Ministry for Primary Industries
Manatū Ahu Matua

Criteria for identifying mānuka honey

A summary of the **mānuka** honey science programme

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Executive summary

Mānuka honey is an iconic New Zealand product that attracts a high retail premium. Overseas markets and New Zealand consumers have raised concerns about the lack of a regulatory definition to provide confidence in the authenticity of the products they are buying.

To date, there is no definition with the scientific rigour and robustness necessary to use in regulation to authenticate mānuka honey when sold as a food. To address this issue, the Ministry for Primary Industries (MPI) initiated the Mānuka Honey Science Programme in 2014 (the Science Programme).

The Science Programme's aim was to develop robust science-based criteria for identifying mānuka honey that could be used to provide product assurances and demonstrate product authenticity in a regulatory context.

To achieve the aim, the Science Programme:
- established plant and honey reference collections;
- identified suitable attributes;
- tested plant and honey samples;
- developed and validated laboratory test methods; and
- analysed and interpreted the data that was collected.

MPI led, designed and managed the Science Programme. It also provided technical input and reviewed all aspects of the work. The Science Programme was helped by contributions from, and consultation with, members of the honey industry. Contracted technical specialists, from both New Zealand and overseas, provided expertise in the areas of plant collection and identification, field work, honey testing, DNA and chemical test development, and validation and statistical analyses.

MPI established two reference collections: plant and honey. The plant reference collection comprised nectar, leaf and pollen samples from over 700 plants from 12 regions in New Zealand and five states in Australia. The plant samples were collected during two flowering seasons: 2014/15 and 2015/16.

The honey reference collection (804 samples) was established using honey sourced from New Zealand suppliers (generally beekeepers or honey producers) and overseas. The New Zealand honey samples were primarily sourced from single apiaries and had not been blended. Suppliers gave MPI a description of the honey type based on flowering at the apiary site. Overseas samples were sourced from a number of countries and were mainly retail products.

The reference collections were used to establish the suitability of candidate attributes for identifying mānuka honey. To provide confidence in the test results, key test methods to detect the attributes of interest were validated to produce accurate and reliable results.

To determine which of the attributes were suitable to authenticate honey as mānuka honey and then further identify the honey as either monofloral or multifloral mānuka honey, a staged statistical approach was used. The first stage involved analysing each individual attribute and their levels in both the nectar and honey data. The outputs of this process reduced the number of candidate attributes to those that were most useful at distinguishing between plant species and honey types.

Further analysis using a classification modelling approach was used to develop the identification criteria. The main benefit of using a classification model is that it enables assessment of numerous attributes and their variability to produce criteria that are straightforward to describe. This characteristic is ideal because it allows for complete transparency.

It is important to highlight that the classification model determined which attributes, and their defined threshold levels, are suitable to separate honey types. These outputs were used to establish threshold criteria for different honey types, including monofloral and multifloral mānuka honey. An important finding of the classification modelling approach was that no single attribute can be used alone to identify mānuka honey but a combination of attributes can be used.

The Science Programme included honey samples from two collection seasons (2014/15 and 2015/16) and archive samples ranging from 2009 to 2014. This approach enabled the influence of a variety of seasonal and environmental conditions on honey composition to be assessed. However, as honey is a natural product that varies with changes in the environment, it will be necessary to be mindful of such factors in the future.

The key finding is that mānuka honey can be authenticated using **FIVE** attributes, in combination, as follows:

1) To authenticate honey as monofloral mānuka honey:
- ≥ 1 mg/kg 2'-methoxyacetophenone; **AND**
- ≥ 1 mg/kg 2-methoxybenzoic acid; **AND**
- ≥ 1 mg/kg 4-hydroxyphenyllactic acid; **AND**
- ≥ 400 mg/kg 3-phenyllactic acid; **AND**
- DNA from mānuka pollen (< Cq 36 which is approximately 3 fg/µL DNA).

2) To authenticate honey as multifloral mānuka honey:
- ≥ 1 mg/kg 2'-methoxyacetophenone; **AND**
- ≥ 1 mg/kg 2-methoxybenzoic acid; **AND**
- ≥ 1 mg/kg 4-hydroxyphenyllactic acid; **AND**
- ≥ 20 but < 400 mg/kg 3-phenyllactic acid; **AND**
- DNA from mānuka pollen (< Cq 36 which is approximately 3 fg/µL DNA).

There was good alignment between the honey types identified by the suppliers and the honey that was identified using the authentication criteria as determined by the Science Programme. For example, 74 percent of honey samples originally identified by the supplier as monofloral mānuka met the monofloral mānuka honey threshold criteria and a further 12 percent met the multifloral mānuka honey threshold criteria. In addition, over 50 percent of samples originally identified by the supplier as multifloral mānuka met the monofloral mānuka honey threshold criteria.

Although beehives may be placed at sites mostly composed of mānuka plants, bees may not always forage from the main flowering plant in the area. Therefore, the classification of some honey types initially identified by the supplier as monofloral or multifloral mānuka honey may change after being assessed under the classification criteria. It is also important to note that most samples came from single-source apiaries and do not reflect blending practices within the honey production supply chain. Therefore, similar correlations with the identification criteria mentioned above may not be evident for products in the market place currently identified as monofloral or multifloral mānuka honey.

1 Purpose of the document

This document provides a high-level summary of the science process and outcomes in establishing criteria to authenticate mānuka honey.

It is not intended to be a full scientific document detailing all of the work done and results produced. The MPI mānuka honey science team will produce further detailed scientific reports resulting from this work programme.

2 Definitions and abbreviations

Word	Meaning
Attribute	Quantitative characteristic common to both the source plant and associated honey.
Blend	Refer multifloral.
Classification model	A statistical approach (CART – classification and regression tree) that uses mathematical relationships to identify groups (eg, honey type) based on common attributes at defined levels.
Cq	Quantification cycles – scientific unit used to represent a test result from a qPCR test.
fg/µL	Femtogram per microlitre – unit of measurement often associated with a DNA test.
HMF	Hydroxymethylfurfural – chemical compound marker 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.
LC-MS/MS	Liquid chromatography tandem mass spectrometry.
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.
mg/kg	Milligrams per kilogram (= parts per million).
Monofloral	Honey with a distinctive combination of attributes 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 attributes 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 an attribute 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 Background

The aim of establishing robust science-based identification criteria for monofloral and multifloral mānuka honey is to provide the following:

- verification of authenticity;
- regulatory assurances in the international marketplace;
- consumer confidence;
- a sustainable platform for the New Zealand apiculture industry to grow in the future.

Reliable identification criteria need to be specific, robust and acceptable to trading partners while also being of minimum burden to industry. In 2014, MPI funded a series of preliminary science projects related to identification of mānuka honey. MPI also undertook an extensive review of published research and available industry information and data. The review highlighted several important issues and strongly indicated a more accurate solution was needed to authenticate mānuka honey. Through this work it was determined that a combination of attributes was likely to provide a more robust solution rather than relying on a single attribute to distinguish mānuka honey from other honey types and help mitigate against fraud. This initial work helped scope and inform the larger Mānuka Honey Science Programme[1].

The current industry-based approaches for authenticating mānuka honey are unable to provide accurate and specific identification criteria. The most common industry approach is based on the presence of methylglyoxal. This chemical, however, is not unique to mānuka honey and is unstable during the shelf life of the product (unless carefully temperature controlled). Methylglyoxal may be useful for medical applications (eg, topical application of medical grade honey), but it is unsuitable as an attribute to authenticate mānuka honey when sold as a food.

A second approach to authenticating mānuka honey is based on the presence of pollen. However, traditional laboratory techniques present challenges when distinguishing between pollen from mānuka and pollen from kānuka plants. This limitation means current tests using microscopy (visual identification) may not accurately reflect the dominant floral source. Newer techniques, such as DNA tests, can address this issue.

[1]Refer to MPI Technical Paper No: 2014/23 – *Science and characterising mānuka honey: Current and future science to support a definition* (www.mpi.govt.nz/document-vault/4147).

4 Objectives

The aim of the Science Programme was to develop robust science-based criteria suitable for authenticating mānuka honey and identifying it as either monofloral or multifloral mānuka honey as derived from *Leptospermum scoparium* (mānuka) when sold as a food.

To achieve the aim, the Science Programme had the following phases:

1) Selecting the attributes.
   - Identification and selection of potential attributes to identify mānuka honey.
2) Establishing plant and honey reference collections to ensure selected attributes are fit for purpose.
3) Developing accurate and specific test methods.
   - Development and validation of test methods to detect the selected attributes in plant and honey samples.
4) Analysing data and establishing identification criteria.
   - Refinement of the number of attributes, data analyses, classification modelling and establishing the identification criteria and associated threshold levels.

5 Scope

The scope of the Science Programme is as follows:
- mānuka honey as derived from *Leptospermum scoparium* JR Forst and G Forst, 1776;
- mānuka honey from New Zealand when sold as a food.

Out of scope are:
- producing identification criteria relating to the geographic origin of mānuka honey;
- producing identification criteria inclusive of *L. scoparium* honey from Australia, or other countries.
- mānuka honey for medical (eg, topical) applications;
- producing evidence to support health claims associated with mānuka honey;
- mānuka honey for cosmetics.

6 Selecting the attributes

The Science Programme began by focusing on identifying and determining the potential usefulness of selected attributes to distinguish mānuka honey from other honey types.

Several factors were considered when assessing the suitability of the attributes.

- The critical importance of demonstrating the relationship to the source plant:
  - Are the attributes linked to nectar and pollen of *L. scoparium*?
    - These are the biological materials a bee transfers to a hive.
  - Are the attributes only found in the mānuka plant and/or are they also found in other *Leptospermum* species or plants involved in New Zealand honey production?
- Levels found in honey:
  - Do the levels of the attributes enable separation of different honey types?
- Ease of detection and quantification:
  - Are there suitable laboratory test methods that could be developed and validated to detect and quantify the target attributes?
- Stability of attributes:
  - Are the attributes influenced by different temperatures over time?
- Regional and seasonal variation:
  - Are the levels of the attributes consistent or different across regions of New Zealand and seasons?
- Likelihood of fraud and adulteration:
  - Is it possible for the combination of attributes to be defensible against fraud?
- Attributes historically used by industry:
  - Are methylglyoxal and dihydroxyacetone suitable attributes?
- Attributes traditionally used to describe a monofloral honey under CODEX Alimentarius:
  - Are physico-chemical parameters, such as colour, conductivity and thixotropy, suitable?
  - Is pollen as determined by microscopy a suitable attribute?

6.1 IDENTIFICATION OF CANDIDATE ATTRIBUTES

While international guidelines (CODEX Alimentarius) provide parameters on which monofloral honeys can be identified, the specifications have focused mainly on European honeys. Significant advances have also been made in science that allow for the use of laboratory techniques that are more objective and consistently produce reliable test results.

To produce criteria for identifying mānuka honey from a regulatory perspective, it was important initially to cast the net wide on the number of attributes that could be evaluated. This enabled a large body of information to be produced so informed decisions could be made on what combination of attributes were best suited to identify mānuka honey (monofloral or multifloral).

Attributes linked to nectar and pollen were investigated because these are the main types of biological material that bees could transfer to a hive from *L. scoparium*.

6.1.1 DNA (pollen)

The presence of pollen is an attribute used to identify numerous honey types around the world. The method traditionally used (microscopy) to identify pollen, however, has challenges when it comes to distinguishing between pollen grains of mānuka and kānuka plants. Microscopy also

has limitations in a commercial sense because it does not allow for high throughput and requires specialist expertise.

To combat the limitations of microscopy, a DNA approach that allows for high throughput and high specificity was selected to detect plant DNA from pollen present in honey. Separate DNA markers from mānuka and kānuka plants were identified as useful attributes. This work involved the development and validation of a new laboratory test. The DNA markers are not intended to be compared with traditional percentage pollen counts as the test method used to detect the DNA markers is very different to using a microscope. The use of the DNA marker is to provide a quantifiable confirmation of mānuka and/or kānuka DNA as derived from pollen in a honey sample. It is not to be used to estimate relative abundance of mānuka or kānuka in comparison with total pollen from the numerous plant species that might be found in the honey.

6.1.2 Chemical (nectar)

A literature review identified an initial list of chemicals that were considered for further evaluation. This list was mainly composed of chemicals that could be useful for establishing if a honey is or is not mānuka. Some of the included chemicals related to kānuka as well and were included to help provide further information on honey samples tested in the programme. Many of these chemicals had been previously identified by others as having a potential role in identifying honey, however, the scientific work was often only associated with a small number of samples.

The Science Programme investigated the following chemical attributes previously identified by others:

- 2'-methoxyacetophenone;
- 2-methoxybenzoic acid;
- 3-phenyllactic acid;
- 4-hydroxyphenyllactic acid;
- 4-methoxyphenyllactic acid;
- abscisic acid;
- dihydroxyacetone;
- kojic acid;
- leptosperin;
- linalool oxide;
- lumichrome;
- methyl syringate;
- methylglyoxal;
- syringic acid.

Stability of the chemical attributes

All chemical attributes were examined in laboratory controlled conditions for their stability over time (500 days) and under different temperatures (4°C, 20°C and 35°C). Six mānuka honey samples were tested.

Summary details of the attributes, their presence and stability in plant and honey samples can be found in Table A (see Appendix 1).

7 Plant and honey reference collections

The Science Programme established extensive reference collections of plant and honey samples. These reference collections ensured sufficient numbers of plant and honey samples representing mānuka, related plants, and relevant plant species from throughout New Zealand were all traceable to origin and available for analysis of the candidate attributes.

7.1 PLANT REFERENCE COLLECTION

A reference collection of plant specimens from New Zealand is now archived in the New Zealand National Forestry Herbarium. The establishment of a reference collection enabled comparison between attributes found in *L. scoparium* and those found in other related and relevant plant species.

Nectar, leaf and pollen samples from over 700 plants were collected from which 509 specimens were tested using a variety of laboratory test methods. The nectar samples were used for the chemistry aspects and the leaf and pollen samples for the DNA aspects of the work. The samples came from 12 regions in New Zealand and five states in Australia (see Table 1, Table 2 and Figure 1). From New Zealand, 29 species of plants are represented in the collection. From Australia, 5 species of *Leptospermum* are represented, including *L. scoparium*. The plant samples were collected during two flowering seasons: 2014/15 and 2015/16.

Taxonomic identification of new species is challenging particularly when a limited number of discrete identification characteristics are available to inform the analyses. MPI recognises the challenge with distinguishing species within *Leptospermum* and *Kunzea* genera (see Section 2: Definitions) and is funding a project to provide further clarity on the taxonomy of both taxonomic groups in New Zealand. If differences are identified within the two taxonomic groups, no substantial impacts on the outcomes of the Science Programme are expected. The main aspect is that *Leptospermum* and *Kunzea* will remain separate, and the Science Programme has collected sufficient sample numbers, from multiple geographic regions, to be able to assess the influence of any taxonomic changes on the outcomes of this Science Programme.

Table 1: Plant species collected from New Zealand from two flowering seasons: 2014/15 and 2015/16

Species	Common name	Number collected
Echium vulgare	viper's bugloss	12
Ixerba brexioides	tawari	15
Kunzea ericoides/K. robusta	kānuka	69
K. sinclairii	Great Barrier Island kānuka	3
K. tenauculis	geothermal kānuka	3
Knightia excelsa	rewarewa	11
Kunzea spp. hybrid	*Kunzea* spp. hybrid	3
Leptospermum scoparium[#]	mānuka	152
L. grandifolium	mountain tea tree	1
L. laevigatum	coastal tea tree	3
L. lanigerum	woolly, silky tea tree	2
L. morrisonii	tea tree	11
L. myrtifolium	swamp tea tree	3
L. obovatum	tea tree	7
L. petersonii	lemon scented tea tree	6
L. polygalifolium	Tantoon, yellow tea tree	10
L. rupestre	alpine tea tree	1
Leptospermum sp.	NA	5
Metrosideros excelsa	pōhutukawa	25
Metrosideros umbellata, M. robusta	northern and southern rātā	12
Nothofagus solandri[*]	honeydew	21
Restia sp.	NA	1
Thymus vulgaris	thyme	3
Trifolium repens	white clover	39
Ulex europaeus	common gorse	16
Weinmannia racemosa	kāmahi	23

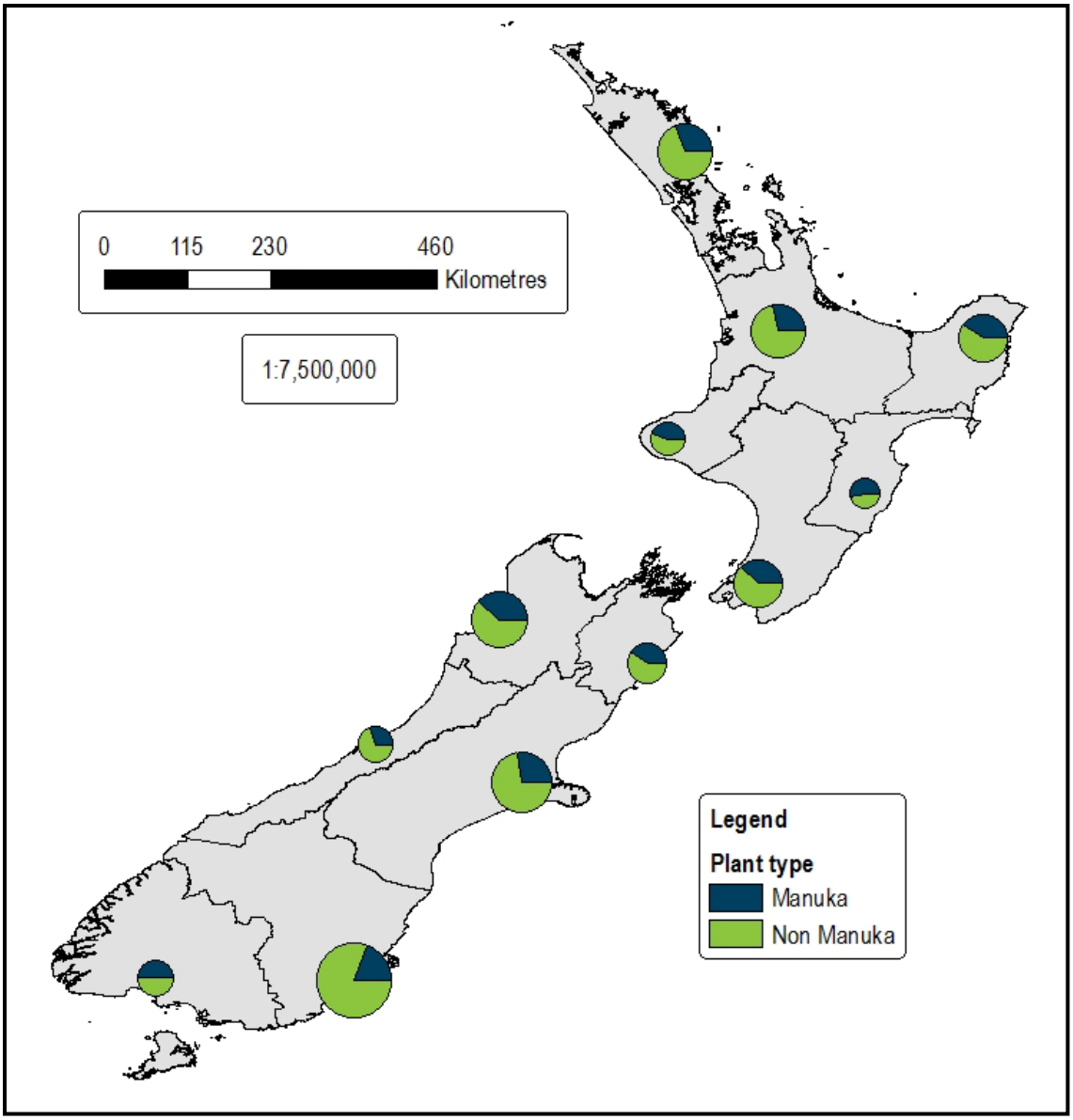
Notes: NA = not applicable.
[#] 2015/16 samples were collected from five different habitat types from each region, where possible.
* The *Nothofagus* genus has recently been split into four genera with two present in New Zealand: *Fuscospora* and *Lophozonia*. *N. solandri* has been assigned to *Fuscospora*. The sample collected from *Nothofagus solandri* is not a nectar sample but a sample of the liquid excreted from insects feeding on the phloem sap of the tree rather than the nectar taken from the flower.

Table 2: Plant species collected from Australia from two flowering seasons: 2014/15 and 2015/16

Species	Common name	Number collected
Leptospermum grandifolium	mountain tea tree	3
L. laevigatum	coastal tea tree	8
L. liversidgei	olive tea tree	9
L. polygalifolium	Tantoon, yellow tea tree	10
L. scoparium	mānuka	12

Note: No pollen or leaf material was imported into New Zealand, nectar was imported and held within a transitional facility.

Figure 1: **Distribution of mānuka and non-mānuka plants collected from across New** Zealand from two flowering seasons: 2014/15 and 2015/16



Note: Pie chart sizes for each region are relative to the total number of samples from across New Zealand.

7.2 HONEY REFERENCE COLLECTION

Honey samples were collected from two honey production seasons (2014/15 and 2015/16) and, where available, from the previous five seasons.

Where possible, honey samples were collected from single apiary sites and known geographic locations (see Figure 2). Additional information on samples was also collected to examine the influence of other variables, such as storage conditions and extraction methods.

In total, 804 honey samples were collected, of these 778 were considered suitable for testing (see Table 3 and Table 4). Honey samples included New Zealand honey deemed to be from both mānuka and non-mānuka floral sources, as well as honey from Australia and other countries.

Table 3: Honey samples sourced and tested from New Zealand

Honey type as identified by the supplier	Number of samples
Bush	10
Bush blend	18
Clover	61
Clover blend	15
Honeydew	15
Kāmahi	22
Kānuka	21
Kunzea species	9
Monofloral mānuka	273
Multifloral mānuka	74
Mānuka honeydew	3
Multifloral	65
Other monofloral	9
Pōhutukawa	7
Rātā	5
Rewarewa	6
Tawari	23
Thyme	14
Tussock grassland	3
Viper's bugloss	3
Willow honeydew	4

Note: 2014/15 samples = 141; 2015/16 samples = 350; archive samples = 169.

The 118 honey samples sourced from outside of New Zealand were from at least 16 countries (see Table 4). The floral source of each sample was not always identified.

Table 4: Honey samples sourced and tested from countries other than New Zealand

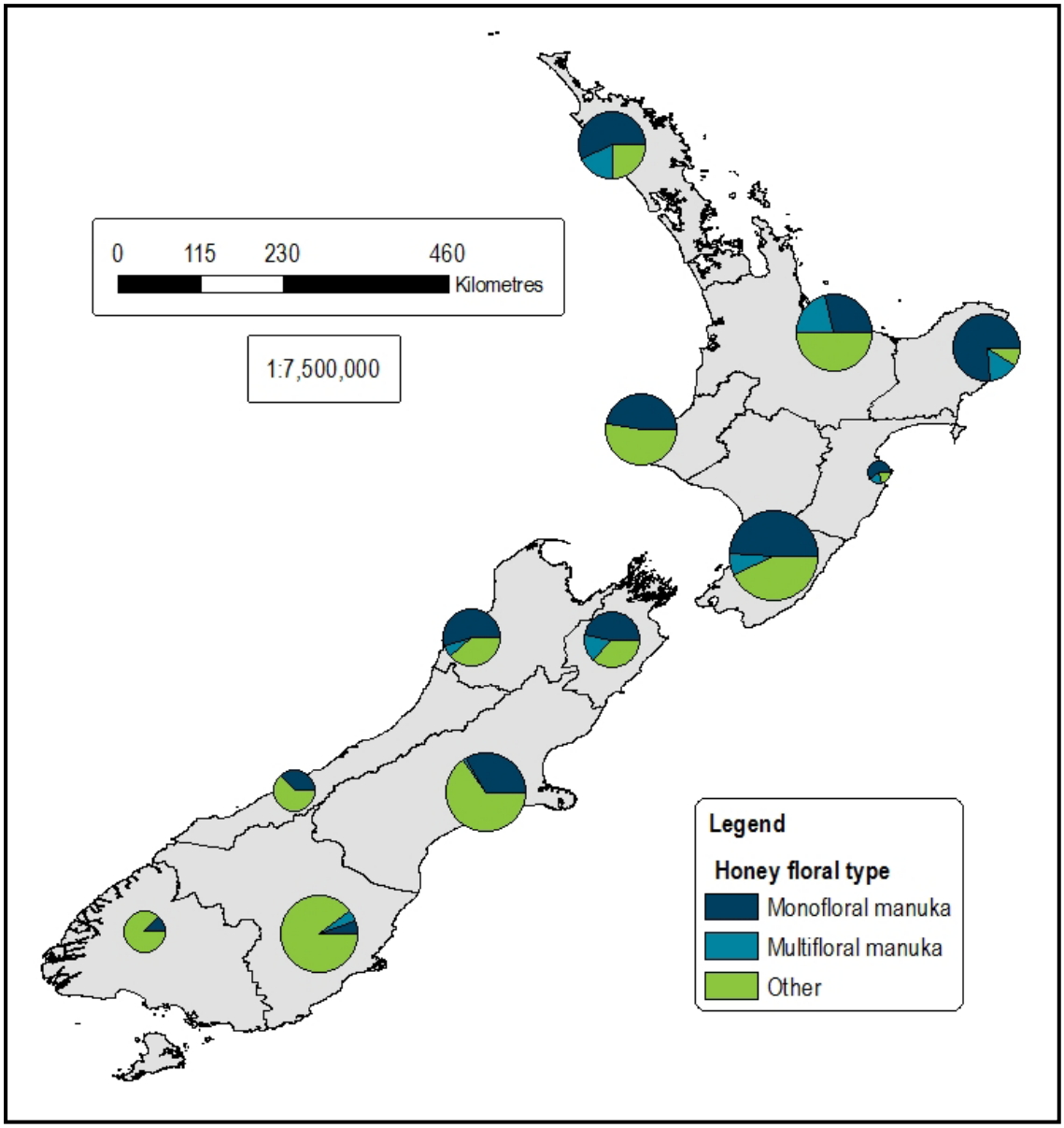
Country	Number of samples
Australia*	47
Botswana	2
Brazil	2
Canada	4
China	13
Germany	2
India	1
Italy	1
Mexico/Brazil	1
Republic of South Africa	2
Swaziland	4
Tanzania (and Zanzibar)	5
United Kingdom	1
United States of America	20
Vietnam	3
Zambia	10

Note: *Honey samples sourced from Australia were from regions where *Leptospermum* species are known to grow.

7.2.1 Standardising the names of honey types tested

To ensure consistency and valid comparison of samples, supplier descriptions of the honey types provided were standardised. The plant source and honey type were amended following defined rules that were used for honey samples collected. As an example, where several species of plants were described as contributing to a honey sample, the sample was labelled as "multifloral honey". When *L. scoparium* was used in addition to other plant species, the sample was labelled as "multifloral mānuka". Alternatively, when *L. scoparium* was described as the main plant species, the sample was labelled as "monofloral mānuka".

Figure 2: Distribution of honey floral types collected across New Zealand as originally described by the beekeeper or honey supplier (unless specified, the remaining honey floral types are displayed under the category “Other”).



Note: Pie chart sizes for each region are relative to the total number of samples from across New Zealand.

8 Test methods

The plant and honey samples were tested using a variety of laboratory methods. While some methods are already established, others needed to be developed and validated specifically for the Science Programme. The test methods were developed by contracted experts from New Zealand and overseas with technical input and review by MPI.

The methods already established were primarily focused on honey quality parameters (eg, sugars, HMF and moisture). Test methods that required new development and validation were the detection of the DNA markers and the chemical attributes of interest. Test methods were validated considering requirements for accuracy, precision, sensitivity, selectivity, repeatability and reproducibility. The success of the validation process was reliant on having an appropriate quality control and quality assurance framework in place.

A brief explanation of how the DNA and chemical test methods work can be found in Appendix 1.

9 Summary of statistical analyses

The extensive information generated by the Science Programme was used to determine identification criteria for monofloral and multifloral mānuka honey. The information was analysed using internationally accepted statistical techniques to ensure the criteria selected by the process were robust and capable of:

- assessing the relationships between attributes;
- evaluating the influence of temperature and time on attributes;
- exploring the variability in the attributes across regions; and
- examining the traceability of the attribute to the source plant (nectar for chemical attributes, pollen for DNA test).

Further details of this are given in Appendix 1.

All statistical analyses were performed using a process that allows for full transparency and documentation of the steps performed.[2]

The outcomes of the statistical analyses demonstrated mānuka DNA and the following four chemicals as robust attributes to authenticate mānuka honey: 2'-methoxyacetophenone, 2-methoxybenzoic acid, 4-hydroxyphenyllactic acid and 3-phenyllactic acid. Further summary information from the outcomes of the statistical analyses used to assess the attributes are given in Appendix 1 (see Table A).

A classification modelling approach[3] (CART – classification and regression tree) was the most suitable method of analysis for determining the identification criteria for mānuka honey because:

- test results for several different attributes were available and needed to be assessed in combination;
- the identification criteria needed to be related to the attributes tested;
- the identification criteria needed to be straightforward, transparent and easily interpreted;
- the outputs would enable an unknown honey sample to be authenticated as monofloral or multifloral mānuka honey.

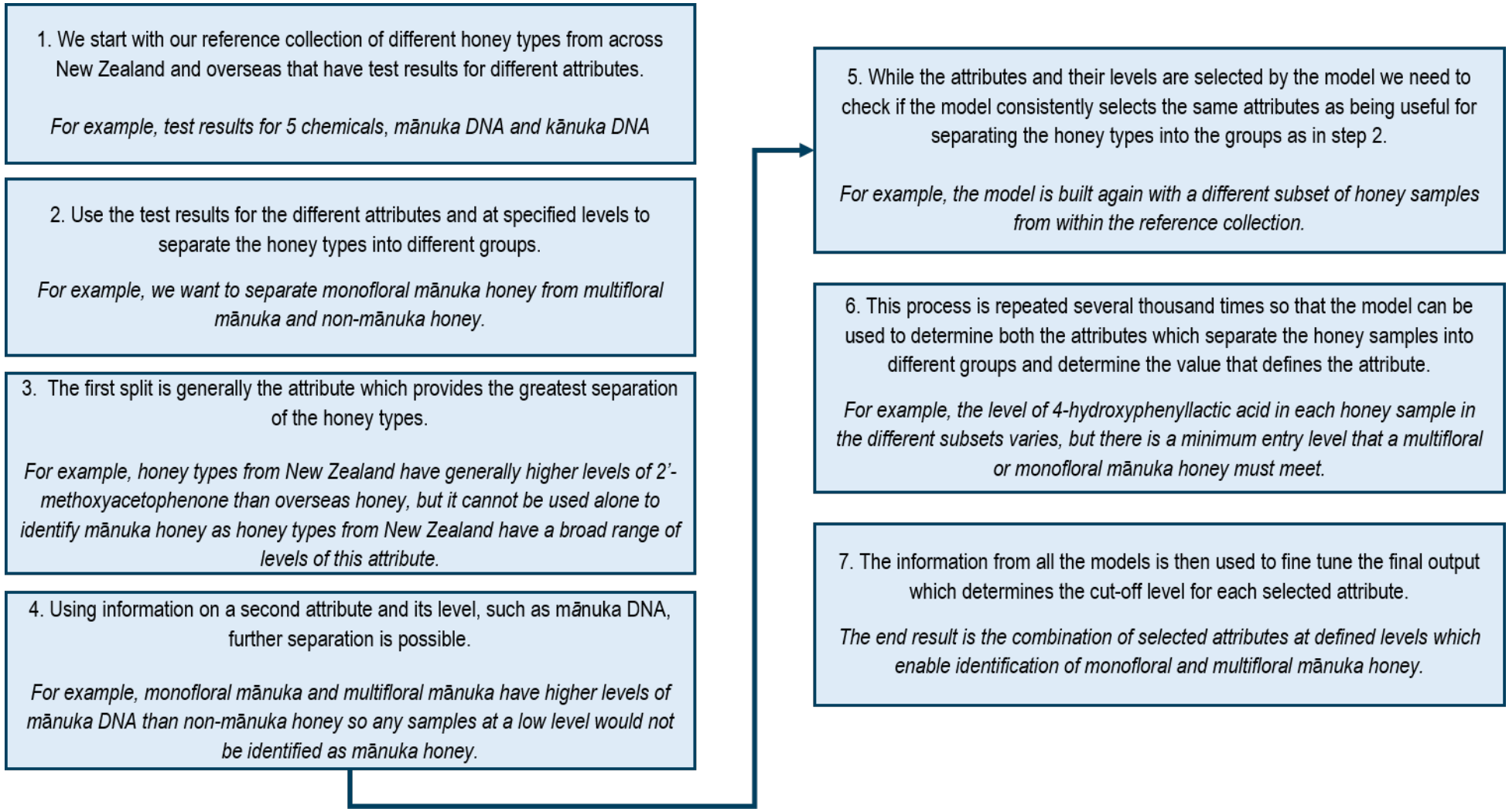
The classification modelling approach is shown with an example in Figure 3, with further details provided in Appendix 1. The classification modelling approach involves:

- exploring patterns in the specific attributes of the honey (chemical and DNA attributes);
- examining the variation in the levels of each attribute across the honey samples to determine how similar each honey sample is to another;
  - this establishes how important each attribute, in combination with others, is for separating mānuka honey from other honey types and the best threshold level to generate this separation.

[2] All statistical analyses were carried out using R: A language and environment for statistical computing version 3.2.0 (www.R-project.org/). Depending on the analysis required, different R packages were used.

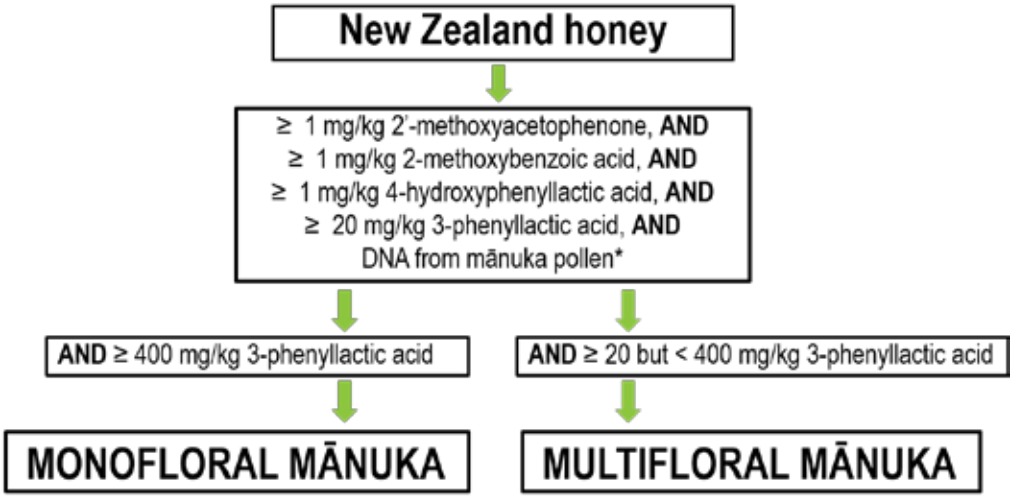
[3] Breiman, L, Friedman, J H, Olshen, R A and Stone, C J (1984) *Classification and Regression Trees*. Chapman and Hall/CRC. Therneau, T, Atkinson, B and Ripley, B (2015) Rpart: Recursive Partitioning and Regression Trees. R package version 4.1-9.

Figure 3: Classification modelling approach used to determine criteria for **authenticating mānuka honey and** identifying **monofloral and multifloral mānuka** honey



The results from running all of the classification models determined the combination of attributes and their levels to authenticate mānuka honey and identify monofloral and multifloral mānuka honey (see Figure 4).

Figure 4: **Combination of attributes required to separate mānuka honey from other honey types and to identify monofloral and multifloral mānuka honey**



Note: *DNA level required is < Cq 36, which is approximately 3 fg/µL.

9.1 IDENTIFYING **MĀNUKA HONEY** – MONOFLORAL AND MULTIFLORAL

The criteria were tested on all honey samples in the reference collection and compared with the original identifications given by the supplier (see Table 5). It can be seen that 74 percent of honey classified as monofloral mānuka by the supplier was identified as monofloral mānuka after applying the criteria. Importantly, 56 percent of honey classified as multifloral mānuka by the supplier was identified as monofloral mānuka using the criteria.

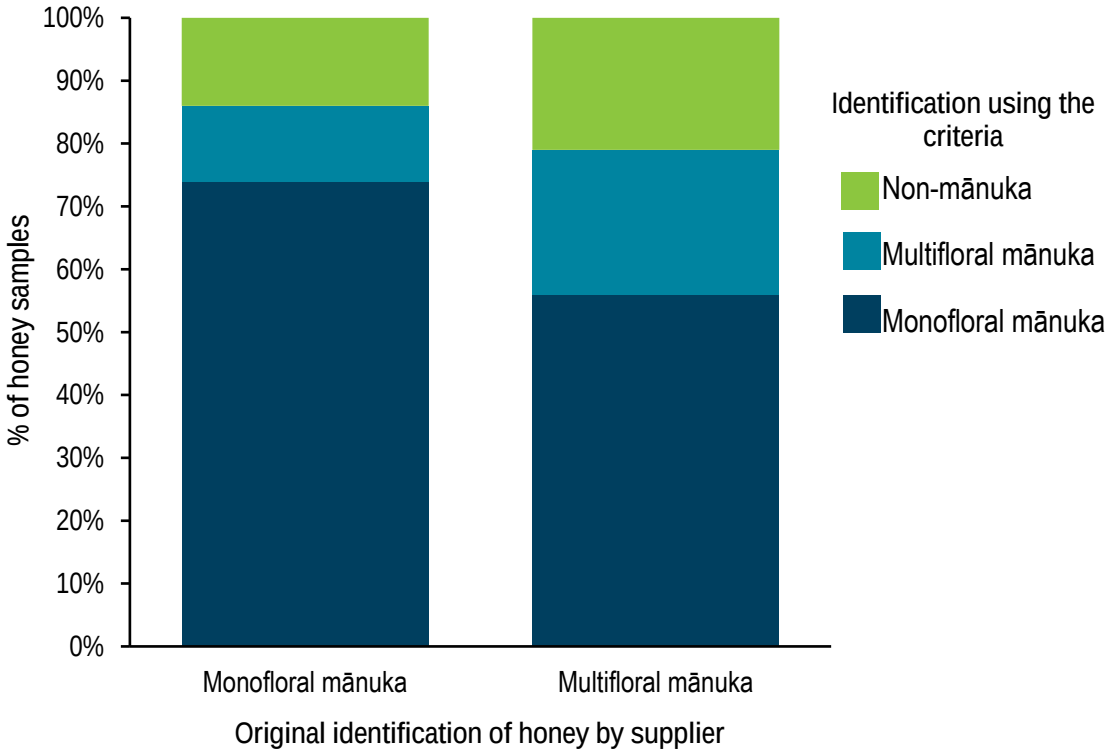
After applying the identification criteria, some honey samples were identified differently from the original identification given by the supplier. This is not surprising given suppliers are likely to have sometimes identified the honey samples differently depending on their understanding of their product. In addition, the complexity of bee foraging behaviour and the wide range of potential source plants at an apiary site can make initial identification of the honey type challenging. For example, bees may forage from other plant species at an apiary site even when mānuka is the most abundant flowering plant at the site. Another representation of applying the identification criteria to honey originally classified as monofloral and multifloral mānuka by the supplier is given in Figure 5.

Table 5: Comparison of original supplier identifications of honey samples against the criteria

		Identification using the criteria		
Supplier identification	No. samples	Not **mānuka** (%)	Multifloral **mānuka** (%)	Monofloral **mānuka** (%)
Monofloral mānuka	273	14	12	74
Multifloral mānuka	95	21	23	56
Kānuka	30	60	17	23
Non-mānuka (eg, clover)	262	88	12	<1
Overseas honey*	118	100	0	0

Note: *Further testing of *L. scoparium* honey from Australia is likely to identify honey that meets the criteria.

Figure 5: Outcomes of applying the identification criteria to honey samples supplied as **monofloral or multifloral mānuka honey**



10 Main findings of the science programme

The main outputs of the Science Programme are:

- establishment of an extensive reference collection of both plant and honey samples;
- development and validation of two new tests to detect and identify the target attributes (chemical and DNA) required to identify mānuka honey – the test methods produce reliable and accurate results when appropriately validated;
- successful application of a classification modelling approach to develop identification criteria for authentication of a premium food product that is complex in composition and has natural variation.

The main findings of the Science Programme are:

- Monofloral and multifloral mānuka honey can be identified and separated from other honey types according to robust scientific criteria.
- Authentication criteria for mānuka honey include a combination of four chemicals and a DNA marker.
- Dihydroxyacetone and methylglyoxal are unsuitable for the identification of mānuka honey when sold as a food.
- No single attribute evaluated in this programme can be used in isolation to identify mānuka honey.
- Monofloral and multifloral mānuka honey that meet the identification criteria are being produced across New Zealand. (Note, this finding is at the apiary level and may not reflect the various types of products in the market place.)

The identification criteria to authenticate mānuka honey developed from the Science Programme are the culmination of three years of systematic and thorough science. The criteria can be applied by both industry and regulators. Verification against the identification criteria will result in the New Zealand mānuka honey industry being able to assure authentication of mānuka honey products in the market place.

11 Acknowledgements

MPI greatly appreciates the efforts of beekeepers, hobbyists and honey companies that contributed both their time and samples to the Science Programme. Without these contributions, the outcomes of the Science Programme would not have been representative of the diversity of New Zealand honey. We also appreciate the efforts of those who sourced honey samples from overseas.

MPI wishes to acknowledge the following scientific service providers for their contributions to the three-year science programme.

- *Sample collection and plant identification*
  - Scion, New Zealand
  - SPS Biosecurity, New Zealand
  - University of the Sunshine Coast, Australia
  - Peter de Lange, Principal Scientist, Department of Conservation, New Zealand
  - Peter Wilson, National Herbarium of NSW, Royal Botanic Gardens, Australia
  - Emily Moriarty Lemmon and Alan Lemmon, Florida State University

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  - dnature Diagnostics and Research Ltd, New Zealand
  - Scion, New Zealand
  - Rachel Chalmers, New Zealand
  - GNS Science, New Zealand
  - Veritaxa, New Zealand

- *Chemical testing*
  - National Measurement Institute, Melbourne, Australia
  - Analytica Ltd, New Zealand
  - University of the Sunshine Coast, Australia
  - GNS Science, New Zealand

- *Statistical analysis*
  - BioSS (Biomathematics and Statistics Scotland), United Kingdom
  - Scion, New Zealand

Appendix 1

SUMMARY OF THE DNA TEST

The DNA method uses a technique called quantitative polymerase chain reaction (qPCR). Essentially, qPCR is a way of copying specific sections of target DNA enough times so detection and quantification are possible. For the purposes of the Mānuka Honey Science Programme, DNA is recovered from plant pollen present in the honey and then qPCR is used to specifically detect the target attribute (eg, mānuka plant DNA).

The ManKan™ PCR method was designed and validated to detect three different target sections of DNA (target DNA) in the same test.

1. DNA from the different types of plant pollen in the honey sample.
   This is the methods' internal control and is used to provide confidence that the DNA recovered from the honey sample is of good quality and good laboratory practice has been followed. At a minimum, a valid test result requires this part of the assay to be positive.
2. DNA from *Leptospermum scoparium* (Man) pollen in the honey sample.
3. DNA from *Kunzea ericoides/robusta* (Kan) pollen in the honey sample. (Note, kānuka is not required for the identification criteria of mānuka honey.)

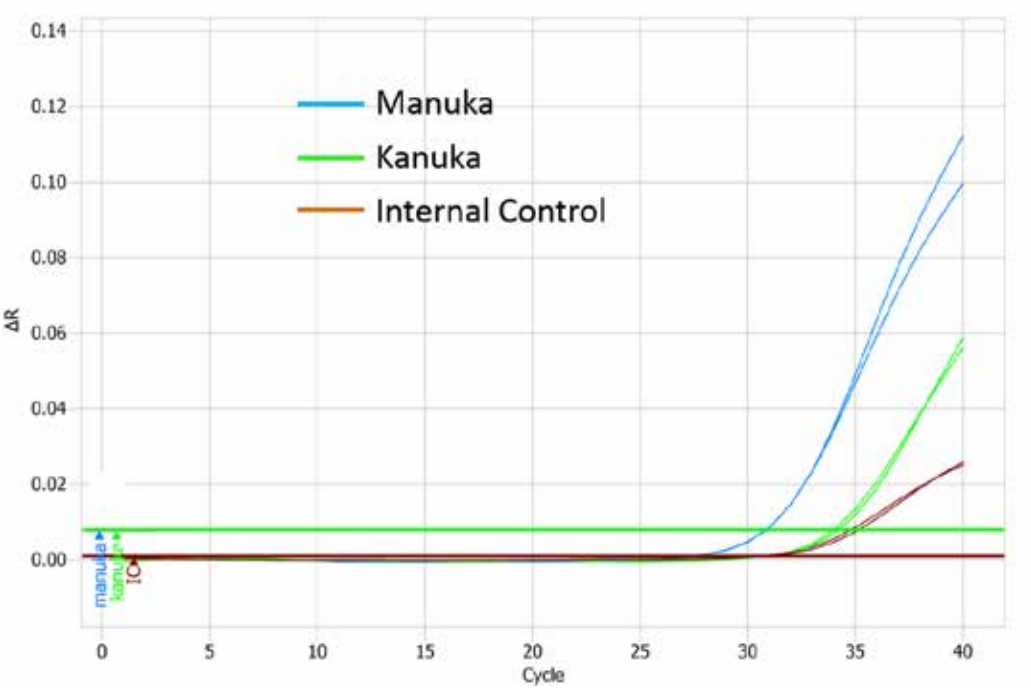
The qPCR assay requires a reaction mixture that includes:

- primers – short pieces of single-stranded DNA that can be matched to the target plant species DNA;
- probes – similar to primers but labelled with a fluorescent dye;
- nucleotides – building blocks needed to help make copies of the target DNA;
- enzyme – needed to help make new strands of DNA that can be matched to the target sequence;
- buffer – solutions that provide optimal conditions for the reaction to occur in;
- template DNA – DNA from the honey sample that is double stranded.

Once all of the components are mixed together, a reaction is started using a PCR machine that heats the mixture to approximately 95°C for a few minutes to separate the double stranded DNA (DNA from the honey sample). The reaction mixture is cooled to about 60°C, during this time the primers and probes set about copying the target DNA by binding to the matching areas of template DNA. The process of heating and cooling is referred to as a cycle and can be repeated up to 40 times. Each time a cycle is completed and the primers and probes successfully bind to the target DNA (eg, mānuka) in the honey sample, a fluorescent signal is produced. The PCR machine measures the level of fluorescence. If the target DNA (eg, mānuka plant) is present in the honey sample, the level of fluorescence will increase as the number of cycles increases.

Like most laboratory tests, the result is reported with a particular unit or description. Historically, pollen counts have been determined by microscopy and reported as the number of pollen grains per 10 grams of honey. The qPCR test is reported based on quantification cycles (Cq). Unlike most quantitative tests where the higher the number the greater the concentration, qPCR is the opposite, the smaller the number, the greater the concentration of the target DNA.

Figure A: Example of a test result from a single honey sample con**taining both mānuka and kānuka DNA**



Note: The point at which the increasing lines cross the horizontal green line is used to provide the test result reported as Cq. This result can be interpreted because there is more mānuka DNA (lower Cq) present in the sample than kānuka (higher Cq). The internal control is used to provide confidence that the DNA recovered from the honey sample is of good quality and good laboratory practice has been followed. The horizontal lines represent the set threshold levels.

SUMMARY OF THE CHEMICAL TEST

The chemical test uses a technique commonly referred to as liquid chromatography tandem mass spectrometry (LC-MS/MS). Briefly, samples of honey are diluted with an appropriate solvent and measured by the LC-MS/MS to determine the amount of each attribute present.

LC-MS/MS

The technique of combining liquid chromatography and mass spectrometry has been used in analytical chemistry laboratories for over 30 years.

In principle, a liquid extract of the test sample is injected into a pressurised system. Chemicals in the liquid extract are separated because of their different properties when applied to a fixed medium and then washed with a liquid solvent or mixture of solvents.

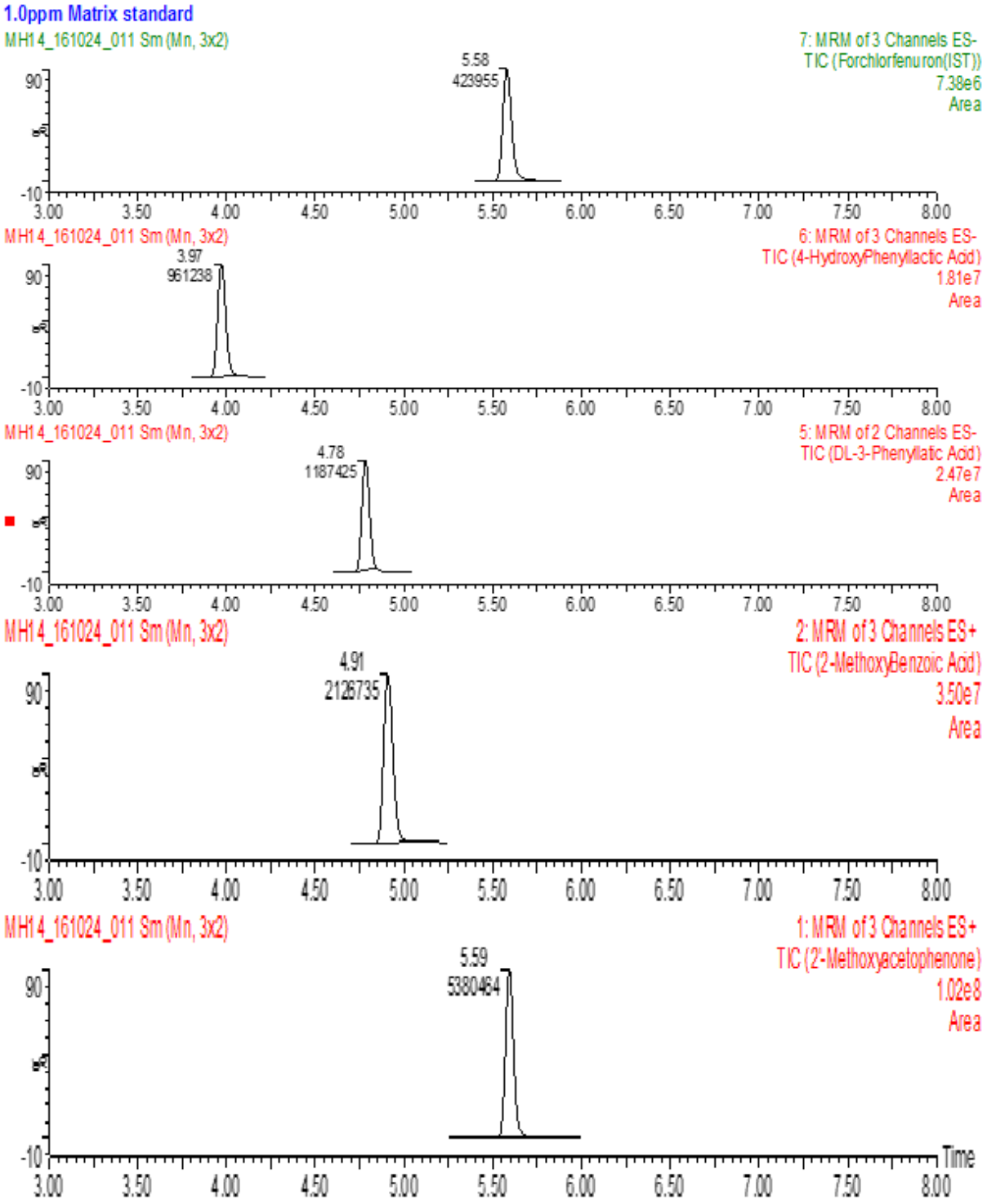
As the different components are washed off the medium they are vaporised and given an electric charge. These charged molecules (ions) are passed into a vacuum and subjected to magnetic forces to separate them. In tandem mass spectrometry, the separated ions are further broken down and the fragments are again subjected to magnetic forces to separate them. A detector records the charge induced or current produced when an ion passes by or hits its surface.

Graphs of detector response over time (chromatographs) are made and measured to calculate the signal intensity. Relative signal intensity is used to calculate the amount of the target attribute (chemical) present against known standards.

Reporting units

Target attribute (chemical) results are reported as the ratio of chemicals measured as a proportion of the sample as received. Commonly, this is reported as milligrams of chemical per kilogram of sample (mg/kg), which can also be expressed as parts per million (ppm).

Figure B: Example of a chromatograph from a single honey sample containing 1 mg/kg (ppm) of each chemical



Note: The chemical used as an internal standard (IST) (in green) is not expected to be present in tested honey. It is used as part of the LC-MS/MS analysis to provide confidence that the chemicals recovered from the honey sample are of good quality and good laboratory practice has been followed.

STATISTICAL ANALYSES

Stage 1 of statistical analyses: plant and honey samples from the first season

The first stage involved analysing the test results from the plant and honey samples collected in 2014/15 to help refine the number of candidate attributes to be further investigated.

In regard to the chemical attributes, their potential usefulness was assessed by:

- analysing the nectar data for chemicals to determine from which plant species and at what concentrations the chemicals could be found;
- analysing the honey data for chemicals to determine from which honey types and at what concentrations the chemicals could be found.

In regard to the DNA attributes, their potential usefulness was assessed by determining that the mānuka and kānuka DNA could be detected independently at specific levels and that DNA is not detected from other related and relevant plant species.

Stage 2 of statistical analyses: all plant and honey samples collected

The second stage involved statistical analyses to explore the datasets and identify possible limitations. This second stage included:

- analysing chemical data of the nectar from a second season (2015/16) to compare with findings from 2014/15;
- exploring the levels of the attributes across regions and from different collection years.
  - honey is a natural product and so its chemical attributes are expected to vary, however, attributes are unlikely to be suitable if they are only found in specific regions or produced during specific years;
- evaluating the stability of the chemicals by looking at how their levels change with increasing temperature and time;
- examining the influence of potential sources of attribute variability, such as habitat type, collector bias for the nectar data, and extraction and storage methods for the honey data.

The Science Programme evaluated many potential influences on the attributes and their effects on the levels found in honey. It is important to recognise, however, that other unknown factors could potentially influence the selected attributes and their levels in both plant and honey samples. For example, the potential effect of ongoing climatic changes on the production of the selected attributes by the plant is unknown at this stage.

By using the analyses from the first and second stages, the number of candidate attributes used to determine the identification criteria for monofloral or multifloral mānuka honey was reduced to the mānuka DNA marker (tested using ManKan^{TM}) and the following four chemicals (tested using LC-MS/MS): 2'-methoxyacetophenone, 2-methoxybenzoic acid, 4-hydroxyphenyllactic acid and 3-phenyllactic acid (Table A summarises the results of the statistical analyses and the justifications they provided for inclusion of the selected attributes in the classification model).

Table A. Assessment of the results of statistical analysis of attribute levels in plant and honey samples from 2014/15 season. Attributes shaded in grey were included in the classification modelling approach in stage 3 and additional test results from 2015/16 (plant and honey) and archive honey samples were also analysed

Attributes	Found in mānuka plants	Only found in mānuka plants	Levels in mānuka plants can be used to separate it from other plant species	Levels in mānuka honey can be used to separate it from at least one other New Zealand honey type	Levels in mānuka honey can be used to separate monofloral from multifloral mānuka honey	Relatively stable over time and under increasing temperature
DNA marker – **mānuka**	Yes	Yes	Yes	Yes	No	Yes
2'-methoxyacetophenone	Yes	Yes	Yes	Yes	No	Yes
3-phenyllactic acid	Yes	No	Yes	Yes	Yes	Yes
2-methoxybenzoic acid	Yes	No	Yes	Yes	Yes	Yes
4-hydroxyphenyllactic acid	Yes	No	Yes	Yes	Yes	Yes
Methyl syringate	Yes	No	Yes	Yes	No	No
Leptosperin	Yes	No	Yes	Yes	No	No
Kojic acid	Not detected	No	Not detected	Yes	Yes	NA
Methylglyoxal	Not detected	No	Not detected	Yes	Yes	No
Dihydroxyacetone	Yes	No	Yes	No	No	No
DNA marker – **kānuka**	No	No	Yes	No	No	Yes
Syringic acid	Yes	No	No	No	No	NA
4-methoxyphenyllactic acid	Yes	No	No	No	No	NA
Lumichrome	Yes	No	No	No	No	NA
Abscisic acid	Yes	No	No	No	No	NA
Linalool oxide	No	No	No	No	No	NA
Mānuka pollen by microscopy	Yes	Yes[§]	NA	NA	No	NA
Physico-chemical attributes*	NA	NA	NA	No	No	NA

NA are either not applicable, not tested for or not assessed as these attributes were not suitable to distinguish mānuka plant and honey from other plants and honey.

[§] Pollen from mānuka cannot be distinguished from kānuka using microscopy

* Including colour, conductivity and thixotropy

Note: Honey types referred in the table reflect the *original identification* used by the supplier..

DEVELOPING THE CRITERIA TO IDENTIFY MĀNUKA HONEY

Criteria to authenticate honey as mānuka honey were developed by building a series of classification models to analyse the available data as follows:

- A baseline honey classification model was built using the mānuka DNA marker and the four selected chemicals. Initially, honey samples collected in 2014/15 were used to build the baseline model while samples collected in 2015/16 were used to test the baseline model.
- The baseline model assigned each honey sample to a specific honey type by developing criteria that, if followed in order and at the set attribute threshold level, separate honey samples according to honey type.
- The selected criteria established by the baseline model are not dependent on whether or not a honey sample was assigned to the same honey type as originally identified by the supplier.
- The criteria established by the baseline model were applied to both the data used to build the model (2014/15 samples) and the data from the other collection season (2015/16 samples).
- The results were reported as the percentage of honey samples that were assigned the same honey type as they were originally identified and those that were now assigned to a different honey type.
- The influence of collection location on the honey type assignment was also assessed. For example, were there honey types from specific regions whose original honey types were always reassigned to a different honey type?

The data were examined in depth to ensure the identification criteria produced by the modelling process were robust and the model outcomes were reliable. Significant changes were examined and compared with the output baseline honey classification model including:

- building the model using samples from different collection seasons;
- splitting the complete dataset many times to generate new sample sets to build and test the classification model;
- grouping the number of honey types into different numbers of categories. A maximum of seven and a minimum of three honey types were examined. For example, the honey types could be assigned to monofloral mānuka, multifloral mānuka or non-mānuka honey;
- building models with fewer attributes to determine the level of importance of a specific attribute at separating the different honey types;
- investigating assumptions regarding the correct identification of a honey sample by the supplier;
- using only honey samples sourced from the North Island to build the classification model and applying the criteria to honey samples from the South Island and vice versa.