Ministry for Primary Industries
Manatū Ahu Matua

Response to submissions on MPI's proposed definition for mānuka honey

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1 Executive summary

In April this year MPI proposed a definition for mānuka honey based on the work of a robust and systematic interdisciplinary 3 year science programme. Following the release of the proposed definition, we drafted and consulted publicly on a proposed General Requirement for Export (GREX) which would implement the definition for all mānuka honey exported from New Zealand.

During the consultation period, MPI published summary information on the science programme and all the data that underpinned our proposed definition. We also hosted a number of interactive workshops around the country to enable stakeholders to seek further information and share their views. Interested parties were invited to provide written feedback on the proposed definition and the GREX. We received 120 written submissions.

MPI completed a thorough scientific analysis of all the information and data provided by submitters to further assess the suitability of our definition prior to adoption into regulation. All the concerns raised were documented and analysed by MPI technical specialists.

This document provides a summary of submissions received on the mānuka honey definition during the April – June 2017 consultation period, along with MPI's assessment and response.

Although submitters were generally supportive of the need for a regulatory definition for mānuka honey, many raised concerns around MPI's proposed definition. Common concerns raised related to the selection of markers and their levels, the DNA test, and the potential for the MPI definition to allow the blending of non-mānuka honey to meet the threshold levels in the definition. Some industry submitters also proposed that aspects of their own funded science should be considered as an alternative definition, including the use of different markers, at different levels.

MPI's role as a regulator means our priority is to ensure that export rules for mānuka honey are based on a robust and accurate scientific definition. This is key to maintaining New Zealand's strong reputation as a supplier of high quality food and animal products that command a premium in the market place.

As a result of our assessment, we determined that there was insufficient scientific evidence provided to support the majority of the concerns raised in submissions. Where evidence was supplied, it often lacked the necessary rigour to back up the points being made by submitters e.g. through limitations in the way research was designed, limited numbers of samples tested, questions over the methodological approach used to evaluate test results.

One concern raised by several submitters was that it would be possible to blend some honeys together to meet the monofloral mānuka honey definition. To test this concern, a thorough assessment of theoretical blending scenarios was undertaken by MPI, using our existing database of honey samples. The assessment identified that there were a small proportion of scenarios where there was an opportunity to blend kānuka honey samples with multifloral mānuka honey samples to produce a blend that met the monofloral definition. This requires availability of honeys with the exact levels of the markers blended together at exact quantities.

To minimise this opportunity, MPI will increase the level of 2'-methoxyacetophenone to 5 mg/kg in our definition. 2'methoxyacetophenone is a chemical which can be directly

attributed to *Leptospermum scoparium.* This increases the amount of nectar from the mānuka plant in the honey.

The identification criteria to authenticate mānuka honey (either monofloral or multifloral) are:

- ≥ 5 mg/kg for 2'-methoxyacetophenone (2'-MAP)
- ≥ 1 mg/kg for 2-methoxybenzoic acid (2-MBA)
- ≥ 1 mg/kg for 4-hydroxyphenyllactic acid (4-HPA) and;
- ≥ 20 mg/kg 3-phenyllactic acid (3-PA) and;
- DNA from mānuka pollen (< Cq 36 equivalent of 3.2 fg/µL DNA).

To further separate honey as either monofloral or multifloral mānuka honey, 3-PA is required:

- Monofloral mānuka honey = ≥ 400 mg/kg 3-PA,
- Multifloral mānuka honey = ≥ 20 but < 400 mg/kg 3-PA.

MPI's definition for monofloral and multifloral mānuka honey provides a robust defendable scientific solution to the concerns that have been raised regarding authenticity. Our final definition and resulting export requirements will give confidence to both consumers and trading partners in the authenticity of New Zealand mānuka honey.

2 Definitions and abbreviations

Word	Meaning
Blend	Refer multifloral.
Classification model	A statistical approach (CART – classification and regression tree) that uses mathematical relationships to identify groups (e.g. honey type) based on common markers at defined levels.
Cq	Quantification cycles – scientific unit used to represent a test result from a DNA test (qPCR test).
fg/µL	Femtogram per microlitre – unit of measurement often associated with a DNA test.
HMF	Hydroxymethylfurfural – chemical that provides information on whether a honey has been exposed to excessive heat or has aged.
Honey types	Term used to describe honeys derived from different floral sources, for example, monofloral mānuka, clover, rata, multifloral mānuka.
Kānuka	*Kunzea ericoides/Kunzea robusta* Since the start of the Science Programme, the *Kunzea* genus in New Zealand has been revised to increase the number of taxa from four to ten. The six newly described species were all previously placed in *K. ericoides* var. *ericoides.* The plant commonly referred to as kānuka, widely distributed throughout both the South Island and North Island, is now *K. robusta* under this revision. In this document, *K. ericoides* and *K. robusta* are both referred to under the common name kānuka**.**
Leptospermum species	Species in the genus *Leptospermum* in the Myrtaceae family.
Mānuka	*Leptospermum scoparium* JR Forst and G Forst, 1776. Mānuka has variable growth forms, with some regional 'forms' identified, although most have not been officially described**.**
Marker	Quantitative characteristic common to both the source plant and associated honey.
mg/kg	Milligrams per kilogram (= parts per million).
Monofloral	Honey with a distinctive combination of markers at specified levels that indicate the honey is predominantly derived from one plant species.
Multifloral	Honey derived from multiple floral sources. A combination of distinctive markers from the named floral source are still present but at levels lower than a monofloral honey.
qPCR	Quantitative polymerase chain reaction – a type of DNA test.
Related plant species	Plant species that are part of the same genus as *L. scoparium*.
Relevant plant species	Plant species associated with honey production in New Zealand.
Specificity	An assessment of how a marker can differentiate a particular plant species when compared against other relevant and related plant species.
Supplier	Supplier of honey samples, for example, beekeeper, hobbyist, honey companies.

3 Summary of submission analysis and high-level response to key concerns on MPI's proposed **definition for mānuka honey**

3.1 PROCESS USED TO ANALYSE SUBMISSIONS

In order to systematically evaluate submissions, they were grouped under the following topics (the approximate number of submissions raising the issue is provided in brackets):

- Scope of programme (4 submissions)
- Selection of markers (18 submissions)
- Addition of other markers (59 submissions)
- Sampling design (11 submissions)
- DNA test (35 submissions)
- Threshold levels (25 submissions)
- Stability (7 submissions)
- Statistical analysis (5 submissions)
- Blending (22 submissions)
- Other issues (21 submissions)

After identifying the key issues in the submissions, MPI followed a thorough and systematic approach to assess the issue of concern. In doing this, a number of questions were posed to help guide the assessment of the issue of concern. These included:

- Was there any supporting evidence provided?
- If evidence was provided, what was the evidence?
    - For example: publications, test results of samples, presentation, expert reviewer etc.
- Has enough information been provided to evaluate the evidence?
    - For example: Were there full details of test results including sample information?
- How robust was the evidence?
    - For example: If data was provided, can it be considered representative of the New Zealand honey industry and honey producing regions? Was a sampling plan or rationale followed to collect the data? Is there any bias in the data provided? Were the test methods valid that were used to test the samples?
- Were analyses of data used to make conclusions or interpret findings?
    - For example: If analyses were presented, were they appropriate for the type of data and have they been applied and interpreted correctly? MPI reanalysed data when necessary using the appropriate methodology and assessed the findings.

3.2 SUMMARY OF RESPONSES TO KEY ISSUES

Industry issue of concern	MPI response
Kānuka was not included under the common name mānuka	Testing of both kānuka and mānuka nectar and DNA showed that the plants were different and should not be included under the same common name.
Markers in the definition are not unique	Whilst unique markers are useful, other characteristics of shared markers such as concentration can be equally useful for defining a honey.
Leptosperin, DHA and MG to be included	Other markers were shown to be more useful for defining mānuka honey from New Zealand when compared against leptosperin, DHA and MG.
Not enough plant and honey sample collected	The number of both plant and honey samples collected were adequate for the intended purpose. This was reflected in the result of the statistical analyses which confirmed that sample numbers were appropriate in development of the definition.
DNA test does not work on 'high grade mānuka honey"	The DNA test performed reliably across a wide range of honey samples of varying ages during testing conducted in the MPI science programme and at a MPI recognised laboratory. Concerns that DNA was affected by MG were unfounded. A modification that was made to the test improved testing outcomes for industry samples. It is important to note that sample quality and sample collection are an important factor for any laboratory test.
Threshold levels are either too high or too low	A robust statistical approach was used to determine the threshold levels. Samples from across New Zealand were used to determine the levels, which were tested using honey representing 7 production seasons. This ensured appropriate thresholds for honey produced by the New Zealand industry were used in the definition.
Blending to meet the definition	MPI determined that there was a possibility of blending multifloral mānuka with kānuka honey to produce a blend that meets the monofloral definition providing the honeys have certain specifications and are combined in certain ratios. As this is a concern to MPI, a mitigation that severely minimises this possibility is to increase 2'-MAP to $\geq$ 5 mg/kg for both monofloral and multifloral definitions.

4 Response to submissions on MPI's proposed definition for **mānuka honey**

4.1 SCOPE OF PROGRAMME

4.1.1 **Concerns that kānuka (*Kunzea ericoides*) is not included alongside *Leptospermum scoparium* under the common name mānuka.**

4.1.1.1 General comment

In 1983, Thompson reported that *Kunzea ericoides* was a separate genus and species to *Leptospermum scoparium*. However, some submitters (four) claimed that these two plant species can both be referred to as mānuka. MPI designed specific aspects of the science programme to answer this question. Analysis of both DNA information from the plants and chemical profiles of the two nectars indicated sufficient differences to support their separation. They did not have enough features in common to be grouped together under the common name mānuka.

4.1.1.2 Submissions

Evidence provided in one submission to support the inclusion of kānuka with mānuka included citation of Walsh (1978). The reference was written before kānuka was renamed as a member of the *Kunzea* genus (Thompson, 1983) so it is still referred to as *Leptospermum ericoides*. This reference is referred to as "very significant" in the submission as it is noted as providing a beekeeping perspective on usage of mānuka as a common name. It is unclear how this reference supports the inclusion of kānuka with 'mānuka' as the description of kānuka (also referred to as tree mānuka) describes a plant that produces nectar under different conditions and produces different coloured pollen to that produced by mānuka. Further, the reference is outdated as it predates the Thompson (1983) publication.

4.2 SELECTION OF MARKERS

4.2.1 Concerns that the proposed definition includes non-unique markers

4.2.1.1 General comment

Some submitters raised concerns that markers used as part of the proposed definition were not unique to the mānuka plant or mānuka honey.

A desire for "unique" markers has been a common theme for many interested parties throughout the duration of the MPI science programme. To our knowledge, no markers have been demonstrated as "unique", as that would require sampling and testing an extensive number of plants, both related and unrelated species, from across the world.

A number of markers were assessed within the science programme to determine their presence or absence in different plant species as well as whether or not their levels could enable separation. Based on information from the science programme, 2'-methoxyacetophenone (2'-MAP) and the DNA marker were only found in *L. scoparium*. Other markers included in the proposed definition were also found in other nectars and honeys. However, this did not detract from their usefulness in developing a proposed definition made up of multiple markers as their levels contributed to the identification and distinction of mānuka honey from other honeys of interest.

MPI undertook a robust process to select the most suitable combination of markers to define mānuka honey. At a high level, MPI considered the following criteria to determine the usefulness or suitability of the chemical and DNA markers:

- presence/absence in mānuka plants
- presence/absence in other related and relevant plants
- whether or not levels in plants and associated honey could be used for separation
- stability of the markers over time and temperature.

After the first season of testing (2014/15) detailed analysis of the data was used to determine which markers would be further investigated. MPI did not continue to test for markers that did not meet the required criteria.

The first step in evaluating the potential suitability of the markers was to determine how successfully a marker can identify and differentiate a particular plant species when compared against other plant species. This ability was confirmed for the DNA markers using plant specimens from herbarium reference collections. The mānuka and kānuka DNA markers were highly specific for *L. scoparium* (mānuka) and *K. ericoides* (kānuka) respectively and found across the different regions of New Zealand. However, results for the chemical markers were not as clear cut because some of the chemicals of interest were found in plant species other than mānuka. Instead it was important to assess the variation in levels of each chemical marker in both the nectar and honey samples from the 2014/15 collection year to answer the following questions:

1. Does mānuka nectar have significantly greater or lower levels of a marker than the nectar from other plant species?
2. Does mānuka honey have significantly greater or lower levels of a marker compared with different honey types?
3. Was the chemical marker stable over time and at different temperatures?

A suite of statistical models compared the levels of each chemical between different plant species and honey types as well as assessing chemical stability, regional and temporal variation. This analysis was used to inform which chemical markers were to be further evaluated during the second season of sample collection in the MPI science programme.

4.3 ADDITION OF OTHER MARKERS

4.3.1 Submissions that leptosperin be included in the proposed definition

4.3.1.1 General comment

Inclusion of leptosperin in a definition of mānuka honey has been a strong theme amongst industry throughout much of the MPI science programme, and was proposed by a number of submitters.

Leptosperin was first discovered by a researcher in Japan in both mānuka and jellybush honey (Kato, 2012). This original work showed that leptosperin is not unique to mānuka. When assessing other published studies on leptosperin they are typically based on small datasets, samples are not representative and statistical methods used to analyse data are not appropriate for the type of data (Kato *et al.*, 2014; Kato *et al.*, 2016; Oelschlaegel, 2012). These factors limit the use of the findings from these studies as part of a regulatory definition.

To address the limitations, MPI carried out a robust evaluation of leptosperin by collecting not only honey samples, but also samples of other key plant species involved in honey production in New Zealand and Australia. MPI's evaluation of leptosperin using plant and

honey data (2014/2015) identified several limitations with using leptosperin (refer to Section 4.3.1.2 and Section 4.7). Further, comparison against other markers showed that other markers were more suitable for usage in a regulatory definition.

4.3.1.2 Submissions

Some submitters stated that leptosperin is of value as it is unique to mānuka honey. However, scientific publications describe its presence in other related plant species in Australia (Bong *et al*., 2017a). The MPI science programme confirmed this and found it in other *Leptospermum* species (both in New Zealand and Australia) and in kānuka and kāmahi plants in New Zealand.

While the MPI finding of leptosperin in non-*Leptospermum* plants is different to other reported findings, this could be attributed to a number of reasons:

- Differences in nectar collection - the MPI science programme applied the same nectar collection method across species. MPI is aware that some industry work has used different nectar collection methods for different plant species, thus compromising comparisons between species.
- Plant species and number tested – the more representative and diverse the plants tested the more likely that differences will be found.
    - Also note that nectar samples collected from plantations resulting from selective breeding would not be representative of the natural population of mānuka plants.
- In addition to leptosperin being detected in other plant species, the foraging behaviour of bees within natural environments means that all honey is made from a mixture of nectar from different plant species. Therefore, markers found in mānuka honey (even those only detected to date in *L. scoparium* plants) are likely to be present in other honey types.

A number of submissions called for leptosperin to be included in the proposed definition either as an addition to proposed markers or as a replacement. Several also requested including leptosperin in the proposed definition for mānuka honey at specified levels of ≥ 100 mg/kg for monofloral mānuka and ≥ 63 mg/kg for multifloral mānuka honey. No data was supplied in submissions to demonstrate how these proposed threshold levels were determined. In order to consider the inclusion and level of a chemical marker in a regulatory definition scientific information would be needed to show how this chemical and the associated threshold were evaluated.

MPI agrees that leptosperin may be a useful marker for some purposes. For example, as levels of leptosperin are typically higher in *Leptospermum* type honeys from Australia, a difference in concentration as indicated by leptosperin testing could be useful for separation of New Zealand and Australian *L. scoparium* honey. If leptosperin was to be included in a regulatory definition for mānuka honey from New Zealand, an upper limit would likely be required. Leptosperin may be of more value for a generic *Leptospermum* honey definition for New Zealand and Australia. However, this would need further investigation over an extended period of time.

4.3.2 Submission that dihydroxyacetone and/or methylglyoxal be included as additional markers

4.3.2.1 General comment

DHA and MG are highly unstable and change over time. This is described in scientific publications (Grainger, 2016a, 2016b, 2016c).

Both DHA and MG were evaluated as part of the MPI mānuka honey science programme. MPI concluded that the widely-acknowledged limitations relating to stability prevent their use as regulatory markers, primarily because of the length of time honey may be in market. This is consistent with the view put forward by UMFHA's at their "This is mānuka honey" symposium in August 2016, noting stability and adulteration issues.

The level of DHA in the nectar of *L. scoparium* plants was found to be significantly influenced by the habitat the plants were growing in. Therefore, some authentic mānuka honey would not meet a definition that included DHA as bees could be collecting nectar from *L. scoparium* plants without any or with low levels of DHA.

DHA and MG are also present in Australian *Leptospermum* plants and honeys at greater concentrations than New Zealand plants and honeys. This suggests that if DHA and/or MG were to be included in a regulatory definition for mānuka honey from New Zealand, an upper limit would likely be required to assist distinguishing New Zealand and Australian honey.

4.3.2.2 Submissions

The limitations of MG and DHA as markers were recognised in a number of submissions, particularly around adulteration and stability concerns. However, a number of these submissions stated these issues can be dealt with on the basis of current understanding and use by industry.

Some submissions proposed a value for MG of 100 mg/kg for the proposed monofloral definition. One submission also suggested a value for MG of 150 mg/kg for the proposed monofloral definition. However, scientific explanations on how these levels were determined were not provided and without such evidence, possible inclusion in a regulatory definition cannot be considered.

4.3.3 Submissions for inclusion of markers not assessed as part of the science programme

4.3.3.1 General comment

MPI has previously engaged with industry on the need to evaluate markers other than those in the definition with the same scientific rigour as those evaluated and currently included in the proposed MPI definition. Suggestions for the addition of new markers need to be backed by robust data that is equivalent to that used by MPI and that meets credible and recognised scientific standards. To assess if these standards have been met, MPI needs full transparency about the way scientific data has been collected and analysed.

4.3.3.2 Submissions

Some markers put forward in submissions as being suitable for determining the authenticity of mānuka honey were the fluorophores lepteridine (3,6,7-trimethyl-2,4(1H-3H)-pteridinedione) (Daniels *et al*., 2016; Beitlich *et al*., 2016) and 6, 7-dimethyl- 2,4(1H,3H)-pteridinedione (Beitlich *et al*., 2016). These markers were not evaluated within the MPI mānuka science programme and we note that lepteridine has only been proposed in the published scientific literature since 2016. Unfortunately, there was insufficient evidence provided to support the potential inclusion of new markers.

We offer the following analysis on the proposal to include lepteridine:

- Lepteridine as a marker has only recently been proposed (Daniels *et al*. 2016, Bong *et al*. 2017a, 2017b, Lin *et al*. 2017). Although some submitters claim that it is unique to mānuka honey, lepteridine has been found in kānuka honey and Australian *Leptospermum* honeys as well as mānuka honey.

- We note a newly published study in support of lepteridine that suggests a level of 2.1 mg/kg to identify mānuka honey (Bong *et al*., 2017b) in combination with other markers. However, this publication does not provide the data and analysis needed to support the inclusion of lepteridine in a regulatory definition.

- MPI considers there are several constraints associated with the Bong *et al*., 2017b research paper that limit the adoption of the outcomes in a regulatory setting. These include:
    - A limited number of samples were collected for each plant species and for many of the honey types, therefore, the samples are unlikely to be representative of either plants or honey in New Zealand. A greater diversity of plant species and honey types would need to be studied to support the conclusions made in the research paper.
    - The test method used lacks specificity and is more appropriate as a screening tool in a research environment. Test methods that support export assurances require great specificity and extensive validation.
    - The statistical analysis used to support the selection and proposed level for lepteridine was not appropriate for the type of data available and the statistical methods need to be applied correctly to have confidence in the findings.
        - MPI recommends that data is normalised before analysis to meet the requirements of the statistical method used.
        - The research paper reports that correlations were determined by regression analysis, however MPI would advise that correlations should be determined by assessing the appropriate correlation coefficient.
        - Principal component analysis (PCA) was used to determine the unique chemical features for the honey types, however MPI advises that PCA should not be used in place of a classification or cluster analysis of honey type.

The research paper presents a combination of markers to identify mānuka honey, however the levels of the markers have been assessed in isolation rather than in combination. Currently, there is limited scientific information available to MPI on the two fluorophores suggested in the submission as potential markers for mānuka honey (Beitlich *et al*. 2016). Further, the

samples used are not reasonably representative of honey produced in New Zealand and no levels are suggested.

4.3.4 Submissions on adding hydroxymethylfurfural (HMF)

HMF is a quality parameter for all honey types to determine if the honey is old or has been subject to excessive heating. As a quality parameter, it is not appropriate in an authentication definition for a specific floral source.

4.3.5 Submissions to include organoleptic properties, physico-chemical properties and pollen as determined by microscopy

Several submissions requested the inclusion of organoleptic, physico-chemical properties, and pollen as determined by microscopy, in the proposed definition. MPI assessed these properties as candidates for determining mānuka honey authenticity early on in the science programme and excluded them from the proposed definition for the following reasons:

- Testing is subjective.
- Separation of honey types cannot be achieved with a high level of confidence.
- Test methods are difficult to standardise and validate.
- Methods are typically reliant on specialist expertise that is not transferable (for example, experts to judge the taste or aroma of a honey).
- There are limitations regarding method accreditation and throughput capacity.

It is important to note that many of these practical reasons for excluding subjective test methods are universal to choosing tests that are appropriate for regulatory purposes.

4.3.6 Submissions to include negative markers found in non-**mānuka New Zealand plants**

Negative markers are those not found in association with *L. scoparium* plants, but are found associated with other species of New Zealand plants. Therefore, the markers' absence or presence at low levels in honey predominantly sourced from *L. scoparium* might be used for authentication purposes.

The potential for negative markers was considered as part of the design of MPI science programme. However, none were identified as being suitable with regards to separating relevant honey types. If useful negative markers had been identified, the sampling approach would have required the collection of more plant and honey types that contained the negative marker of interest. For example, if the negative marker was in clover, then we would have targeted a greater number of clover plant and honey samples so we could determine levels of the negative for use in a definition.

4.4 SAMPLING DESIGN

4.4.1 Concerns about regional, seasonal and site-specific variation of markers

4.4.1.1 General comment

As part of the MPI science programme, both plant and honey samples were collected from across New Zealand representing different regions, habitats and two different collection

seasons. Variation due to these factors was a paramount consideration when developing the proposed mānuka honey definition.

The degree of variation was not measured specifically for each region or season as this would require a very different, and even more intensive, sampling regime. For example, we did not set out to measure the average or minimum level of 3-PA in mānuka plants in one region versus another. We also used archived samples to assess any temporal trends in application of the proposed definition (none were observed).

4.4.1.2 Submissions

Some submissions questioned the adequacy of the sampling design used in the MPI science programme. However, this was not clearly supported by the information provided in the submissions. The sampling design ensured as full a representation of New Zealand honey types as practical and reasonable within the time constraints of the MPI science programme. We are unaware of any other sample databases in New Zealand that provide the same level of rigour, standardisation and representation.

4.4.2 Concerns that the taxonomic variation in mānuka and kānuka has not been fully considered

As new scientific evidence becomes available, taxonomic descriptions of plants can change. In view of this, MPI ensured that an extensive reference collection of plants was established and plant specimens archived such that any future taxonomic changes could be assessed in terms of influence on the proposed definition.

The MPI science programme collected and tested a number of mānuka and kānuka plants from New Zealand and Australia. This enabled MPI to assess whether or not genetic variation within species influenced the selection of markers and associated levels in the proposed definition. MPI considers the taxonomic variation within the two species has been well accounted for within the programme.

4.4.3 Concerns that sample numbers were too small

Some submissions stated that sample numbers were too small. No evidence was provided in submissions to support this claim.

All science programmes evaluating biological systems where there is considerable natural variability are challenged by the question of adequacy of sample numbers. The MPI science programme collected over 700 plant samples and 800 honey samples from across New Zealand and other countries. MPI considers the sample size collected for both plant and honey samples was adequate for the purpose ascribed and the statistical analyses illustrate this adequacy in development of the proposed definition.

4.5 DNA TEST

4.5.1 **Honeys considered 'high grade mānuka' by the industry not passing the DNA test**

4.5.1.1 General comment

During the consultation phase, some industry representatives raised concerns that some of their "high grade mānuka honeys" were passing the MPI chemistry tests, but not the DNA test component of the proposed mānuka honey definition. MPI assumes that "high grade" relates to methylglyoxal (MG) or Unique Mānuka Factor (UMF).

MPI took the following actions to address these concerns:

- Assessing the extent of the observations reported by the industry;
- Investigating possible causes of the unexpected test results;
- Modifying laboratory treatment of samples.

4.5.1.2 Assessing the extent of the observations by industry

Only 3% of samples in the MPI reference database passed the chemistry test for mānuka honey and failed the DNA test. This did not raise concern in the science programme as it is not unusual to find a very small number of samples that behave differently. Additionally, commercial testing conducted at a MPI recognised laboratory showed that only 7% and 2.4% of samples passing the monofloral and multifloral chemical tests respectively failed the DNA test using the *original* method. On this basis, MPI concluded that the concerns raised by industry potentially affected a small proportion of honey samples.

Anecdotal reports from some industry members suggested that there was a high percentage of honey passing the chemistry test but failing the DNA test. However, quantitative evidence of the extent of the problem was not provided. At a minimum, such evidence would need to be derived from a representative honey database that included test results from a recognised laboratory using validated test methods and samples that had been collected appropriately.

4.5.1.3 Discussion on possible causes of 'high grade mānuka' not passing the DNA test

Many reasons were put forward by industry and other parties to explain why some honey samples that were expected to pass the original DNA test but did not, however, none of these explanations are conclusive as outlined further below.

Explanations put forward by industry in submissions as to why the DNA test was not working included that:

- the honey is 'high grade mānuka' based on existing industry grading systems
- the honey is old
- the honey has high HMF (not always qualified)
- the honey has high MG
- DNA is not stable in honey

MPI also notes that the quality of the honey itself, or the way the sample is collected, could possibly result in samples not testing according to industry expectations of 'high grade mānuka'.

4.5.1.4 Submissions

One submission graphed results of honey samples tested using the *original* DNA test method and MG levels (no raw data was provided) and another submission quoted this work. It was concluded that the increasing concentration of MG with an increasing Cq value was statistically significant. This conclusion is opposite to that made from similar MPI data analyses. We note that:

- The use of the *modified* DNA test is likely to reduce the reported strength of the relationship between increasing MG and Cq values even further, making a significant result even less likely.
- From the samples tested in the MPI science programme, MPI knows that mānuka DNA is very easily detected in samples with high MGO values and this implies that the suggested "inhibition" is not consistent.
- The range of MG values in the samples tested by the submitter and MPI was very similar (0 – 1000 mg/kg of MG in the MPI data; 0 − 1250 mg/kg of MG in the submission). However, samples presented in the submission failed the DNA test (assuming samples are capped at 37 Cq value) at a variety of MG levels (~100-1250 mg/kg).
- MPI also makes the following observations:
    - Test results were from a laboratory not recognised by MPI at the time of testing.
    - It is not clear if the test results were valid i.e. the internal control passed.
    - The origin and floral source of the samples is unknown.
    - The sample size looks adequate from the graphs and the statistical parameters are included in the results, but little information is provided on the representativeness of the samples.

MPI received additional information from the same submitter that when using the *modified* DNA test there is no longer any evidence of an influence of MG on the DNA concentration in a honey sample.

This same submission also presented the test results of honey samples using the *original* DNA test method and their corresponding HMF values in a graph. A similar trend to MG was observed by the submitter with a statistically significant relationship found between HMF and Cq value. The MPI science programme also found a significant relationship between Cq value and HMF. However, the relationship was not a simple linear one as the samples where no mānuka DNA was detected were in the middle of the HMF range of the samples tested, and some samples with much higher HMF values showed clear detection of mānuka DNA.

MPI notes that:

- From the data presented by the submitter, it seems the values for HMF have been rounded rather than using raw test results, however, the reasoning for this has not been given.
- There is evidence of a potential relationship between Cq value and HMF, however, test results using the *modified* DNA test would need to be assessed.
- In addition, honey samples with a greater range of HMF values than provided would be needed to further assess the relationship.

Another submission presented test results in a graph for honey samples with the DNA concentration measured in femtograms (not Cq values) and HMF.

MPI notes that:

- No information was provided with the graph to determine the origin of the honey samples tested.
- Results from the analyses suggest that changes in HMF concentration can be used to explain the changes in DNA concentration. Due to limitations associated with how the statistical analyses performed, this conclusion cannot be supported.

Other information in the submission included the presentation of results of an experiment where honey samples and washed pollen from honey samples were incubated at 27°C after addition of two different concentrations of MG and DHA. The results were used to show that the DNA concentration, measured using the original DNA test, is negatively affected by MG concentration.

MPI raises the following regarding the information presented:

- It is assumed synthetic DHA/MG was added, however, we cannot be confident that natural and synthetic DHA/MG would have the same influence. During the synthetic manufacture of chemicals different levels of purity and quality can be achieved and this can produce different results in an experiment when compared against natural forms of the chemical.
- The experiment involves adding both MG and DHA at the same time which means that you cannot evaluate if MG alone is causing the apparent DNA test failure.
- The graphs presenting the results require further work as trend lines are incorrectly fitted to the data.
- No statistical analyses have been carried out on the data in support of the conclusions.
- No rationale is provided to justify the temperature, incubation time period, or range of DHA/MG values used in the experiment.
- No information about the origin of the honey samples used in the experiments is provided; only that they meet the proposed multifloral honey definition and have low concentrations of MG and DHA. Details of what values of MG and DHA qualify as "low" are not provided.
- No information is given around subsampling of the honey and how it was confirmed that a similar DNA concentration was present in all subsamples prior to incubation and addition of the DHA/MG used in the experiment.
- The washed pollen and honey were incubated for different time periods.

Some submissions cited scientific publications to support the claim that MG degrades DNA. The majority of the cited publications are focused on understanding the role of MG as naturally produced in the human body in association with adverse human health effects. It is unclear how the submitters determined that the outcomes of these papers can be applied in the context of the DNA test apparently not working because of high MG levels in honey. The DNA that is detected using the DNA test is contained within the mānuka pollen that is present in the honey. When the DNA is recovered from the pollen, the honey is first centrifuged to concentrate the pollen. The pollen is then washed to remove unwanted materials and is then broken up, inhibitors removed and the DNA purified.

One publication cited in the submissions was by Murata-Kamiya & Kamiya (2001). This study investigates the potential of MG to cause cross-links with DNA and proteins, specifically focusing on cross-linking between DNA and human DNA polymerase and how

this may inhibit DNA replication within cells in the human body. The paper proposes that DNA polymerase activity is inhibited by MG. If this mechanism was operating in the context of the mānuka DNA test, the DNA polymerase which enables the amplification of the target DNA would not work; meaning no DNA would be detected. The mānuka DNA test independently, but simultaneously, amplifies DNA from mānuka pollen and other plant pollen (internal control). For MG to inhibit the DNA polymerase activity, as the submissions suggest by citing this publication, then DNA test failures for both DNA targets (mānuka and internal control) would be expected for most samples. MPI has not seen evidence of this.

Other publications put forward in submissions in relation to MG and DNA include:

- Kang (2003) investigated oxidative damage of DNA by the reaction of MG with amino acids and proposed that this mechanism may be linked to several diverse biological processes including mutagenesis, aging, carcinogenesis, and diabetic complications.
- An & Kang (2013) investigated whether ferritin enhances DNA cleavage by the reaction of MG with lysine. It is unclear how this study relates to the concerns raised with the DNA test in submissions given the focus is on ferritin and not on MG.
- Imlay *et al*. (1988) discuss the role of oxidative stress in the damage of DNA. No specific mention of MG is made. However, many of the modes of action could be expected to be similar in MG induced cell damage.
- Ruckriemen *et al*. (2017) discuss the reaction of MG with proteins in the context of mānuka honey, with no specific mention of DNA.

It is unclear how submitters used the information in these publications to show that MG was impacting upon the mānuka DNA test.

4.5.1.5 Modification of DNA extraction method

In response to industry concerns regarding 'high grade mānuka' honey, MPI made a minor modification to one of the reagents used in the DNA extraction method. The modification involved the addition of a reagent called Proteinase K. In DNA extraction, proteinase K has the role of breaking down contaminating proteins that impact upon the target DNA being detected. The modified DNA extraction method then underwent a verification process involving commercial laboratories. Honey samples were supplied by several companies to assist in the verification process and MPI is appreciative of their support.

MPI expects that this modification will considerably reduce the proportion of 'high grade mānuka honeys' previously observed by industry to be passing the chemical test but not the DNA test, assuming the test is performed appropriately.

4.5.1.6 Conclusion

In regard to submissions on reasons why honey might fail the DNA test while passing chemical testing, the following summary comments are provided by MPI:

- Samples of 'high grade mānuka' honey tested jointly by MPI and industry had a variety of mānuka Cq values so restriction to 'high grade mānuka' was not substantiated.

- Honey samples tested from previous collections seasons (2009 − 2014) in the MPI science programme and older honey tested by industry passed the DNA test, so an "older" honey will not necessarily fail the DNA test provided it is of good quality.

- Honey samples tested in the MPI science programme suggest that honey with a high HMF value may be more likely to fail the DNA test, but this is not a simple relationship. Assessment of industry data also suggests a similar outcome. Both MPI and industry consider honey samples with HMF levels greater than 40 mg/kg to be of poor quality.

- Analysis of data from the MPI science programme and industry data did not support a quantitative relationship between the level of MG in the honey and the Cq value (further details provided in a later section).

4.5.2 Concerns about the stability of DNA markers

4.5.2.1 General comment

MPI tested a range of honey samples that had been stored by industry across a number of years under a variety of variable conditions. This included 160 New Zealand honey samples, representing honey from 2-7 years of age from a number of different floral sources. Industry had communicated to MPI that the typical shelf life of a retail honey is between 5-7 years; honey older than this can have quality issues due to increasing HMF levels.

The DNA test worked successfully on the majority of the archive honey samples. Some samples (<10%) tested negative for the internal control in the DNA test, however the majority of these were from a single supplier, so there may be a reason specific to this supplier.

MPI notes that the internal control component of the multiplex qPCR targets a DNA sequence common to most plants. The information on the internal control was used to assess stability of the DNA in the context of the archive collection which represented multiple floral types. As the majority of samples across the archive collection tested positive for the internal control, DNA stability was not considered an issue. The archive samples are representative of the various industry practices. In addition, the assessment of DNA stability using these samples caters for more variables than a laboratory controlled experiment that only allows for a limited number of variables to be assessed.

4.5.2.2 Submissions

Several submissions queried the stability of the DNA marker. One submission provided results of mānuka Cq values and mānuka DNA concentrations using the *original* DNA test on seven honey samples stored at 20ºC and 27ºC for up to 881 days. The work was not completed at a MPI recognised laboratory. From details in the submission, it appears that the objective of the stability trial was not to assess the stability of DNA over time, instead it was to assess the stability of chemical markers. The original experimental design would have required different considerations and set up if the objective was to assess the stability of DNA as derived from pollen. Thus we are of the view that the results need to be interpreted with caution.

Other points noted by MPI are as follows:

- While graphical summaries of the data are given, these tended to be as means across samples against time and this may not be an appropriate statistical approach.

- There are some unusual features in the data set that raise issues of data quality and experimental work. For example, one sample tested negative for mānuka DNA for two consecutive time periods (554 and 723 days) and then positive at 881 days. This could suggest a number of potential issues including original sample homogeneity, sub-sampling and/or test repeatability.

4.6 THRESHOLD LEVELS

4.6.1 Concerns that threshold levels for markers were inappropriate

4.6.1.1 General comment

The threshold levels for all markers used in the proposed monofloral and multifloral mānuka honey definition were determined using the CART modelling approach. This approach assesses the data on each marker at the same time to determine the usefulness of the marker for separating different honey types. The approach also determines the level of each marker that when used in combination provides the greatest degree of separation between honey types.

Some submitters' concerns were based on an assumption that the method used to determine the threshold levels was to take the average level of the marker in either the plant or honey samples collected. This is not the approach that MPI used. Such an approach would not be appropriate given the likely variation in the markers both within, and between, different plant species and honey types[1].

Changing the levels of any marker in the proposed mānuka honey definition requires a balance between the number of honey samples that would be wrongly classified as mānuka and the number of honey samples that would be wrongly classified as non-mānuka. The CART modelling approach enabled this balance to be assessed in a systematic and comparative way.

4.6.1.2 Specific proposals to change thresholds

Some submissions stated that the level of 3-phenyllactic acid (3-PA) is too low for the proposed multifloral mānuka honey definition and it should be increased. MPI notes that:

- No suggested levels were provided in the submissions, although at the science technical workshop one suggestion was that 3-PA for a multifloral mānuka honey should be greater than or equal to 100 mg/kg.
- MPI assessed increasing the level of 3-PA to 100 mg/kg and this had a minimal impact on the number of samples which would be classified as multifloral mānuka using the MPI honey data. MPI is not aware of a scientific justification to increase the threshold to 100 mg/kg in comparison with other potential thresholds such as 75 or 150 mg/kg.

Some submissions stated that the level of 3-PA in the proposed definition is too high for the monofloral mānuka honey definition and it should be decreased. Other submissions state it is too low and should be increased. MPI makes the following observations:

[1]MPI notes that the average level of a marker can be used for assessing the potential usefulness of that marker at separating mānuka from different plant species or separating mānuka honey from other honey types. However, appropriate statistical analyses must be used rather than calculating average levels directly from the data or using graphical methods alone.

- One reason given to justify a decrease in the 3-PA level was that the current threshold was too high to account for regional variation in mānuka honey. Graphs of mean levels of markers for some honey samples from different regions were provided in the submissions. However, the submissions did not include the information required to determine the validity of comparing these honey samples and did not show evidence of the analyses carried out to reach the submitter's conclusion.
- Regional variation in 3-PA and all other markers, in both nectar and honey samples, was assessed as part of the MPI science programme. Whilst variation was observed, this was not cause for concern as the variation in the levels of the markers were above the threshold set by the CART modelling approach.
- MPI contends that, instead of using mean levels of markers, assessing the classification of honey samples per region is more appropriate. For example, if all mānuka honey samples from one region failed the proposed definition then we could assess if this was due to the threshold level of one of the markers. No such regional variation in classification was observed when applying the proposed mānuka honey definition.

Different submissions stated opposite views for justification of both increasing and decreasing the 3-PA level for monofloral mānuka honey. Some state it should be decreased as some industry honey currently labelled as monofloral are failing to meet the proposed monofloral mānuka honey definition. Others state it should be increased as the current level enables too many samples to be identified as monofloral mānuka honey.

4.6.2 Submissions regarding threshold levels being too close to limit of detection

4.6.2.1 General comment

When analysis methods are first used, the laboratories (operating under ISO17025) using them rigorously test them to determine the performance limits of the methods with their equipment. For example, they will determine the concentration range in which the test reliably performs, the lowest concentration where a positive identification can be made (the limit of detection or LOD), the lowest concentration that can be measured with an acceptable precision (the limit of quantification or LOQ), and the between and within-day repeatability. Laboratories use information from this method validation process to determine how they will use the method in routine testing, such as establishing limits of reporting (LOR).

Depending on the test method and the laboratory's client requirements, the LOD and LOR can be the same, however, the LOD is commonly lower. The LOR is normally equal to or greater than the concentration that can be measured with an acceptable precision (the limit of quantification or LOQ). Both the chemical and DNA test methods have established LOR and LOD (lower than LOR).

Due to slight variations in how any test is performed, the equipment used or the environment, the same sample is likely to produce slightly different results each time it is tested. This is expected, however, there is a limit to the level of acceptable variation, referred to as uncertainty of measurement (UoM) or measurement uncertainty (MU). The uncertainty of measurement is determined by the laboratory during its initial validation of a test method. If a replicate result falls outside the established UoM, this indicates to the laboratory that the unexpected result must be investigated.

Laboratories typically have quality control samples at critical concentration levels (such as LORs or decision limits) that allow them to reliably measure at these concentrations.

4.6.2.2 Submissions

Some submissions have raised concerns that the proposed mānuka honey definition has threshold values for the chemical and DNA markers that are at the limit of detection. MPI contends that the uncertainty of measurement for that concentration is more important and the uncertainty of measurement would affect any given threshold. To give context, many regulatory tests worldwide set threshold limits close to LOR.

One submitter detailed a scenario to support their contention that repeated testing on a sample would mean there is 50/50 chance that the result would be above or below the threshold. For example, the submission proposed that an uncertainty of ± 25% at 1 mg/kg would mean that 95% of the time a sample could have a value between 0.75 mg/kg and 1.25 mg/kg (assuming the uncertainty is a 95% confidence interval, however, this is not stated in the submission). The submission concluded that there was a 50/50 chance that a repeat test could pass or fail the threshold. MPI makes the following observations:

- The submitter's statement around a "50/50 chance" only applies to samples with a true concentration of 1 mg/kg, as the reported value will be greater than 1 mg/kg for 50% of the time. MPI considers that this statement is not appropriate for honey samples when applied to true values (actual real value of the sample) greater or below the threshold value.

- For any sample with a true value on any threshold for any test (not just tests for mānuka honey), assuming a symmetric uncertainty range around the threshold, there will always be a 50/50 chance of that sample being higher or lower than the threshold.

Some submissions stated that as threshold levels for markers in the proposed mānuka honey definition are at the limit of reporting, then by incorporating uncertainty, there is a 33% chance of a "borderline honey" passing the threshold. This is based on an underlying assumption for this estimate that the levels of each marker are independent. This approach is incorrect and in MPI's view this is highly unlikely because a true mānuka honey is likely to have high values for each marker. MPI also questions the underlying assumption that the uncertainty of measurement is the same for all markers.

Some submissions suggested that the threshold levels used in the proposed mānuka honey definition should incorporate the uncertainty of measurement for each marker. For example, instead of a honey sample needing a level greater than or equal to 1 mg/kg for a marker, then a honey sample would meet the criteria if the test result was between 0.8 mg/kg and 1.20 mg/kg.

However, MPI considers that applying an acceptable range for a specific marker based on an individual laboratories MOU is not an appropriate approach as it may not be transferable across laboratories. Regardless of the threshold there will always be some honey that will only just fail the threshold even if it was set at 0.8 mg/kg.

4.7 STABILITY

4.7.1 Concerns about the stability of chemical markers

4.7.1.1 General comment

During the MPI science programme, the stability of the chemical markers was investigated using laboratory controlled experiments. The levels of the chemical markers were measured in six mānuka honey samples and then each sample was stored at three temperatures (4, 20 and 35°C). The levels of the chemicals were measured again 68 days later. This time point was used to help inform which chemical markers were further evaluated in samples from the second season of the MPI science programme. Each sample was tested in triplicate at each temperature and time point to ensure the level of the chemical measured was representative of the sample.

MPI analysed the data to determine if there was a statistically significant difference (95% confidence level) between the levels of the chemical markers as a result of the storage conditions studied. For the purposes of this experiment, a chemical was judged as not being stable when:

- a statistically significant difference was found between the level measured at the start of the experiment and the level when stored at one or more of the temperatures. This difference could be an increase or a decrease in the level of the chemical marker.
- a statistically significant difference between the levels of the different chemical markers at the start of the experiment and 68 days later.

Consistency in observed increases or decreases in the levels of a chemical was also evaluated. For example, did all samples behave in the same manner for each chemical or did some samples increase whilst others decreased? The overall results from this work have been presented in the MPI science programme summary document.

All four chemical markers in the proposed mānuka honey definition were deemed to be stable over increasing temperature and time. The MPI science programme also found the starting concentrations of the samples used in the stability trial did influence the degree of change observed for each marker.

In addition, the MPI science programme tested a range of honey samples that had been stored by industry, under a variety of variable conditions, across a number of years. This involved testing 160 archive New Zealand honey samples representing honey from 2-7 years of age from a number of different floral sources. Industry had communicated to MPI that the typical shelf life of a honey is between 5-7 years as honey older than this can have quality issues due to increasing HMF levels. The chemical markers could be detected, and at high levels, in the archive honey samples identified as mānuka honey by the supplier. The archive samples are also more representative of the various industry practices than conditions studied in a stability experiment. The detection of the chemical markers at levels which meet the proposed mānuka honey definition in these samples caters for more variables than a laboratory controlled experiment that only allows assessment of a limited number of variables.

4.7.1.2 Submissions

Two submissions provided results of stability trials for levels of the chemical markers included in the proposed mānuka honey definition and leptosperin. Both submissions stated that these results indicate that leptosperin is stable at increasing temperatures and time

periods. They also stated that several of the markers used in the proposed MPI definition are not stable.

MPI is of the view that the two stability trials referred to in the submissions did not apply appropriate statistical analyses for the type of data. There are also inconsistencies in the methodology used and the way information was presented. For example, sample identification numbers suggest other samples may also have been analysed at least for part of the experiments, but results for these samples have not been included in the information to MPI.

While both submissions provided graphical summaries of the data, this tended to be as means across samples against time. This is problematic as the individual samples are in fact often very different from one another and this is masked by taking a mean. To test these concerns with the two stability trials, MPI re-analysed the data using linear mixed effects models. As information was limited in the submissions, MPI made certain assumptions when re-analysing the data:

- Samples had been stored and homogenised appropriately prior to testing.
- Control samples were measured prior to storage at the two temperatures and all were measured under the same laboratory conditions.
- Sub-samples taken at designated time points had been held at appropriate archive storage temperatures in suitable storage containers and had undergone limited freeze thaws.
- Data corresponded to single individual subsamples rather than means of replicates.

The data presented in both submissions include measurements across multiple time points and for time periods longer than a year. This enables a percentage change to be calculated either per annum or per time period studied. MPI did not consider a percentage change calculation appropriate for the MPI stability trial. Therefore, a direct comparison using a percentage change is not possible, but general trends can be compared.

4.7.1.3 Submission 1

The data were from 7 honey samples stored at 20°C and 27°C and tested at four/five time points over 811 days. The rationale for the selection of storing at 27°C is not clear, but this temperature was not studied in the MPI science programme. This temperature is not high enough as storage temperatures of honey during harvest, post-harvest, processing and transit to point of sale will be higher. Although the honey is unlikely to be stored for long periods at high temperatures (>30°C), this could influence the concentration of the markers in the honey.

MPI reports the following results and observations:

Marker	Analysis by submitter in submission 1	Analysis by MPI of data in submission 1	MPI Summary comments
4-HPA	• No statistically significant change when stored at 20°C. • Statistically significant increase by 4.5% per annum when stored at 27°C.	• No statistically significant change when stored at 20°C. • Statistically significant increase by 4.6% per annum when stored at 27°C.	• Agree with conclusions made in the submission that 4-HPA is stable with time and temperature.
3-PA	• No statistically significant change when stored at 20°C. • Statistically significant increase by 1.6% per annum when stored at 27°C.	• No statistically significant change when stored at 20°C. • Statistically significant increase by 1.7% per annum when stored at 27°C.	• Agree with conclusions made in the submission that 3-PA is stable with time and temperature.
2-MBA	• Statistically significant increase by 5.9% per annum when stored at 20°C. • Statistically significant increase by 9.7% per annum when stored at 27°C.	• Statistically significant increase by 5.4% per annum when stored at 20°C. • Statistically significant increase by 9.5% per annum when stored at 27°C.	• Although lower percentage change found by MPI analyses, these are similar to the submitter's conclusions.
2'-MAP	• Statistically significant decrease by 17% per annum when stored at 20°C • Statistically significant decrease by 12% per annum when stored at 27°C	• Decrease by 15.5% per annum when stored at 20°C, but not statistically significant. • Decrease by 7.9% per annum when stored at 27°C, but not statistically significant.	• Data more complex than other markers indicating a straight line trend is inappropriate. • MPI contends from its own analysis of the data presented in the submission that 2'-MAP is stable over time.
Leptosperin	• Statistically significant decrease of 4.4% per annum when stored at 20°C. • Statistically significant decrease of 4.3% per annum when stored at 27°C.	• Statistically significant decrease of 4.6% per annum when stored at 20°C. • Statistically significant decrease of 4.7% per annum when stored at 27°C.	• Both estimates (4.6% and 4.7%) are close to the submitter's suggested threshold of lack of stability at 5%.

This submission reports that the criterion for stability is provided as when a non-significant trend is found or a significant trend of 5% or less change per annum. No rationale is provided for the 5% cut-off threshold and why this would be acceptable. From MPI's analyses of the submitter's data, several significant trends of changing concentration over time were found, including for leptosperin. However, using the submitter's 5% criteria only 2-MBA would be considered as not stable. The change in 2-MBA was a small increasing trend, which would mean that a sample would still meet the proposed definition over the shelf-life of the honey.

4.7.1.4 Submission 2

For this submission, 10 samples were stored at 37ºC and sampled at four time points up to 444 days. MPI reports the following results and observations:

Marker	Analysis by submitter in submission 2	Analysis by MPI of data in submission 2	MPI Summary comments
4-HPA	• No statistically significant change	• No statistically significant change	• Agree with conclusions made in the submission that 4-HPA is stable with time and temperature.
3-PA	• No statistically significant change	• No statistically significant change	• Agree with conclusions made in the submission that 3-PA is stable with time and temperature.
2-MBA	• Statistically significant increase by 8.8% over 444 days	• Statistically significant increase by 7.9% per annum	• Although lower percentage change found by MPI analyses, these are similar to the submitter's conclusions. • Evidence of a small increasing trend with time when stored at 37°C.
2'-MAP	• Statistically significant decrease by 20% over 444 days	• Decrease by 8.2% per annum, but not statistically significant.	• Data more complex than other markers indicating a straight line trend is inappropriate. • MPI analysis of results differs from that in the submission as the original analysis does not account for the substantial variability in the data set. • MPI contends from its own analysis of the data presented in the submission that 2'-MAP is stable over time.
Leptosperin	• Decrease of 0.1% over 444 days, but not statistically significant.	• Increase of 0.65% per annum, but not statistically significant.	• MPI analysis of the data presented in the submission agrees with finding by the submitter. • However, a small increase rather than a decrease in concentration was observed.

From submission 2, the only significant trend over time was for 2-MBA, and again this was a small increasing trend meaning that levels are unlikely to drop below the proposed threshold level over time. No decrease in leptosperin was observed over time, which is different from that observed from the stability trial in submission 1.

4.7.1.5 Publications provided in submissions

Submission 2 also provides an abstract of a paper which has been submitted for scientific publication with authors affiliated to the submitter (Bong *et al.*, 2017b). It is not clear if the stability data provided is the same as that referred to in the abstract, but similar conclusions are made. Despite the conclusion made by the submitter that 2'-MAP is unstable, it is still

recommended by the authors of the paper as a useful marker for mānuka honey. This suggests that criteria for marker selection were not applied consistently during the same research study.

Two publications were specifically quoted in this submission for consideration by MPI as evidence for the stability of leptosperin and they are evaluated below:

- Kato (2014) investigated the stability of leptosperin as part of a study to determine leptosperin and methyl syringate as suitable markers for mānuka honey. As part of the stability trial, honey samples were stored at two temperatures (37ºC and 50ºC) for 30 days and samples tested at 6 time points. The publication concludes that leptosperin is stable. MPI contends that further evidence would be needed to confirm this:
    - The total number of honey samples tested for the stability trial is not clear. Mānuka honey samples used in other sections of the paper are detailed, but they are not specifically mentioned for the stability trial.
    - No data are presented, with only a graphical summary provided. Information is not well labelled, no uncertainty ranges are provided, sample sizes are not reported and it is not clear if means were plotted or if the plot is of an individual honey sample.
    - No statistical analyses are performed on the data.
    - The publication states that methyl syringate decreased within 30 days at both temperatures, but leptosperin was relatively stable. There is no evidence in the paper as to what is meant by the term "relatively stable" and a percentage change is only provided for methyl syringate.
    - The MPI science programme found methyl syringate to increase significantly (at 95% confidence level) when stored at temperatures of 35ºC for 66 days. An increase in methyl syringate was also observed by Bong *et al*. (2017a). Although it is possible to have different findings in comparison with Kato (2014), it is surprising to find a change in concentration in a completely different direction.
- Bong *et al*. (2017a) investigated the stability of leptosperin as part of a study on leptosperin as a detectable fluorophore in *Leptospermum* honeys. Honey samples were stored at 37ºC and subsampled at three time periods up to and including 444 days. The publication concludes that leptosperin remains "relatively constant" throughout the study period. MPI contends that further evidence would be needed to confirm this:
    - Limited information is provided on the origin of the honey samples and why they were selected.
    - No details are given about the homogenisation procedure prior to subsampling.
    - Mean percentage change is provided in a graphical summary with one standard error around the mean reported. This does not enable the between-sample variability at each time point to be assessed and the data are not provided elsewhere in the paper.
    - The starting levels of leptosperin are not reported for the samples, therefore, it is unclear if this factor influenced the reported stability.
    - The statistical analyses are not adequately described for the stability data and are not appropriate for the type of data.
    - The level of test validation is not known.

From the stability trials and the publications, different conclusions are evident on the stability of leptosperin under increasing temperature and storage time. Assuming all other factors are equal, this suggests that determining the changing levels of leptosperin is dependent on the samples selected for the trial and whether they can be considered representative of honey produced in New Zealand.

Given the concerns and limitations detailed above, MPI concludes that there is insufficient evidence to have confidence that leptosperin is stable over time.

4.8 STATISTICAL ANALYSIS

4.8.1 Submission that independent determination of the floral source used to describe the honey samples is required

4.8.1.1 General comment

For the MPI science programme reference collection, honey samples were sourced from single apiaries in New Zealand representing seven production years. A single apiary site was defined as a known geographic location at which one or more bee hives were placed and honey was harvested, extracted and stored from this site without blending with honey from other areas. Samples were obtained directly from beekeepers (including professional and hobbyist beekeepers) and honey packers prior to blending. Suppliers were asked to nominate the main floral source of the honey samples.

MPI asked each supplier to provide traceability data on every honey type such as apiary site location, start and end date of flowering period and harvest date. Storage temperature and length of storage period were also documented to ensure full sample traceability. This information was used to ensure a valid comparison of the honey samples could be made.

Honey samples from other countries were also sourced either directly from retail or using local contacts. As such full traceability information about these samples or at times the main floral source was unknown. However, as *Leptospermum* is absent or uncommon in most of the overseas countries from which samples were collected, with the exception of Australia, these samples were considered as one group. Samples from Australia were considered separately due to the native distribution of *Leptospermum* species of plants in Australia.

The purpose of the MPI science programme was to define mānuka honey because there was no existing means to achieve this in a regulatory context. MPI designed the science programme to make maximum use of information about the source plant (e.g. DNA from pollen and chemicals found in nectar) to provide information on the associated honey.

The CART modelling approach used to develop the proposed definition was not dependent solely on the named honey type of each sample. The levels of the markers and the similarity of the data was used to determine whether the samples should be grouped together or not. Further, the sensitivity of the CART model output to the honey types and the samples included in the model were analysed as part of the programme. The CART modelling approach determines which honey samples are authentic or not by using the marker data from all honey samples rather than using a pre-processing step to remove certain or unwanted data prior to analysis.

4.8.1.2 Submissions

Some submissions raised a concern that the identity of honey samples in the MPI honey reference collection may be incorrect. It was suggested that an independent determination of the floral sources should have occurred prior to using the data from the honey samples to develop the proposed definition.

Several methods were suggested as additions to the MPI methodology and these were evaluated as follows:

- Apply multivariate analyses techniques using all markers measured in the science programme to identify honey samples which are similar and the potential for others to be considered as outliers.
    - MPI assessed a range of potential markers as part of the science programme and some of these were found to be unsuitable for separating mānuka honey from other honey types. Therefore, they cannot be used with confidence to independently confirm the floral source of a honey on their own or in combination.
    - MPI contends that this approach does not ensure that the same separation of mānuka honey from other honey types will occur using a subset of the markers, as can be achieved through the CART model.
- Use pollen as measured by microscopy to verify the floral source.
    - Pollen as measured by microscopy was evaluated as part the MPI science programme and found to be unsuitable for use in a mānuka honey definition. Some studies have suggested that mānuka can be differentiated from kānuka pollen using microscopy. However, MPI considers that this cannot be done with sufficient confidence for regulatory application.
- Use taste testers to determine the floral source.
    - It is not scientifically robust to use one taste tester for over 800 honey samples and therefore a panel of tasters would need to be established. A tasting panel would require substantial resources to establish, but would not provide accurate verification of the floral source of a honey sample.

4.8.1.3 Conclusion

It is unclear from the information in the submissions how suggested methods to "independently" determine the floral source would materially strengthen the scientific evidence underpinning the proposed MPI definition.

4.9 BLENDING

4.9.1 Submissions that blending honey can achieve the proposed **mānuka honey definition**.

4.9.1.1 General comment

Any honey definition using markers set at thresholds which are publically known can be blended to, irrespective of the markers used. This would also apply to any definition created using leptosperin, MG, DHA or any other approach used. This is due to the nature of honey production and the activity of bees. Naturally, all honey will contain both chemical and DNA markers that come from a number of different plant species. Bees will visit a number of different plants to collect both nectar and pollen and return to the hive. This means even

monofloral honey will contain nectar and pollen from more than one floral source. Markers which may only be found in specific plant species (e.g. mānuka) will still be present in honey predominantly of a different floral source (e.g. kānuka). It is not surprising to find some mānuka markers in other honey types, this is simply a reflection of the variability found in a natural product.

Blending is routine practice for many industries to meet certain product specifications. The honey industry routinely blend varieties of honeys together for a number of reasons:

- Increase the volume for a particular batch by blending 'like' with 'like' from different apiary sites.
- Dilute a honey that has high concentrations of the required markers to meet the minimum threshold levels for particular grading systems.
- Dilute contaminates such as HMF or tutin.

Unfortunately blending also occurs as part of fraud, adulteration and mislabelling practices. Having a robust scientific definition for mānuka honey is only one aspect needed to address acceptable blending practices. Other information must also be assessed such as harvest declarations, traceability and testing records of honey that comprise a batch.

Using a combination of markers (chemical and DNA based) makes it more challenging to accurately blend honey types to meet the proposed definition for mānuka honey. The proposed definition includes one DNA marker specific to *L. scoparium* and a chemical marker which we have also found to be specific to *L. scoparium* in the context of the MPI reference database.

4.9.1.2 Submissions

Several submissions raised concerns that the proposed MPI mānuka honey definition is susceptible to blending to meet the definition. Blending may be used to increase volumes through diluting monofloral mānuka honey or to compromise authenticity.

A first response is to attempt to estimate the scale of blending of concern that might occur. However, this remains largely unknown from the submissions. A number of factors will influence this including:

- The availability of honey samples that when blended produce the required end product specifications.
- The influence of seasonal and environmental changes on available honeys with the required specifications for blending.
- Economic incentives.
- The individual practices of industry members.
- The impact that blending will have on markers that are part of industry grading systems.

One submission included an evaluation of a limited data set to predict what might happen when different honey types were blended together. However, many of the samples considered as non-mānuka by the submitter contain significant levels of some of the markers in the proposed MPI mānuka honey definition. If you were to blend these honeys with a honey that meets the proposed definition, the final product may still meet the proposed definition as the levels may remain above the proposed thresholds.

Other submissions provided examples of theoretical blending of honey samples for which there were known test results. While this approach illustrates the possibility of blending to achieve mānuka honey, MPI is of the view that this work does not shed light on the possible scale of potential blending for the following reasons:

- In general, a small number of samples of unknown provenance and selection criteria were blended.
- In the larger theoretical blending experiment, the values of samples that were blended together were based on a mean value of each marker for a specific honey type. MPI contends that the means are unlikely to be representative of the diversity within a honey type and do not enable the difference between samples to be accounted for[2].
- The raw data showed that many of the honey samples would be reclassified using the proposed MPI definition as either multifloral or monofloral mānuka honey. Therefore, MPI contends that the blending scenarios explored in the submission do not appropriately assess blending concerns raised by industry about the proposed MPI mānuka honey definition.
- The blending experiments did not state important assumptions around how the samples were blended together. MPI assumed the values of each chemical were mixed using a simple linear calculation using the proportions blended as weights.

Two submissions detailed results where testing of physically blended honey samples was carried out. One of these provided data from a limited number of non-mānuka samples that were blended together to produce a monofloral mānuka honey. While this shows a potentiality, MPI contends that more comprehensive scientific work will be needed to establish the likelihood and feasibility of such blending results, taking into account appropriate criteria for sample selection and standardised blending scenarios.

In another submission, a blending scenario is explained using two samples. The supplier identified their two samples as kānuka and bush honey, however MPI notes that the kānuka sample met the proposed monofloral definition. This honey supplier claimed that there was very little *L. scoparium* at the apiary site and that the kānuka and bush honey could be blended together to meet the proposed definition. However, both samples contained substantial amounts of mānuka pollen DNA. In addition, 2'-MAP (only found in *L. scoparium* to date) was also detected in both samples. This provides supporting information that *L. scoparium* was growing in the area where the kānuka and bush honey originated. Therefore an alternative explanation could be that the original sample was in fact mānuka honey according to the proposed MPI definition, and that the source plants in the area had been inaccurately identified.

4.9.1.3 MPI assessment of blending scenarios

In response to industry concerns, MPI explored the blending issue further by conducting numerical blending experiments using New Zealand honey samples from the MPI mānuka honey science programme reference collection (n = 660). The impact of blending honey using a number of different scenarios to create honey that met either proposed mānuka honey definition was assessed. This was done by measuring the proportion of samples that when blended produced honey that met either of the proposed MPI definitions mānuka honey.

[2]MPI recommends that it would be better to perform an experiment where randomly or systematically selected data points were repeatedly obtained from each group in the data set and then combined by some method. This would then allow for variability between samples. However, this is not possible with the data set in the submission, particularly for ling honey as only a single sample was tested. The supplier labelled this sample as "ling/heather/man", but the submitter relabelled it has "ling". Results of blending between a single sample of ling honey (and likely to be a blend with mānuka) and the other honey types should be interpreted with caution.

For the blending scenarios that were assessed, **the non-mānuka honey was always the larger proportion in the blending scenarios** as this reflected the blending scenarios of concern. Two different honey types were blended together in different proportions including: 90/10, 80/20, 70/30, 60/40 and 50/50. The following two honey types were blended together in all five proportions and compared:

Scenarios for blending non-mānuka honeys to meet the proposed mānuka honey definitions

1. Non-mānuka with non-mānuka honey
2. Kānuka with kānuka honey
3. Kānuka with non-mānuka honey

Scenarios for blending mānuka honey with non-mānuka honey to meet the proposed mānuka honey definitions

4. Non-mānuka with monofloral mānuka
5. Non-mānuka with multifloral mānuka
6. Kānuka with monofloral mānuka
7. Kānuka with multifloral mānuka

All possible pairings of the samples were blended, giving the maximum number of blends possible with the MPI reference collection. The total number of blended honeys created per combination ranged from 324 blended honeys to 93,636 blended honeys. The range depended on the total number of honeys in the reference database that met the identification criteria for each scenario being assessed. The seven subsets were created based on whether or not samples would meet the proposed MPI monofloral or multifloral mānuka honey definitions, not what the floral type originally identified by the supplier. Kānuka samples used for the blending scenarios were those originally identified as kānuka by the supplier, but did not meet either proposed mānuka honey definition. Noting there is no scientific definition for kānuka honey.

As this was a theoretical blending experiment it was assumed that:

- Physically blended samples would behave in the same way as in our calculations.
- The chemical markers would combine perfectly in proportion (e.g. a honey sample with 2'-MAP value of 0.5 mg/kg blended in a proportion of 50/50 with a honey sample with a 2'-MAP value of 1.5 mg/kg would produce a mixture with a 2'-MAP value of 1.0 mg/kg).
- Combining mānuka Cq values in the same way as the chemical markers was not considered valid. As such, a conservative approach was taken and the maximum mānuka Cq value was used when combining two samples in whatever proportion they were blended.

Table 1: Summary of results

Blending scenario		**No. of blended honeys created for each of the 5 blending ratios**	**Can you blend to meet the proposed monofloral mānuka honey definition?**	**Can you blend to meet the proposed multifloral mānuka honey definition?**
Blending two non-mānuka (including kānuka) honeys together	1.Non-mānuka with non-mānuka honey	93,636	No	No
	2. Kānuka with kānuka honey	324	No	No
	3. Kānuka with non- mānuka honey	5,184	No	No
Blending a non-mānuka (including kānuka) honey with a mānuka honey	4. Non-mānuka with monofloral mānuka	80,478	Yes, dilution effect* but less will meet the definition	Yes, dilution effect* but less will meet the definition
	5. Non-mānuka with multifloral mānuka	27,846	Yes, but limited possibility	Yes, dilution effect* but less will meet the definition
	6. Kānuka with monofloral mānuka	4,734	Yes, dilution effect* but less will meet the definition	Yes, dilution effect* but less will meet the definition
	7. Kānuka with multifloral mānuka	1,638	Yes, but limited possibility	Yes, dilution effect* but less will meet the definition

* Note: Dilution effect refers to reducing the levels of the markers in the honey by blending, but markers for some of the blends are still at levels which meet the definition.

MPI assessed that blending was only a remote possibility ('No' in the table above) where testing all five ratios of blending honey samples resulted in less than 0.2% of honeys that would meet either proposed mānuka honey definition.

There were a very limited number of potential blending outcomes where the final blend met the proposed monofloral mānuka honey definition. An example of this is where some kānuka honeys were blended with a multifloral mānuka honey resulting in some blends that meet the monofloral definition. However, of the 18 samples in the MPI database (n = 660) provisionally labelled as kānuka by the supplier, this blending scenario was not possible for most of them. Blending would need to be done very deliberately and would be dependent on the availability of specific honeys with certain profiles that when blended at specific ratios produce a final blend that met the proposed monofloral definition.

MPI looked at how this risk can be mitigated. For the blending scenarios where the potential existed, increasing the threshold level of 2'-MAP in the proposed mānuka honey definition

was assessed as a mitigation option. Based on current knowledge, this chemical is specific to *L. scoparium*, therefore, by increasing its levels to ≥ 5 mg/kg, the amount of *L. scoparium* nectar represented in the honey is increased.

Increasing the level of 2'-MAP minimised the potential for blending of a kānuka honey with a multifloral mānuka honey to achieve a honey that meets the proposed monofloral mānuka definition.

4.9.2 Conclusion

Although some submissions claimed that two non-mānuka honey types could be blended to meet the mānuka honey definition, MPI determined from the evidence provided that this is only a remote possibility.

MPI's own analyses of blending indicated that apart from the inevitable dilution potential inherent in any blending situation, the MPI definition severely limited the possibility for creating more mānuka honey by deliberate blending. However, as the possibility exists, increasing the level of 2'-MAP for both definitions minimises this possibility.

4.10 OTHER ISSUES

4.10.1 Submission that the proposed definition does not meet the CODEX Standard for honey

4.10.1.1 General comment

There is no Codex Standard for mānuka honey. The Codex standard was developed for European honeys and as such includes parameters and characteristics that have not been extensively validated for New Zealand honeys. Codex primarily identifies honeys based on organoleptic, physicochemical and microscopic properties corresponding with the origin of the honey. Testing used to determine many of these characteristics is subjective and not suitable as part of a regulatory definition for mānuka honey.

4.10.1.2 Submissions

Some submissions raised concerns that the proposed definition did not provide a percentage of mānuka honey and referenced the "wholly or mainly' statement in the Codex Standard. Codex does not provide a percentage requirement to meet 'wholly or mainly'. In general, the Codex Standard of "wholly or mainly" has been interpreted and applied to pollen counts in honey. This represents the percentage of pollen present in the honey only and does not directly relate to the percentage of nectar contribution.

Based on currently available science it is not possible to determine the percentage contribution of nectar from a specific source plant to a particular honey. At a minimum the following information would need to be generated and validated across regions and seasons:

- Nectar production per plant at an apiary site.
- Nectar contribution from individual plants to the honey.
    - Environmental influences will affect the number of flowers open per plant, the volume of nectar produced by each flower as well as the flowering period for each plant.
- Number of flowers visited by bees per plant per hive.

- Proportion of available nectar collected from flowers by the bees per hive.
- Proportion of nectar transferred from the bees into the honey for each hive.

4.10.2 Submissions suggesting alternative testing methods for identifying monofloral mānuka honey

Different test methods have been suggested in submissions for detecting chemical markers in mānuka honey. In general, the test methods are based on one or a combination of techniques which are used to measure the abundance of many chemicals within a sample using a single test. Graphical outputs (chromatograms) are produced which show each chemical in a sample as a different peak. The outputs of many samples must be collated and the patterns analysed to find chemicals which are similar across honey samples from the same floral source. The chemical may already be known or the chemical may currently be unidentified, in which case further work is required to elucidate the structure, composition and name of the chemical found.

There are several publications referencing these techniques to solve food authenticity problems and laboratories outside of New Zealand do offer these methods as commercial services. Although they all have the potential to be useful for identifying chemicals present in honey, there are currently some key limitations in using these techniques within a regulatory definition as follows:

- Some of these chemicals may already be identified by other techniques, but others may still remain unknown. Identification of the chemical is not straightforward and requires the use of further laboratory processes. A regulatory definition cannot be verified if it is based on a list of unknown chemicals.
- With the potential for many thousands of chemicals to be identified from these techniques, there must be a large database of honey samples representative of both mānuka and non-mānuka honey.
- The large datasets generated by chemical fingerprinting require considerable statistical expertise and complex models for application. Many of these models are not transparent and do not easily translate into defined levels for the chemicals. This means the model would need to be run each time a sample had to be tested to be defined as mānuka honey.
- The techniques are not easily transferrable between different laboratories as they are dependent on the database of honey samples and the software used to detect the chemicals.
- Currently there is limited capability in New Zealand commercial laboratories to use such test methods, particularly NMR (nuclear magnetic resonance). Sending samples offshore for regulatory testing would present several logistical challenges.

MPI recognises that new approaches and associated test methods will have the potential to help support authentication of mānuka honey in the future.

4.10.3 Submission that the proposed definition is not appropriate for future developments in **plant breeding programmes for mānuka**

4.10.3.1 General comment

The scope of the MPI mānuka honey science programme was to develop a definition for monofloral and multifloral mānuka honey when sold as a food product. The scope of the programme did not include current or future plant breeding programmes. The normal practice for producing honey sold as a food product is by placing hives within naturally growing stands of flowering mānuka plants. Plant breeding programmes for mānuka are based on selecting specific characteristics of the mānuka plant and ensuring this characteristic is produced in more individual plants. The plants with these characteristics are then grown in the one area, such as a plantation, where hives can be placed. Specific characteristics selected may include high production of certain chemicals in the nectar, resistance to disease, greater number of flowers or extended flowering periods.

The influence of selecting these traits on the mānuka honey produced, and whether it will continue to meet the proposed mānuka honey definition, is unknown. However, it is unlikely to have a large influence if the same suite of chemical markers continue to be produced and flowers are not physically changed (i.e. still have pollen).

4.10.3.2 Submissions

One concern raised was that breeding programmes may produce plants with male sterile flowers, meaning no pollen would be produced by the mānuka plants. In this case, honey produced from these plants would not meet the proposed mānuka honey definition.

Measuring the level of mānuka pollen DNA in honey was found to be a valuable marker for determining mānuka authenticity. Pollen is commonly used in overseas markets, particularly in the European Union, as an authenticity marker of honey. Pollen is also commonly used to ensure integrity of product such as checking that the honey has not be excessively filtered and that the honey is actually honey and is not sugar syrup. As such, absence of pollen in a honey may not be acceptable in overseas markets.

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