A definition for the authentication of mānuka honey

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Why mānuka honey?

- Honey made by bees collecting nectar from mānuka plants – *Leptospermum scoparium*
- Challenges:
  - No gold standard
  - Claims of health/therapeutic benefits
  - Mānuka plants not isolated
  - Bees forage across large areas
  - Natural product can vary
## Selecting candidate markers

### Nectar chemicals
- **Mānuka markers?**
  - 2’-methoxyacetophenone
  - 2-methoxybenzoic acid
  - 3-phenyllactic acid
  - 4-hydroxyphenyllactic acid
  - dihydroxyacetone
  - methylglyoxal
  - leptosperin
  - syringic acid
  - abscisic acid
  - kojic acid
  - linalool oxide
- **Kānuka markers?**
  - lumichrome
  - methyl syringate
  - 4-methoxyphenyllactic acid

### DNA from pollen
- DNA marker from the mānuka plant
- DNA marker from the kānuka plant

### Physico-chemical
- Colour
- Conductivity
- Thixotropy
Reference collections

Plant collection
• Over 700 plants collected, 509 tested
  – Collected during 2014/15 and 2015/16
  – 12 regions in New Zealand (29 species of plants)
  – 5 states in Australia (5 *Leptospermum* species)

Honey collection
• Over 800 samples collected, 778 tested
  – 660 samples from New Zealand
    • Primarily single apiary sources
    • 2014/15, 2015/16 and archive samples
  – 118 samples from overseas
    • 16 countries
Marker data analyses

Key questions for assessing markers included:

- Only found in mānuka plants (to date)?
- Separate mānuka from other NZ species?
- Separate mānuka honey from other NZ honey types?
- Separate monofloral from multifloral mānuka?
- Stable over increasing time and temperature?
Marker evaluation

- **Factors considered include:**
  - Habitat type for nectar samples
  - Relationships between markers
  - Levels found in different honey types
  - Regional and seasonal variation
  - Honey extraction, storage time and conditions

- **Markers selected for further analysis:**
  - Mānuka DNA marker
  - Kānuka DNA marker
  - 4-hydroxyphenyllactic acid
  - 2-methoxybenzoic acid
  - 2’-methoxyacetophenone
  - 3-phenyllactic acid
Why use classification and regression trees?

• Markers needed to be assessed in combination

• Flexibility to assess outputs with no gold standard

• Identification criteria needed to be:
  – straightforward, transparent and easily interpreted
  – suitable for implementation in regulatory context
Baseline CART model

• Honey type as a 6 level response variable
• Training data: each honey production year plus Australia and non-NZ/Aus samples
• Test data: other honey production year and the archive samples.
• Bootstrap sampling with replacement used to determine:
  – True positives/negatives
  – False positives/negatives
  – Number of times a marker was selected at the first split point
  – Number of times a marker was selected in the CART
Sensitivity of CART outputs

Bootstrap sampling with replacement within each honey type was used to test CART outputs under a range of scenarios:

- different honey production years e.g. 2014/15 vs 2015/16
- different production areas e.g. North Island of NZ vs South Island of NZ
- different numbers of honey types classified e.g. 6 vs 4 classes
- different numbers of markers used to fit the CART
- importance of the test method limit of reporting values in the data
Establishing criteria and testing robustness

• To establish final criteria:
  − 2014/15 data as training set
  − Build CART with both a 4-level and a 6-level response variable
  − Using 3-PA and both DNA markers as other markers had minimal effect
• BUT other markers were selected in the CARTs:
  − add 2-MBA, 4-HPA and 2’-MAP to the criteria and compare classifications
• Robustness:
  − Influence of rounding
  − Systematic bias in laboratory test methods
Final identification criteria

New Zealand honey

- ≥ 1 mg/kg 2’-methoxyacetophenone, AND
- ≥ 1 mg/kg 2-methoxybenzoic acid, AND
- ≥ 1 mg/kg 4-hydroxyphenyllactic acid, AND
- ≥ 20 mg/kg 3-phenyllactic acid, AND
- DNA from mānuka pollen*

AND ≥ 400 mg/kg 3-phenyllactic acid

AND ≥ 20 but < 400 mg/kg 3-phenyllactic acid

MONOFLORAL MĀNUKA

MULTIFLORAL MĀNUKA

*DNA level required is < Cq 36 which is approximately 3 fg/µL DNA.
A detailed series of blending simulations suggested that in a small number of scenarios, a multifloral manuka honey type could be blended with a non-manuka honey type (kanuka) to form a monofloral manuka honey type.

This scenario was prevented by increasing the level of 2’-methoxyacetophenone from 1 mg/kg to 5 mg/kg in the final regulatory definition.
Key findings

- A combination of 5 markers (4 chemical and 1 DNA) can be used to authenticate monoflora and multiflora manuka honey.

- The identification criteria can be used within a regulatory setting as:
  - Based on defendable, robust and transparent science
  - Can easily be used for verification purposes
  - Meet expectations of MPI and overseas authorities
  - Fit for purpose for industry
  - Provide consumer confidence

- Identification criteria can be adapted to accommodate industry practice and potential environmental influences.
Acknowledgements

• **Industry**
  – Supply of honey samples

• **Sample collection and plant identification**
  – Scion Research, SPS Biosecurity, University of Sunshine Coast, Peter de Lange (DOC) Peter Wilson (National Herbarium of NSW), Emily Moriarty Lemmon and Alan Lemmon (Florida State University)

• **DNA & pollen**
  – dnature diagnostics & research Ltd, Scion Research, Rachel Chalmers
  – GNS Science, University of Sunshine Coast, Veritaxa

• **Chemical testing**
  – National Measurement Institute, Melbourne, Analytica Ltd, University of Sunshine Coast, Geological Nuclear Sciences

• **Statistical analysis**
  – BioSS (Biomathematics and Statistics Scotland), Scion Research