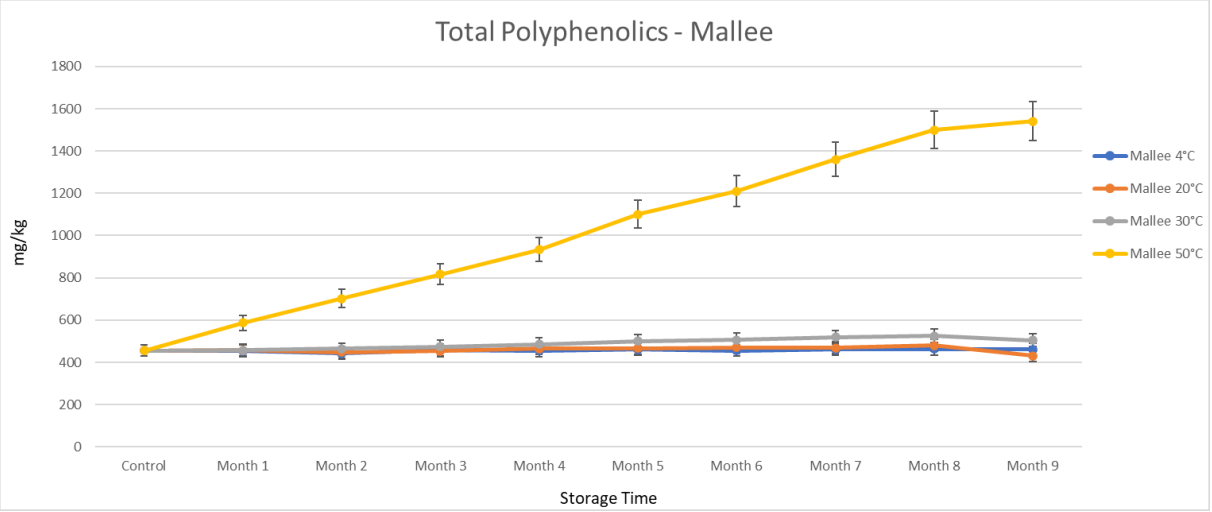

Figure 31 Phenolic content of Mallee honey following exposure to various temperatures.

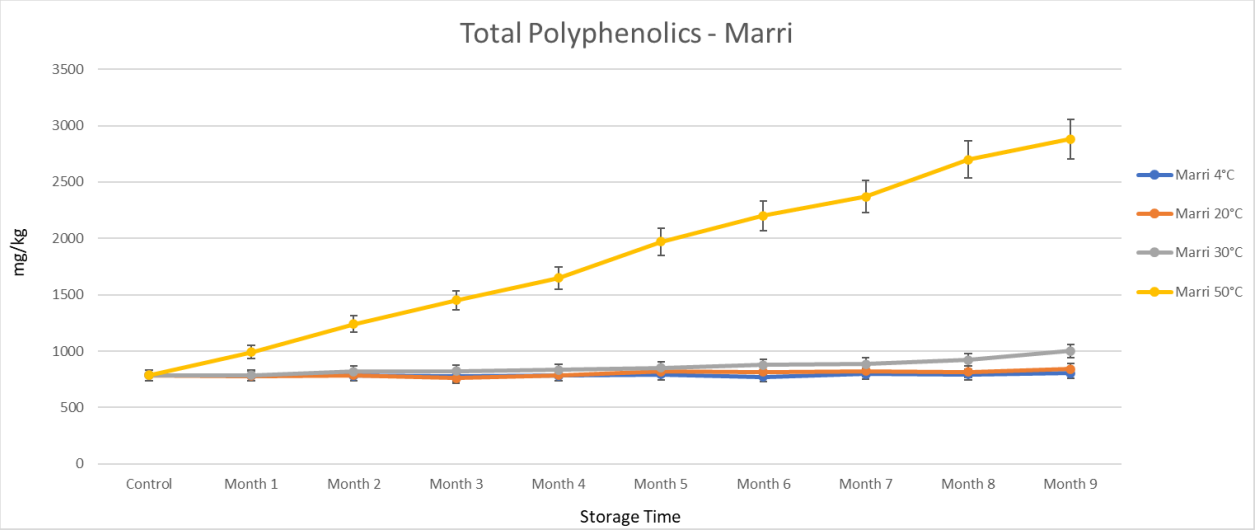

Figure 32 Phenolic content in Marri Honey following exposure to various temperatures.

7.3.8. FRAP

FRAP, as a measure of antioxidant activity, varied significantly for the two different types of honey across the various storage conditions. In Mallee honey, FRAP exhibited no significant variation across the nine months at temperatures of 4 °C, 20 °C and 30 °C. However, at 50 °C, FRAP increased over nine months, with the final activity being more than triple that of the control sample. These finding are detailed in Figure 33.

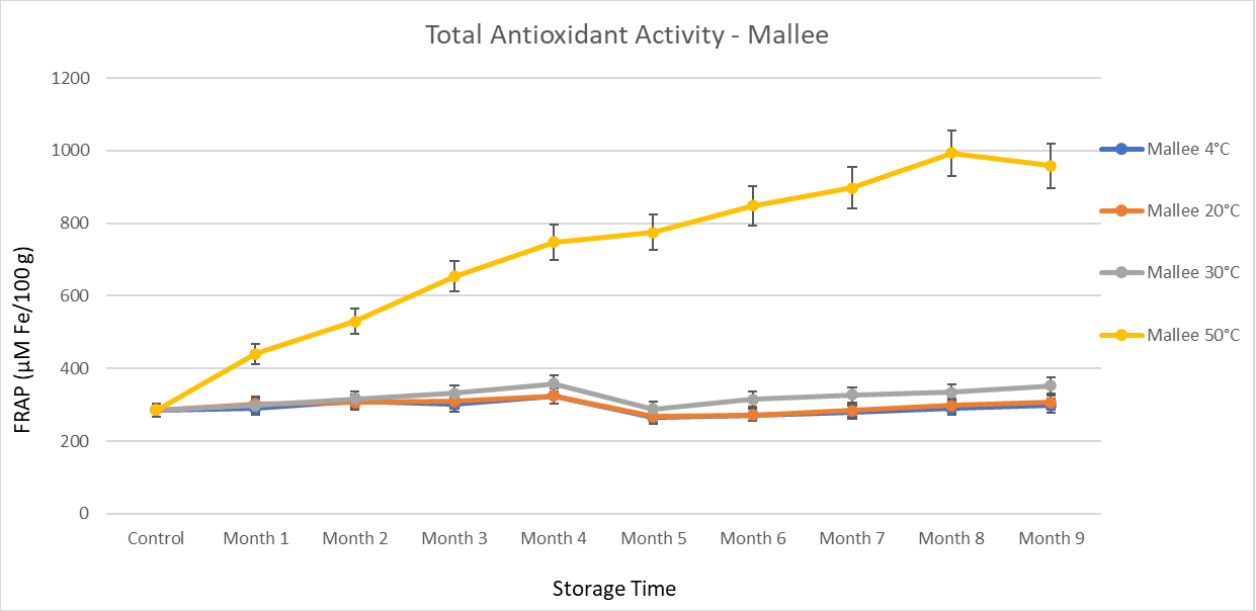

Figure 33 Antioxidant activity (FRAP) in Mallee honey following exposure to various temperatures.

In Marri honey, antioxidant activity was far more variable across the various storage conditions than in Mallee honey. This variation was most pronounced at 50 °C, where the activity fluctuated over nine months, eventually increasing to approximately 20% higher than the control sample.
In contrast, at 4 °C, 20 °C and 30 °C, antioxidant activity decreased approximately 35% over the first month, prior to a prolonged period of stability. However, at 30 °C, the activity remained stable until the end of month eight, before almost doubling over the remaining month. These findings are detailed in Figure 34.

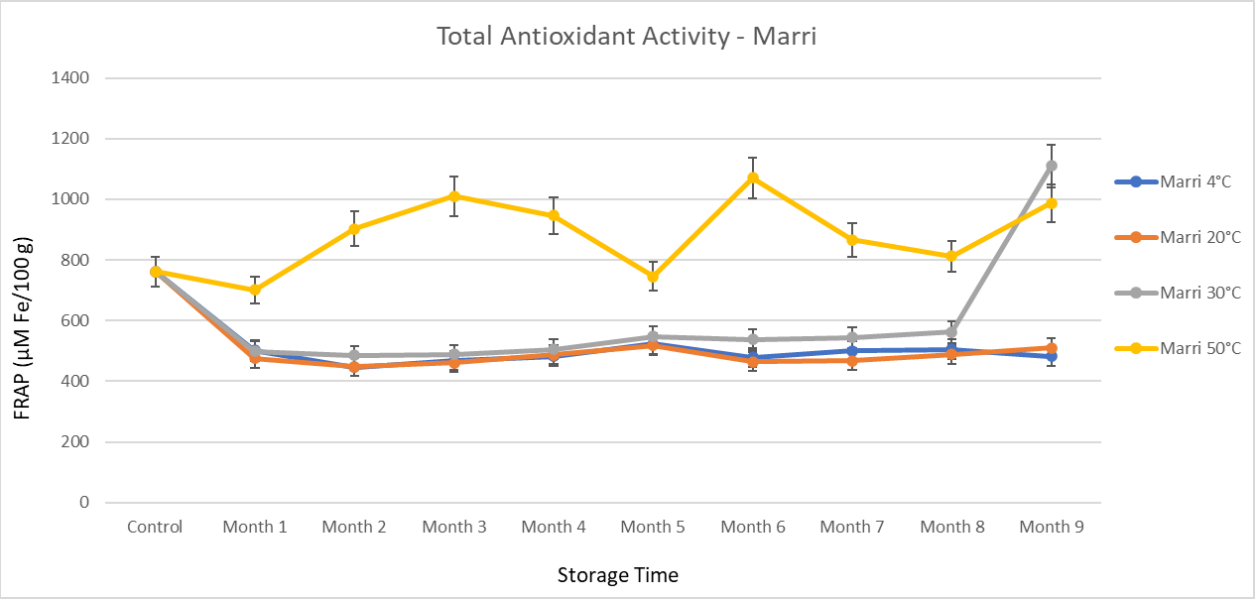

Figure 34 Antioxidant activity (FRAP) in Marri honey following exposure to various temperatures.

The FRAP results are consistent with the total phenolic results detailed previously, particularly with regards to honey samples stored at 50 °C. These findings are consistent with literature studies[6] which indicate that the antioxidant capacity of honey is largely attributable to the

presence of phenolic compounds.

7.4. Conclusion

A long-term storage trial investigating the effect of storage temperature on chemical characteristics of honey has been completed. It was observed that the parameters of moisture and electrical conductivity exhibited no significant variation following storage at temperatures ranging from 4 °C to 50 °C for up to nine months.

Free acidity and pH exhibited minor variation at elevated storage temperatures, potentially due to the reduction of organic acids into alcohols. In contrast, hydrogen peroxide, HMF, FRAP and phenolics exhibited significant variation over the storage period, with this variation more pronounced at elevated temperatures.

Based upon these results, it may be possible to improve the potential health benefits of certain honey varieties by increasing their antimicrobial activity (elevated hydrogen peroxide results) and antioxidant activity (elevated FRAP and total phenolic results).

However, it should be noted that doing so will significantly increase HMF values beyond acceptable levels for consumption, though would not prevent them from being used for topical applications.

8. Long term storage trial 2

8.1. Introduction

A second long-term storage trial was initiated to investigate the effect of temperature exposure over time on several physico-chemical parameters in Jarrah honey. Honey was stored at temperatures ranging from 4 °C to 50 °C for up to seven months prior to the determination of eight compositional parameters.

8.2. Method

Jarrah and Mallee honey samples were dispensed into a series of small glass hexagonal jars. Sample jars were grouped in accordance with intended exposure temperature and placed into laboratory ovens for known exposure periods up to seven months. Once exposure was completed, samples were stored at -20 °C until analysis.

Following completion of exposure, all honey samples were returned to room temperature and analysed in accordance with the methods listed in Table 8.

Table 8 List of analyses and methods performed on honey samples.

Analyte	Method Code	Method Description
Moisture	ORG401	Moisture in honey by refractometry
pH	ORG403	pH/Free Acidity in honey
Free Acidity	ORG403	pH/Free Acidity in honey
EC	ORG404	Electrical conductivity in honey
H_2O_2	ORG171	Determination of Hydrogen Peroxide by Spectrophotometric Method
FRAP	ORG423	Antioxidant Activity (FRAP)
Total Phenolics	ORG426	Total polyphenolic analysis in honey
Oligosaccharides	ORG181	Fructo-oligosaccharides in honey
Total Activity	Outsourced	Testing outsourced to University of Western Australia

Three samples of each honey were stored at each unique exposure condition. Electrical conductivity, hydrogen peroxide and pH analyses were performed on all three of these samples, while the remaining tests were performed on only one sample from each exposure

condition.
For the tests that were performed on all three samples from each exposure condition, the average of the three results was calculated and used for the subsequent data analysis.

8.3. Results and discussion

A brief discussion regarding each of the parameters is included below. Where results are presented graphically, error bars represent the experimentally determined measurement uncertainty of the analytical method.

8.3.1. Moisture

Jarrah honey exhibited minimal variation in moisture content across the various exposure conditions. All results for Jarrah honey were within 2% relative of the control sample result, which was determined at 16.5% moisture.
It was therefore concluded that the temperatures and storage times investigated during this trial had no significant effect on moisture content of Jarrah honey, supporting the results from the previous trial. These findings are detailed in Figure 35.

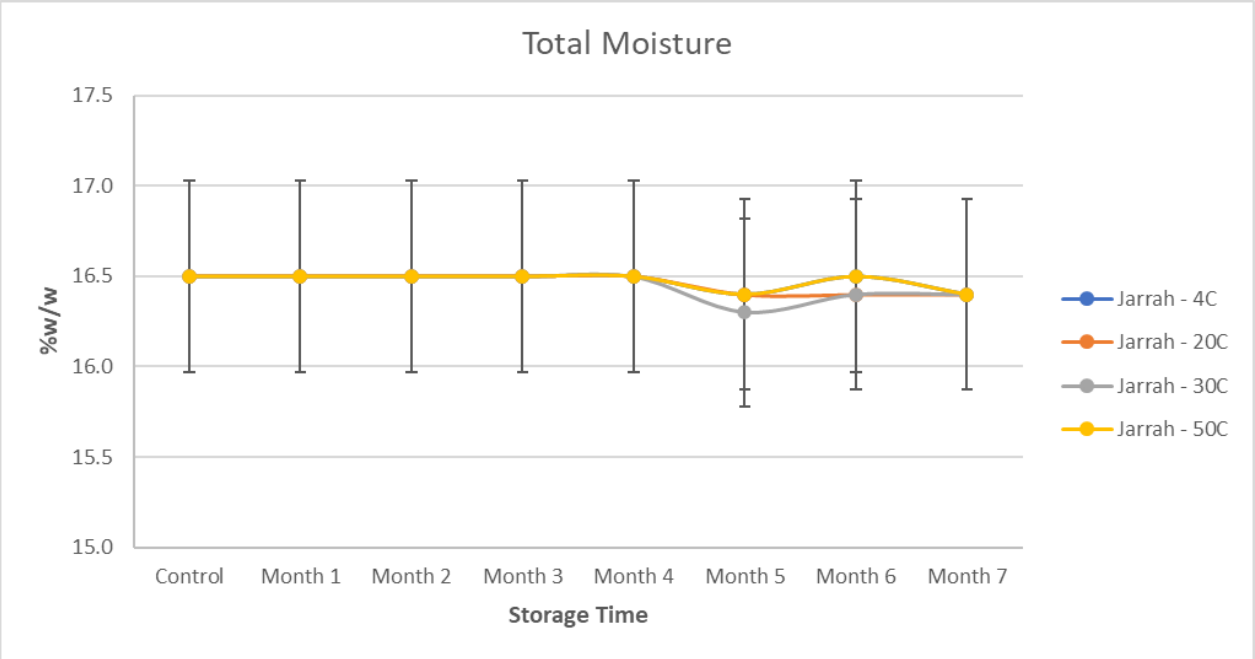

Figure 35 Moisture content in Jarrah honey following exposure to various temperatures.

8.3.2. pH

Jarrah honey exhibited minimal variation in pH across the various exposure conditions, with these findings detailed in Figure 36.

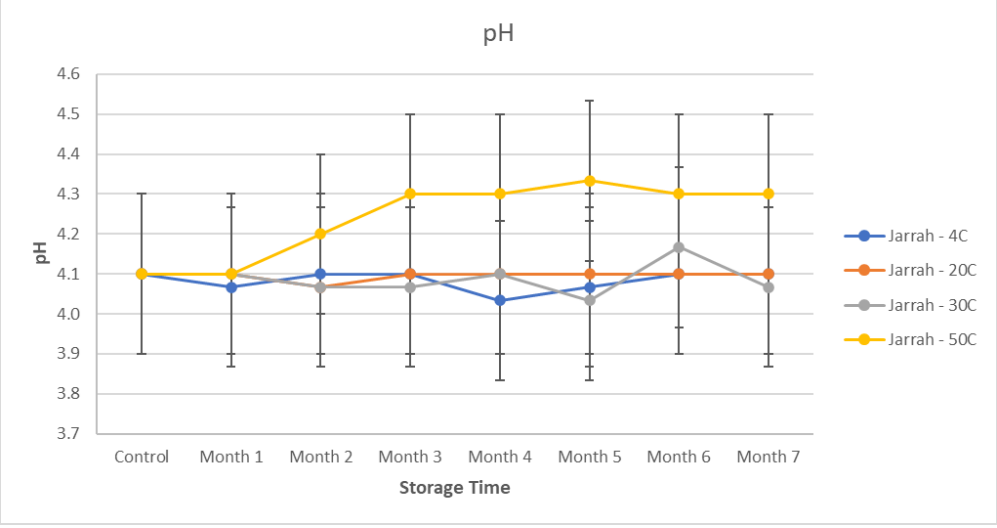

Figure 36 pH in Jarrah honey following exposure to various temperatures.

As observed in the previous long term storage trial a minor increase in pH has again been observed in honey stored at 50 °C, with the decreasing acidity potentially associated with loss of organic acids as discussed previously.

8.3.3. Free acidity

Minor variation was observed in the free acidity of Jarrah honey across the various storage conditions, as detailed in Figure 37. Variations observed for honey stored at temperatures of 4 °C, 20 °C and 30 °C were all minimal and within the measurement uncertainty of the method (10% relative). However, a decrease in free acidity was observed for Jarrah stored at 50 °C, with these results supporting the pH increase illustrated in Figure 36. As with the previous long term storage trial, further investigation regarding organic acids will potentially assist in understanding these results.

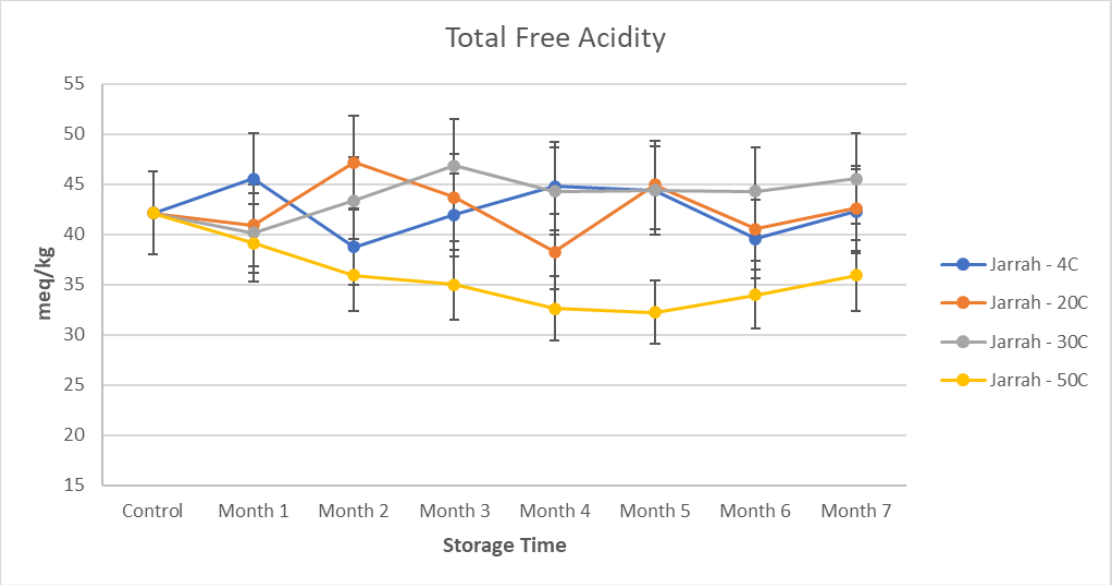

Figure 37 Free acidity in Jarrah honey following exposure to various temperatures.

8.3.4. Electrical conductivity

As detailed in Figure 38, the electrical conductivity of Jarrah honey did not exhibit any significant variation across the various storage conditions.

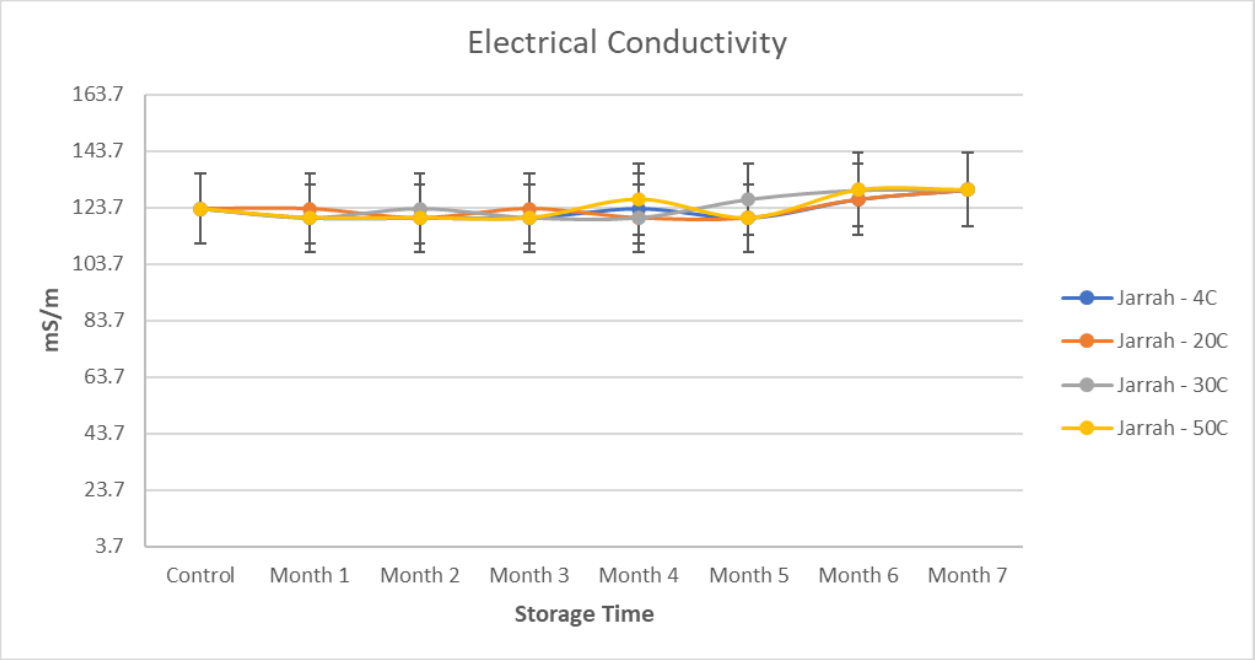

Figure 38 Electrical conductivity in Jarrah honey following exposure to various temperatures.

8.3.5. Hydrogen peroxide

Jarrah honey exhibited a significantly higher hydrogen peroxide content (152 mg/kg) compared to the Mallee and Marri honeys (<10 mg/kg) previously analysed. This discrepancy in hydrogen peroxide levels can be attributed to the relative "freshness" of Jarrah honey at the commencement of the long-term storage trial, with the bulk sample harvested only a short time prior to initiation of the trial.

Variation in hydrogen peroxide levels of Jarrah honey during storage is illustrated in Figure 39. Jarrah honey stored at temperatures of 4 °C, 20 °C and 30 °C remained relatively stable through the first five months of storage, with any variation predominantly attributable to measurement uncertainty (10% relative). During months 6 and 7, a decrease in hydrogen peroxide levels was observed, with a larger drop in hydrogen peroxide levels observed in samples stored at 30 °C compared to lower temperatures.

In contrast to the results detailed above, a significant decrease in hydrogen peroxide levels of Jarrah honey was observed within one month of storage at 50 °C, reaching 6 mg/kg by the second month. Consequently, storage of Jarrah honey at elevated temperatures has the potential to negatively impact hydrogen peroxide production significantly.

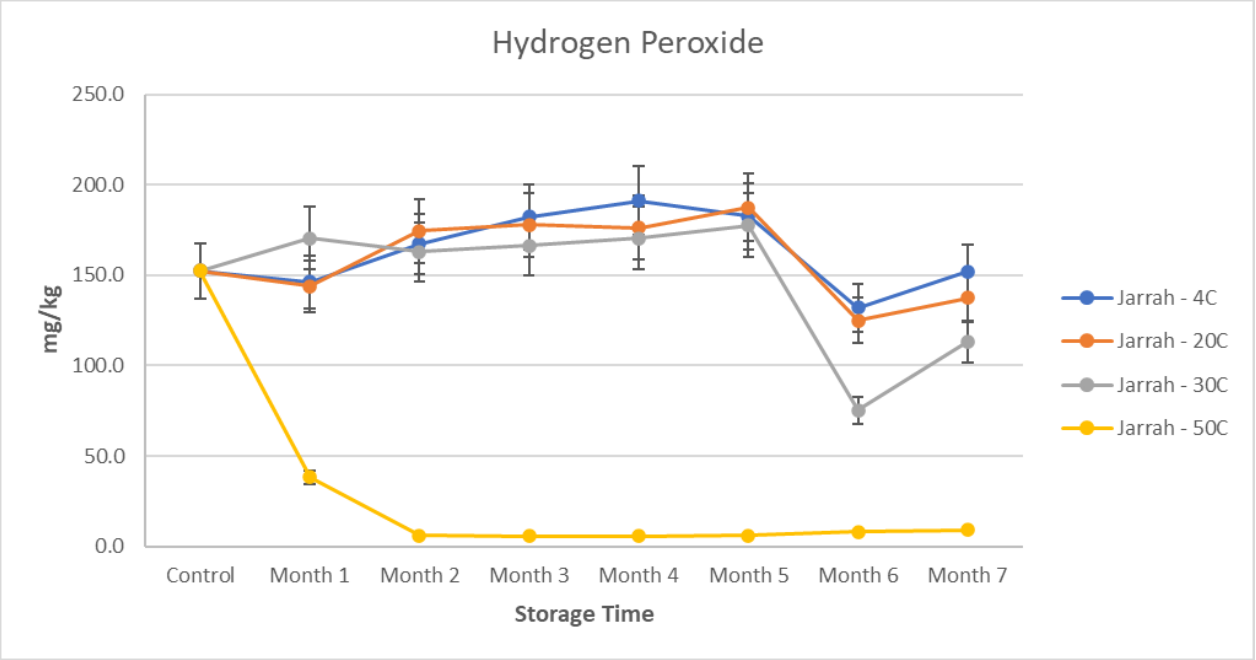

Figure 39 Hydrogen Peroxide in Jarrah honey following exposure to various temperatures.

The previous long term storage trial indicated that hydrogen peroxide levels may increase at elevated storage temperatures. However, when the starting material has a high level of hydrogen peroxide, the opposite effect occurred. Consequently, while elevated storage temperatures may have a slight, positive impact on hydrogen peroxide levels of low antimicrobial activity honey, they have a significant detrimental effect on high activity honey.

8.3.6. Total antimicrobial activity

The effect of storage temperature on the total antimicrobial activity of Jarrah honey is illustrated in Figure 40. The trend across the total activity results is similar to those observed with hydrogen peroxide (Figure 39). These similarities are to be expected given that hydrogen peroxide is recognised as the active ingredient with regards to antimicrobial activity in Jarrah honey. These results demonstrate that total antimicrobial activity will decrease over time, even with cold storage with the degradation rate influenced by the storage temperature.

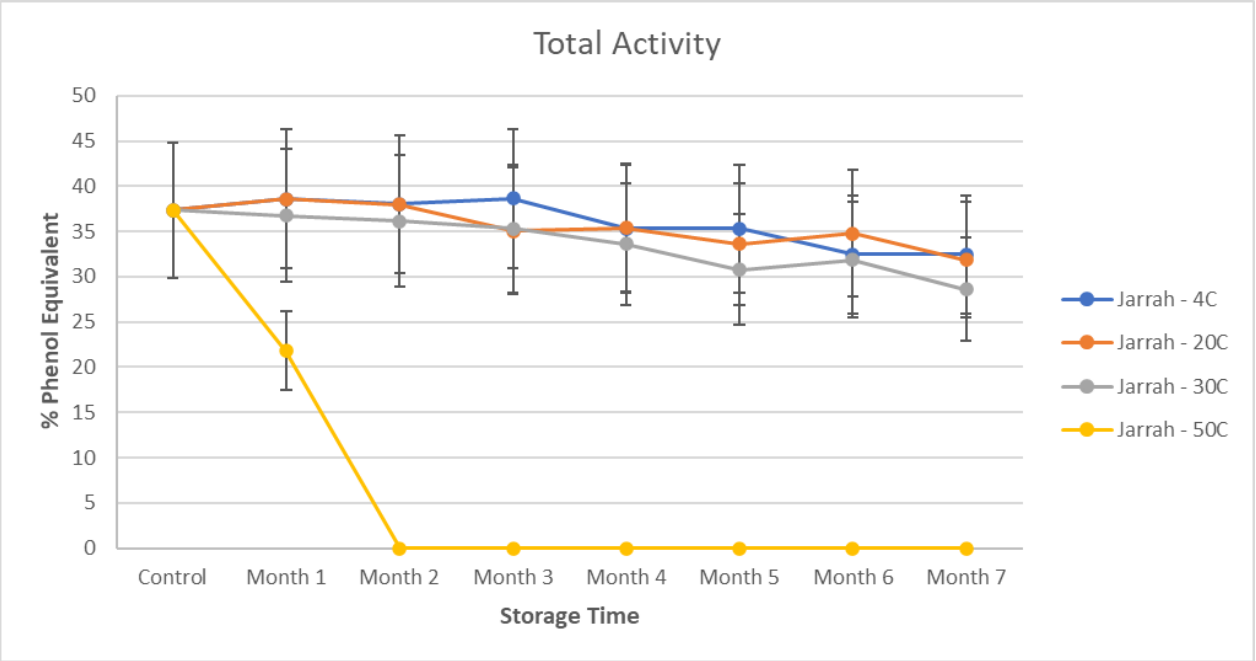

Figure 40 Total Activity of Jarrah honey following exposure to various temperatures.

8.3.7. Oligosaccharides

Fructo-oligosaccharide levels were determined in Jarrah and Mallee honey throughout the seven-month storage trial. The fructo-oligosaccharides were classified based upon their degree of polymerisation (DP), with no samples exhibiting levels of DP5 or DP6 above the limits of reporting (30 mg/kg and 100 mg/kg respectively). DP3 fructo-oligosaccharides in both Jarrah and Mallee honey remained relatively consistent throughout the seven-month storage trial at temperatures up to and including 30 °C. However, at storage temperatures of 50 °C, the levels of DP3 fructo-oligosaccharides steadily decreased (Figure 41). Conversely, levels of DP4 fructo-oligosaccharides were observed to increase over time at elevated temperatures in both Mallee and Jarrah honey (Figure 42).

The decreasing levels of DP3 fructo-oligosaccharides, combined with increasing levels of DP4 fructo-oligosaccharides, may suggest a transfructosylation reaction is occurring at elevated temperatures. This reaction in honey is typically catalysed by the α-glucosidase (invertase) enzyme[7], although invertase activity has been demonstrated to decrease during storage, the enzyme will still remain active in the honey for a significant period of time[8].

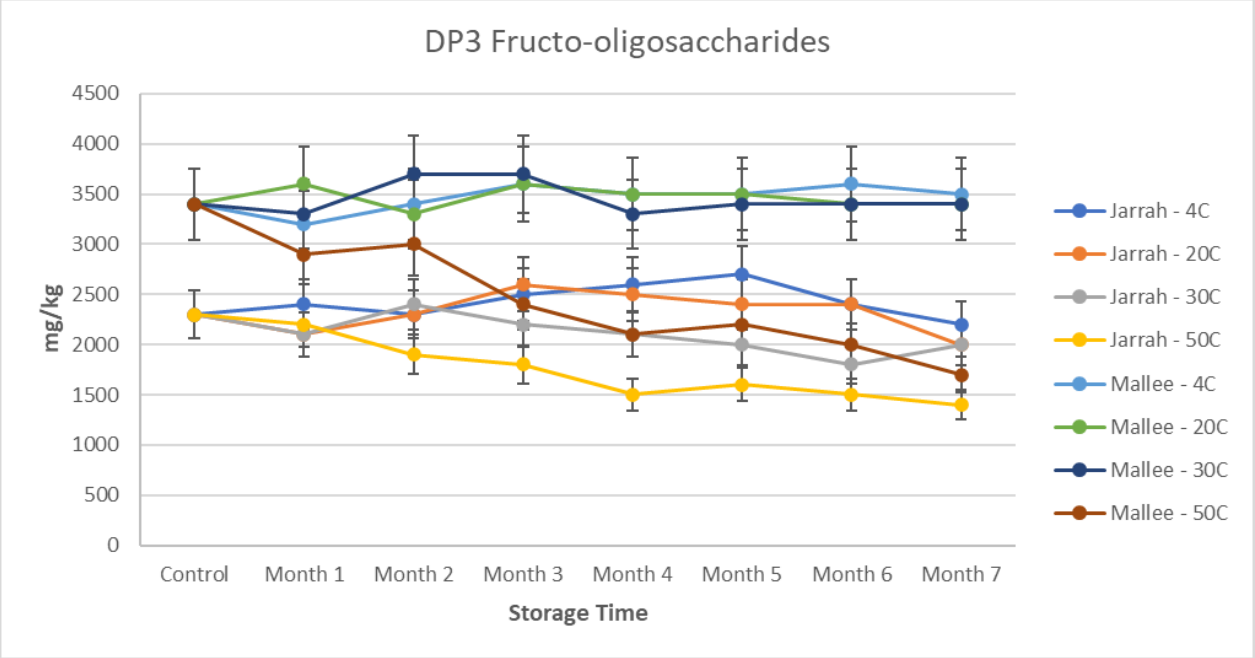

Figure 41 DP3 Fructo-oligosaccharide levels in honey following exposure to various temperatures.

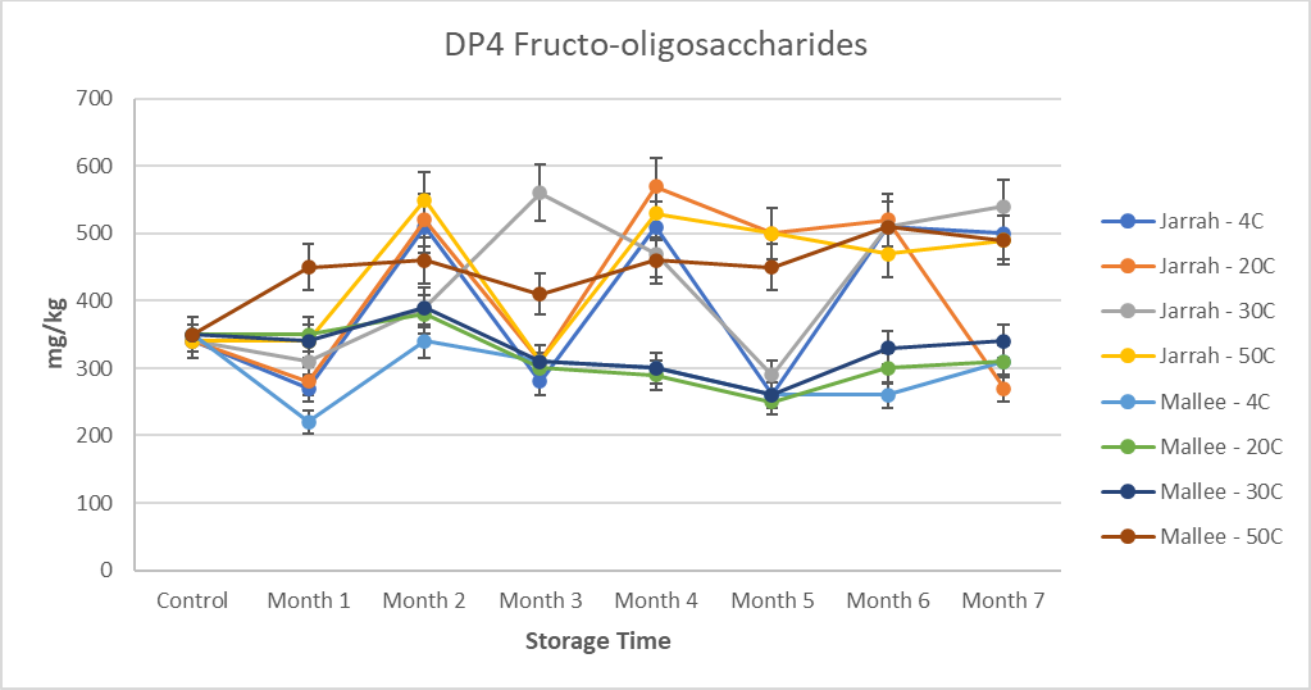

Figure 42 DP4 Fructo-oligosaccharide levels in honey following exposure to various temperatures.

8.3.8. Total phenolics

The total polyphenolic content of Jarrah honey stored at various temperatures is illustrated in Figure 43. At 4 °C and 20 °C, the phenolic content of Jarrah honey exhibited no significant variation over seven months. At 30 °C, the phenolic content of Jarrah honey slightly increased over seven months, with an increase of approximately 10% over the duration of the trial. At 50 °C, the phenolic content of Jarrah honey exhibited a significant increase over the seven-month trial, with results similar to those observed with Mallee and Marri honey in the previous section and likely attributable to the hydrolysis of bound phenolics over time at elevated temperatures in the acidic honey matrix, increasing their availability.

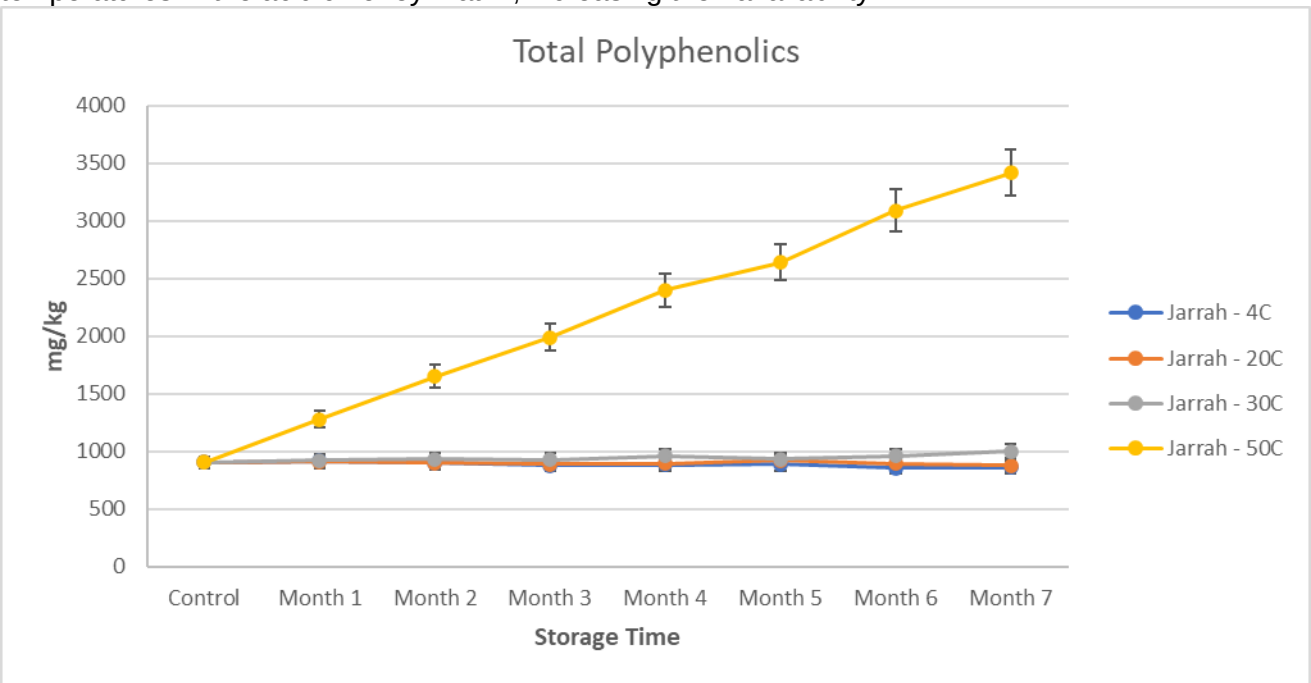

Figure 43 Phenolic content of Jarrah honey following exposure to various temperatures.

8.3.9. FRAP

FRAP was determined in honey as a measure of antioxidant activity during the storage trial. In Jarrah honey, FRAP exhibited no significant variation across the seven months at temperatures of 4 °C, 20 °C and 30 °C. However, at 50 °C, FRAP increased steadily over seven months, with all results illustrated in Figure 44.

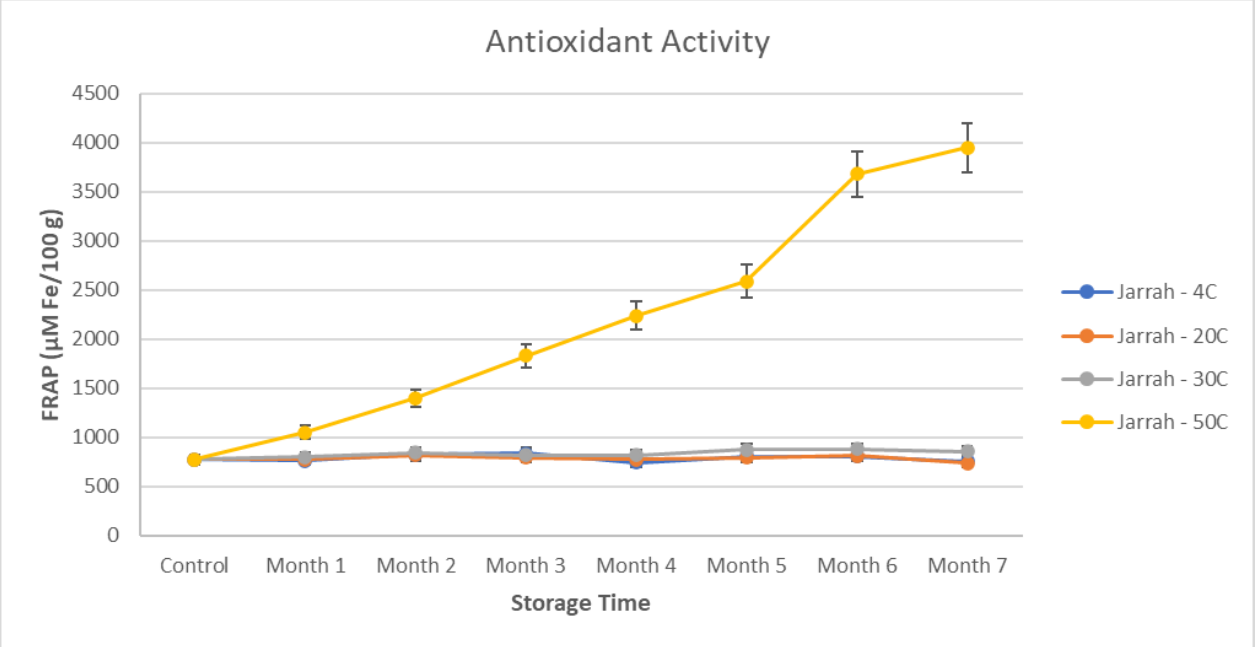

Figure 44 Antioxidant activity (FRAP) in Jarrah honey following exposure to various temperatures.

The FRAP results are consistent with the total phenolic results detailed previously, particularly with regards to honey samples stored at 50 °C. These findings are consistent with literature studies[9] which indicate that the antioxidant capacity of honey is largely attributable to the presence of phenolic compounds.

8.4. Conclusion

A second long-term storage trial investigating the effect of storage temperature on chemical characteristics of Jarrah honey has been completed. It was observed that the parameters of moisture and electrical conductivity exhibited no significant variation following storage at temperatures ranging from 4 °C to 50 °C for up to seven months.

Free acidity and pH exhibited minor variation at elevated storage temperatures, potentially due to the reduction of organic acids into alcohols. Oligosaccharide levels also exhibited minor variation, with elevated temperatures leading to a reduction in DP3 fructo-oligosaccharides and an increase in DP4 fructo-oligosaccharides. In contrast, hydrogen peroxide, total activity, FRAP and phenolics exhibited significant variation over the storage period, with this variation more pronounced at elevated temperatures.

These results once again support the use of heat treatment to improve the antioxidant activity of honey, however doing so in high antimicrobial activity honey such as Jarrah has a significant detrimental effect on total activity due to a reduction in hydrogen peroxide levels. Consequently, this approach should only be considered for low antimicrobial activity honey with consideration of the effects on concomitant or potential increases in HMF.

9. Heat sterilisation

9.1. Introduction

Strict guidelines exist regarding the importation of honey into Western Australia. Sterilisation

of honey products must be performed to protect the industry from diseases found in other States, such as European Foulbrood.
However, this creates an issue for Inspectors from the Department of Primary Industries and Regional Development (DPIRD) who must validate that sterilisation has occurred once honey products are received.
Advice received from DPIRD indicated that the most common sterilisation procedures for honey involved heat treatment under two possible conditions: either maintained at 60 °C for 10 hours or 65 °C for 8 hours.
Consequently, the aim of this trial was to identify if natural chemical markers within the honey could identify that sterilisation had been performed. The trial was designed to monitor honey chemistry affected by heat and examine the changes at two hourly intervals for up to 12 hours.

9.2. Methods

Three varieties of honey were examined, representing a high peroxide-based antimicrobial activity honey (Marri), a low peroxide-based antimicrobial activity honey (Yate) and a high non-peroxide-based antimicrobial activity honey (Manuka).
Following completion of sterilisation, all honey samples were stored frozen prior to returning to room temperature and analysis in accordance with the methods listed in Table 9.
Table 9 List of analyses and methods performed on honey samples.

Analyte	Method Code	Method Description
Diastase	ORG421	Diastase activity in honey
HMF	ORG188F	Carbonyl compounds in honey by LC-MS/MS
Hydrogen Peroxide	ORG171A	Determination of Hydrogen Peroxide by spectrophotometric method
Total Phenolics and Tannins	ORG426	Total polyphenolic analysis in honey
Free Acidity	ORG403	pH/Free Acidity in honey
Total Sugars	ORG155F	Sugars in food and plant material using liquid chromatography

A complete list of samples generated during the trial is detailed in Table 10.

Table 10 List of samples and sterilisation conditions.

Sample Number	Honey Variety	Sterilisation Temp. (°C)	Sterilisation Duration (h)	Sample Number	Honey Variety	Sterilisation Temp. (°C)	Sterilisation Duration (h)
001	Marri	Control	0	049	Yate	65	8
002	Marri	Control	0	050	Yate	65	8
003	Marri	60	4	051	Yate	65	10
004	Marri	60	4	052	Yate	65	10
005	Marri	60	6	053	Yate	65	12
006	Marri	60	6	054	Yate	65	12
007	Marri	60	8	055	Yate	70	4
008	Marri	60	8	056	Yate	70	4

Sample Number	Honey Variety	Sterilisation Temp. (°C)	Sterilisation Duration (h)	Sample Number	Honey Variety	Sterilisation Temp. (°C)	Sterilisation Duration (h)
009	Marri	60	10	057	Yate	70	6
010	Marri	60	10	058	Yate	70	6
011	Marri	60	12	059	Yate	70	8
012	Marri	60	12	060	Yate	70	8
013	Marri	65	4	061	Yate	70	10
014	Marri	65	4	062	Yate	70	10
015	Marri	65	6	063	Yate	70	12
016	Marri	65	6	064	Yate	70	12
017	Marri	65	8	065	Manuka	Control	0
018	Marri	65	8	066	Manuka	Control	0
019	Marri	65	10	067	Manuka	60	4
020	Marri	65	10	068	Manuka	60	4
021	Marri	65	12	069	Manuka	60	6
022	Marri	65	12	070	Manuka	60	6
023	Marri	70	4	071	Manuka	60	8
024	Marri	70	4	072	Manuka	60	8
025	Marri	70	6	073	Manuka	60	10
026	Marri	70	6	074	Manuka	60	10
027	Marri	70	8	075	Manuka	60	12
028	Marri	70	8	076	Manuka	60	12
029	Marri	70	10	077	Manuka	65	4
030	Marri	70	10	078	Manuka	65	4
031	Marri	70	12	079	Manuka	65	6
032	Marri	70	12	080	Manuka	65	6
033	Yate	Control	0	081	Manuka	65	8
034	Yate	Control	0	082	Manuka	65	8
035	Yate	60	4	083	Manuka	65	10
036	Yate	60	4	084	Manuka	65	10
037	Yate	60	6	085	Manuka	65	12
038	Yate	60	6	086	Manuka	65	12
039	Yate	60	8	087	Manuka	70	4
040	Yate	60	8	088	Manuka	70	4
041	Yate	60	10	089	Manuka	70	6
042	Yate	60	10	090	Manuka	70	6

Sample Number	Honey Variety	Sterilisation Temp. (°C)	Sterilisation Duration (h)	Sample Number	Honey Variety	Sterilisation Temp. (°C)	Sterilisation Duration (h)
043	Yate	60	12	091	Manuka	70	8
044	Yate	60	12	092	Manuka	70	8
045	Yate	65	4	093	Manuka	70	10
046	Yate	65	4	094	Manuka	70	10
047	Yate	65	6	095	Manuka	70	12
048	Yate	65	6	096	Manuka	70	12

9.3. Results and discussion

9.3.1. Diastase activity

The diastase number (DN) of Marri, Yate and Manuka control samples was 7.7, 5.45 and 32.05 Schade units respectively. A consistent trend of a decrease in DN over both time and temperature was observed for all types of honey tested.

The decreasing DN with heat treatment, is potentially attributable to denaturation of the enzyme and subsequent decreased α-amylase activity and is consistent with previous results in the literature[10]. An example of this decreasing DN is illustrated in Figure 45, detailing the change in DN for Manuka.

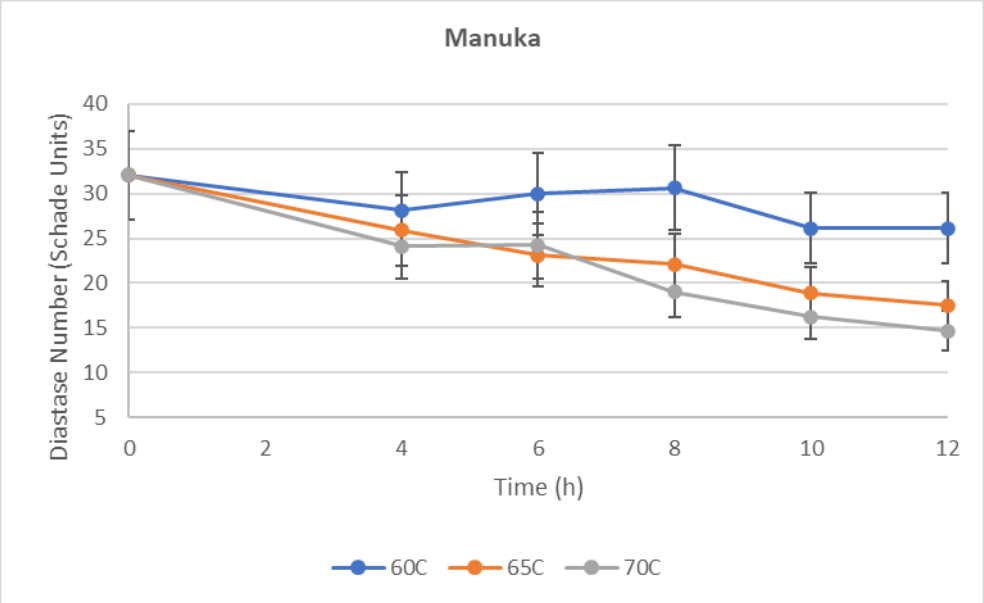

Figure 45 Effect of heat treatment on Diastase Number in Manuka honey.

When comparing the overall change as a percentage value between untreated and sterilised honey (Table 11), a clear difference can be observed between the two sterilisation conditions. The variation arising as a result of sterilisation at 60 °C is substantially lower than the variation arising as a result of sterilisation at 65 °C and, for two varieties of honey, less than the measurement uncertainty of the method (approximately 15%).

Consequently, it would not be possible to definitively identify sterilisation performed at the lower temperature.

Table 11 Percent change in Diastase Number following sterilisation.

Honey Variety	10 hours at 60 °C	8 hours at 65 °C

Marri	5.2%	26.6%
Yate	10.1%	42.2%
Manuka	18.4%	31.0%

A claim of sterilisation at 65 °C may be supported by DN, however alternative time and temperature combinations (4 hours at 70 °C for Marri honey) may yield false positive results. The analysis utilised for DN measurement is challenged by the sophistication and resolution of the analysis, however the use of this measurement shows some promise.
Critical to use of DN is the initial presence of this enzyme and then the subsequent loss of functionality due to denaturation. Use of peptide LC-MS methodology to track the formation of enzyme degradation compounds through this change in functionality may allow a greater understanding of the temperature induced changes in enzyme function that are occurring and therefore allow a better and more authoritative and legally defensible use of this loss of enzymatic function as an indicator. Further work is recommended.

9.3.2. 5-Hydroxymethylfurfural (HMF)

The initial HMF concentration of Marri, Yate and Manuka control samples was 64, 52 and 5 mg/kg respectively. A consistent trend of an increase in HMF concentration over both time and temperature was observed for all types of honey tested.
The increasing HMF concentration with heat treatment is consistent with literature[11] and attributable to the dehydration of fructose and glucose to HMF. An example of this increasing HMF concentration is illustrated in Figure 44, detailing the change in HMF for Manuka.

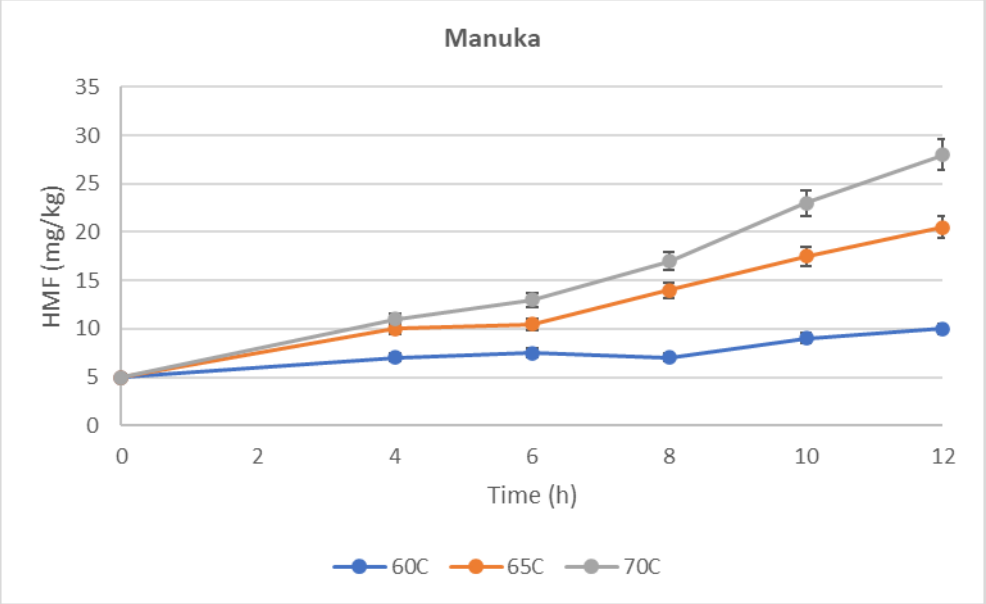

Figure 44 Effect of heat treatment on HMF in Manuka honey.

The overall change as a percentage value between HMF concentrations of untreated and sterilised honey (Table 12) details a significant increase in concentration, in excess of measurement uncertainty of the method (5.7%). However, the relative increase in HMF concentration after sterilisation varies between honey varieties.
Table 12 Percent change in HMF following sterilisation.

Honey Variety	10 hours at 60 °C	8 hours at 65 °C
Marri	21.5%	33.3%
Yate	11.9%	11.1%

Manuka	44.4%	64.3%

Furthermore, equivalent variation in HMF concentration can be achieved by exposing the honey to higher temperatures for a shorter time (4 hours at 70 °C for some varieties).
Consequently, establishing a minimum relative variation to indicate sterilisation does not appear to be possible, though HMF can be used to support claims that some form of heat treatment has occurred.

9.3.3. Sugars

Individual and total simple sugar concentrations of control samples of Marri, Yate and Manuka honey were determined, with fructose concentrations of 43.7, 39.3 and 43.1 g/100 g determined respectively.
A general decrease in fructose concentration was observed over time, supported by literature[12] and the increasing HMF concentrations detailed previously. This decreasing trend is illustrated in Figure 45. However, the relative variation in fructose concentration between sterilised and untreated honey samples was minimal (<3%) and below the measurement uncertainty of the method.

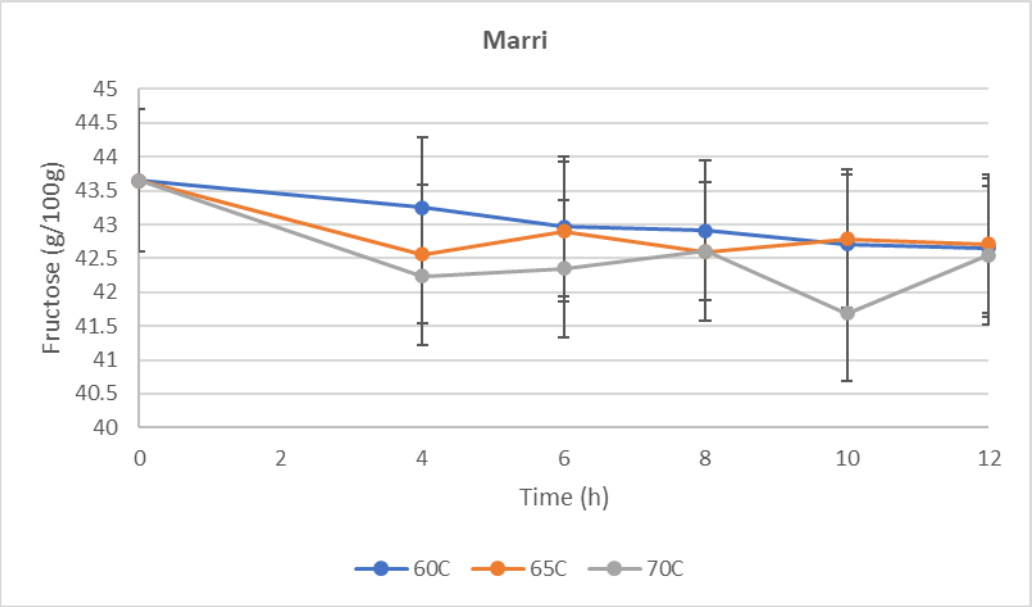

Figure 45 Effect of heat treatment on fructose concentration in Marri honey.

Variation in other individual sugars (glucose, sucrose, maltose) and total simple sugars was also within measurement uncertainty.
Consequently, it would not be possible to use individual or total simple sugar concentrations as a chemical marker for honey sterilisation, given the low variation occurring in a major component of the honey. As the sugars are most likely being dehydrated to HMF, the HMF represents a more discriminating marker of sugar degradation and heat treatment.

9.3.4. Hydrogen peroxide

Hydrogen peroxide values were determined for Marri honey only, as it was the only honey expected to exhibit a peroxide-based antimicrobial activity. The Marri control sample exhibited a hydrogen peroxide concentration of 1.95 mg/kg.
No clear trend in hydrogen peroxide concentrations was observed following exposure of the honey samples to the various temperature conditions over time, with these results illustrated in Figure 46. The overall variation observed over time is less than the measurement uncertainty of the method (10%). Consequently, hydrogen peroxide concentration could not be used as evidence of heat sterilisation of honey.

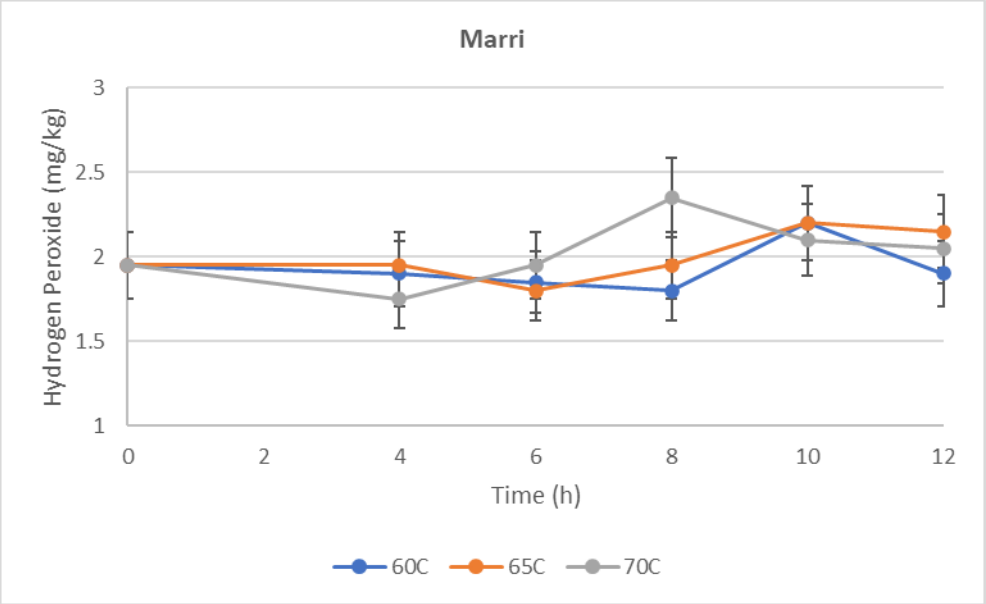

Figure 46 Effect of heat treatment on hydrogen peroxide concentration in Marri honey.

9.3.5. Total phenolics

Total phenolics were determined as Gallic Acid equivalents for Marri honey, with control samples exhibiting a concentration of 758 mg/kg.

A general trend of increasing total phenolic concentration over time may be observed in the results illustrated in Figure 47 . This result is contrary to the existing literature[12], where a decreasing total phenolic concentration may be expected.

However, the result is consistent with recent ChemCentre studies which have detailed increasing total phenolic concentrations in honey following exposure to heat (see previous two sections).

Despite the overall increase in total phenolic concentration, the relative increase resulting from exposure to sterilisation conditions is minor with a 1.6% increase after 10 hours at 60 °C and a 6.1% increase after 8 hours at 65 °C. These minor differences in total phenolic concentration are equivalent to the measurement uncertainty of the method (6%).

Consequently, total phenolic concentration could not be used as evidence of heat sterilisation. Effects of specific phenolic compounds or phenolic adducts may deliver a more definitive and specific indicator.

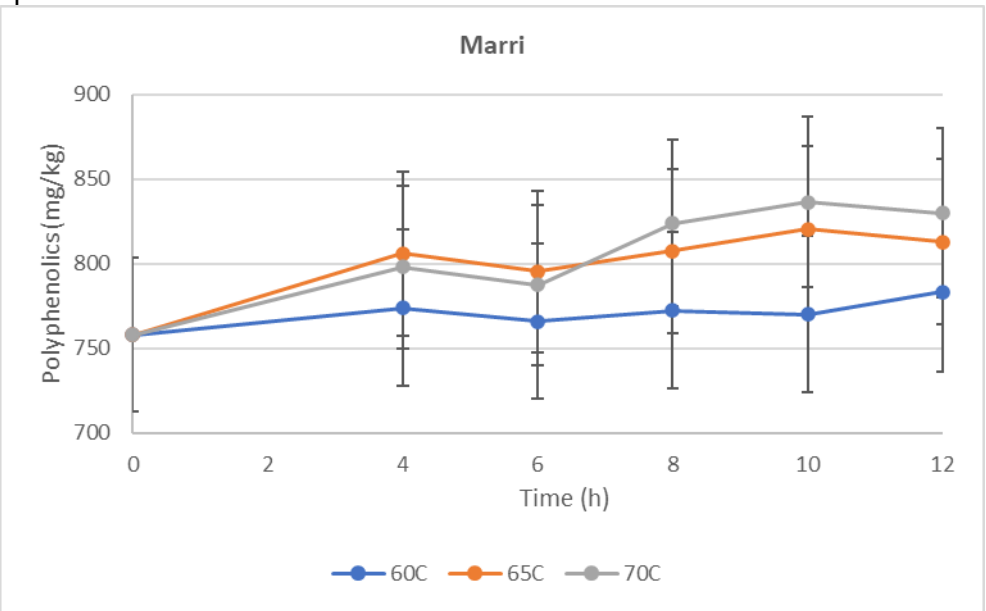

Figure 47 Effect of heat treatment on total phenolic concentration in Marri honey.

9.3.6. Free acidity

Free acidity was determined for Marri honey, with the control sample exhibiting a concentration of 40.5 meq/kg. A general trend of decreasing free acidity was observed with exposure to elevated temperatures over time (Figure 48).

This result is contrary to literature[12] and other ChemCentre studies regarding free acidity of honey and exposure to elevated temperatures. However, the overall variation in free acidity concentrations between the control sample and sterilised samples is approximately 3-7%, below the measurement uncertainty value for the method (10%). Consequently, free acidity could not be used as evidence of heat sterilisation and the trend observed may be within experimental and analytical error for this analysis.

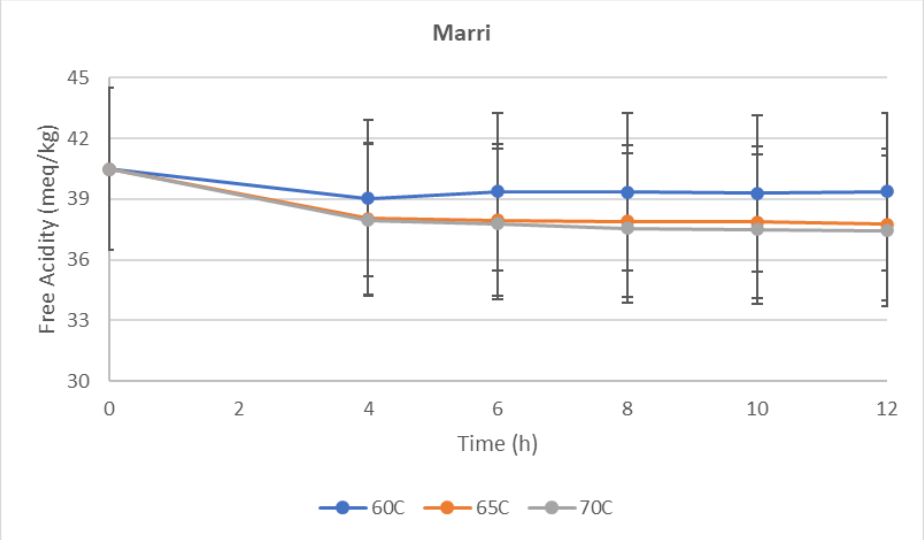

Figure 48 Effect of heat treatment on free acidity in Marri honey.

9.4. Conclusion

Most chemical parameters examined during this study exhibited some degree of variation following exposure to elevated temperatures, with the exception of hydrogen peroxide concentration. However, the exposure time and temperature conditions required to sterilise a honey sample only result in minor variation for several parameters (sugars, total phenolics and free acidity), with that variation falling below measurement uncertainty for the relevant methods. Consequently, it would not be possible to use those parameters to confirm that heat treatment had occurred.

Diastase number and HMF concentration were determined to have exhibited significant variation in heat treated samples, beyond the measurement uncertainty of those methods. However, the degree of relative variation appears dependent upon honey variety and sterilisation conditions, preventing establishment of a single value across all varieties at which it could be concluded that heat treatment had occurred. While it is still possible to use these two parameters and compare treated and untreated samples to establish whether some degree of heat treatment has occurred, it would not be possible to definitively state that honey samples had been sterilised based upon this data alone.

10. References

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