New approach: Chemical and fluorescence profiling of NZ honeys

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Abstract

New Zealand manuka (Leptospermum scoparium) and kanuka (Kunzea ericoides) honeys contain a unique array of chemical markers useful for chemical fingerprinting. We investigated the presence of 13 potential marker compounds in nectars of the major honey crop species. We confirmed that leptosperin, lepteridine, 2-methoxyacetophenone, and 2-methoxybenzoic acid are exclusive to manuka nectar whereas lumichrome is unique to kanuka nectar. 3-Phenyllactic acid and 4-hydroxyphenyllactic acid are present in manuka and kanuka nectars. Leptosperin, lepteridine, 3-phenyllactic acid, and 4-hydroxyphenyllactic acid are chemically stable over prolonged storage, but not 2-methoxybenzoic acid and 2-methoxyacetophenone. Accordingly, lepterospin and lepteridine are definitive chemical markers for authentication of manuka honey. An optimal concentration cut-off was established for the floral source-specific markers: leptosperin (94 mg/kg), lepteridine (2.1 mg/kg), 2-methoxyacetophenone (2.0 mg/kg) for manuka honey, and lumichrome (4.5 mg/kg) for kanuka honey. The use of leptosperin and lepteridine as fluorescence markers for manuka honey authentication is reinforced.

1. Introduction

New Zealand is one of the world’s leading producers of monofloral honeys. The country currently produces approximately 20,000 tonnes of honey annually that is worth more than NZ$300 million (Ministry for Primary Industries, 2017a). Among the principal monofloral honeys are manuka, kanuka, pohutukawa, rata (Myrtaceae), rewarewa (Proteaceae), kamahi (Cunoniaceae), clover (Fabaceae), thyme (Lamiaceae), NZ ling (Ericaceae), vipers bugloss (Boraginaceae), and honeydew (Nothofagaceae) honeys (Stephens, 2006). Manuka honey harvested from the indigenous Leptospermum scoparium is highly sought after and internationally traded at a premium relative to the other honey types. Consumer expectation for true-to-label honeys as well as concerns about the authenticity of New Zealand premium honey products have identified a need for reliable and robust methods for honey authentication.

According to the Codex Standard, a honey has to originate wholly or predominantly from a particular floral source and display the corresponding organoleptic, physico-chemical, and microscopic properties in order for it to be designated by that floral origin (Codex Alimentarius Commission, 2001). It is generally accepted that honey produced in the natural environment is never exclusively derived from one floral species as it is impossible to control the free-flying bees and their forage sources (Winston, 1987). Honey bees commonly forage within one km radius from the hive site, however they have been shown to be capable of travelling up to 14 km from the colony, and nectar may be collected from any floral sources available within the region at that particular time (Eickwort & Ginsberg, 1980; Seeley, 1995; Stephens, 2006). The term monofloral honey therefore describes the predominant nectar source constituting the honey.

The current standard reference method for honey analysis is melissopalynology based on pollen composition (Louveaux, Maurizio, & Vorwohl, 1978). Although widely adopted and traditionally the preferred method, it is laborious and time-consuming, and taxonomic resolution depends to a large extent on the pollen morphological characteristics. The pollen grains of New Zealand manuka (L. scoparium) and kanuka (Kunzea ericoides), for instance, are virtually indistinguishable in a honey medium due to their morphological resemblance (Moar, 1985; Stephens et al., 2010). This difficulty is compounded by the coexistence of manuka and kanuka populations within the same geographical location and their overlapping flowering time (Butz Huryn, 1995; Stephens, 2006).
Other routine honey testing methods such as colour and sensory analysis are also commonly adopted in New Zealand. However, these methods lack reproducibility and rely to a great extent on the expertise of the evaluating panel. Manuka honey, for instance, possesses strong flavour and aroma that can easily mask the organoleptic properties exhibited by other mild honeys such as clover (Stephens, 2006). Consequently, blended or polyfloral honey with little manuka content may be misidentified as manuka honey based on conventional sensory evaluation.

In the past few decades, chemical fingerprinting has become increasingly popular in food authentication research. Honey comprises a complex mixture of chemical compounds that vary with floral origin and storage conditions (Fearnley et al., 2012). In particular, New Zealand manuka and kanuka honeys contain an abundant suite of phenolic compounds that distinguish them from the other honey types. Elevated concentrations of 2-methoxybenzoic acid (o-anisic acid), 2-methoxyacetophenone, and kojic acid are characteristically associated with a manuka honey (Beitlich, Koelling-Speer, Oelschlaegel, & Speer, 2014; Oelschlaegel et al., 2012; Senanayake, 2006; Stephens et al., 2010; Tan, Holland, Wilkins, & Molan, 1988), whereas kanuka honey is characterised by elevated concentrations of 4-methoxypHENylactic acid, methyl syringate, lumichrome, and 4-methoxybenzoic acid (p-anisic acid) (Beitlich et al., 2014; Senanayake, 2006; Stephens et al., 2010).

Manuka honey is additionally characterised by the presence of the dicarboxyl compounds dihydroxyacetone (DHA) and methylglyoxal (MGO). Dihydroxyacetone is inherently present in L. scoparium nectar, whereas MGO forms progressively in honey from DHA (Adams, Manley-Harris, & Molan, 2009). Although nectar-derived and unique to Leptospermum honeys, the use of these compounds as chemical markers can be problematic due to their fluctuating concentrations throughout the honey’s shelf life (Grainger, Manley-Harris, Lane, & Field, 2016; Stephens et al., 2015). Moreover, DHA and MGO are readily available commercially and therefore it is possible in principle to externally supplement non-manuka honeys with these compounds.

In the search for more reliable chemical markers, researchers have discovered three novel compounds in manuka honey: leptosperin1 (Kato et al., 2012, 2014), lepteridine (Daniels et al., 2016), and 6,7-dimethyl-2,4(1H,3H)-pteridinedione (Beitlich, Lübken, Kaiser, Isipryan, & Speer, 2016). Leptosperin is responsible for the unique fluorescence characteristics of manuka honey at ex270–ex365 nm (MM1) (Bong, Loomes, Schlothauer, & Stephens, 2016; Bong et al., 2017), whereas lepteridine and 6,7-dimethyl-2,4(1H,3H)-pteridinedione fluoresce at ex330–ex470 nm (MM2) (Beitlich et al., 2016; Bong et al., 2016; Lin, Loomes, Prijic, Schlothauer, & Stephens, 2017). Both leptosperin and lepteridine are nectar-derived and chemically stable over prolonged storage at elevated temperatures (Bong et al., 2017; Kato et al., 2014; Lin et al., 2017).

Within the ongoing Manuka Honey Science Programme, a New Zealand government initiative to define manuka honey, the New Zealand Ministry for Primary Industries (MPI) recently released new criteria based on chemical fingerprinting and pollen DNA analysis. In the April 2017 report, four chemical markers were proposed for manuka honey: 2-methoxycetophenone, 2-methoxybenzoic acid, 4-hydroxyphenyllactic acid, and 3-phenyllactic acid (MPI, 2017b). Interestingly and despite multiple publications, leptosperin was included in the MPI analyses however it was not recommended as a chemical marker, whilst lepteridine and 6,7-dimethyl-2,4(1H,3H)-pteridinedione appear unexamined altogether.

1 Leptosperin was initially named “leptosin” but was later renamed to avoid confusion with the marine fungus-derived leptosins (Kato et al., 2014).

In the present study, we examined the feasibility of these MPI proposed chemical markers, together with leptosperin and lepteridine, for classification of New Zealand manuka honeys. In addition, we explored potential chemical markers for kanuka honey. We investigated the nectar origin and chemical stability of these compounds and evaluated their potential as chemical markers. The application of chemical and fluorescence analysis to classification of New Zealand honeys is discussed.

2. Materials and methods

2.1. Honey and nectar samples

Nectars were collected through spring and summer 2015/2016. These include manuka (L. scoparium, n = 20), kanuka (K. ericoides, n = 4), rewarewa (Knightia excelsa, n = 5), pohutukawa (Metroserids excelsa, n = 4), clover (Trifolium spp., n = 4), vipers bugloss (Echium vulgare, n = 2), and NZ ling (Calluna vulgaris, n = 4). Honey samples were kindly supplied by Comvita NZ Ltd. and the Unique Manuka Factor Honey Association (UMFAH). These include field-collected and commercially purchased manuka (L. scoparium, n = 113), kanuka (K. ericoides, n = 23), pohutukawa (M. excelsa, n = 3), rata (Metroserids umbellata, n = 3), rewarewa (K. excelsa, n = 6), kamahi (Weinmannia racemosa, n = 5), towai (Weinmannia silvicola, n = 2), tawari (Xeroba brexioides, n = 4), clover (Tri- folium spp., n = 5), vipers bugloss (E. vulgare, n = 3), NZ ling (C. vulgaris, n = 3), thyme (Thymus spp., n = 4), koromiko (Hebe stricta, n = 1), beechnhydew (Fucospora solandri, n = 4), willow honeydew (Salix spp., n = 2), and multifloral (n = 2) honeys. Ten additional manuka and blend honeys extracted from commercial honey drums were supplied by Comvita NZ Ltd. and analysed separately for the stability study. The floral source ascription of these honeys was based on floral source site analysis and beekeepers’ advice on principal flowering species during the major nectar flow.

2.2. Chemicals and reagents

Leptosperin and lepteridine chemical standards were synthesised by the Department of Chemical Sciences at the University of Auckland based on methods described by Aitken, Johannes, Loomes, and Brimble (2013) and Daniels et al. (2016), respectively. 4-Methoxyphenyllactic acid was synthesised by Hangzhou Sage Chemical Co. Ltd. (Hangzhou, China). Kojic acid, gallic acid, 5-hydroxymethylfurfural, 4-hydroxyphenyllactic acid, syringic acid, 3-phenyllactic acid, 2-methoxybenzoic acid, lumichrome, and 2′-methoxycetophenone were purchased from Sigma Aldrich (St. Louis, MO, USA). Methyl syringate was sourced from Alfa Aeser (Lancashire, UK), and 4-methoxybenzoic acid from BDH Chemicals Ltd. (Poole, England). Analytical grade methanol was purchased from Merck (Darmstadt, Germany) and formic acid from Scharlau (Barcelona, Spain). Distilled water was generated using a Sartorius Pro (18.2 MΩ cm) water purification system.

Stock standards for all compounds of interests were prepared in methanol and stored at –20 °C. Further dilutions to give calibration standards for HPLC analysis were carried out with 0.1% formic acid.

2.3. High-performance liquid chromatography (HPLC)

The chemical profile of honey was established using the Dionex Ultimate® 3000 reversed-phase HPLC system (Thermo Fisher Scientific, New Zealand) with diode-array detection (DAD). Honeys and nectars were prepared in 0.1% formic acid. Honeys were diluted to a final concentration of 0.1 g/ml, and nectars to approximately 1° Brix. All diluted samples were centrifuged at 14,500 rpm

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for 5 min, and loaded as 100 µl aliquots onto a 96-well microplate (Greiner Bio-One, polystyrene, conical bottom).

The injection volume was 5 µl. Separation was achieved by gradient elution on a Hypersil GOLD column (150 × 2.1 mm; 3 µm particle size) at a constant flow rate of 0.160 ml/min. The binary mobile phase consisted of 0.1% aqueous formic acid (Solvent A) and acidified methanol containing 0.1% formic acid (Solvent B). A 45 min gradient elution programme was employed: initial (2% B, held 2 min), 5 min (5% B), 15 min (25% B), 25 min (50% B), 31 min (100% B, held 3 min), 35 min (2% B, held 10 min). The column compartment was thermostatically controlled at 32 °C.

The compounds of interest were monitored within the range of phenolic compound absorbance (Rodríguez-Delgado, Malovaná, Pérez, Borges, & García Montelongo, 2001) at 250, 265, 280, and 330 nm. These include kojic acid, gallic acid, 5-hydroxymethylfurural, 4-hydroxyphenyllactic acid, lepteridine, syringic acid, leptosperin, 3-phenyllactic acid, 2-methoxybenzoic acid, 4-methoxyphenyllactic acid, methyl syringate, lumichrome, 4-methoxybenzoic acid, and 2-methoxyacetophenone. Compound identification was based on retention time and ultraviolet-visible (UV–Vis) spectra of respective chemical standards.

Data acquisition and peak integration were performed with Thermo Fisher Scientific™ Dionex™ Chromel™™ 7.2 Chromatography Data System (CDS) software. The concentrations of target compounds were quantified against external calibration curves of respective chemical standards based on integrated measurement of peak area. All compound concentrations in nectars were expressed as normalised weight ratio of compound per 80 °Brix sugar solution in mg/kg.

2.4. Fluorescence spectroscopy

Fluorescence analysis was carried out on a Gemini EM Dual-Scanning Microplate Spectrofluorometer (Molecular Devices Inc., Sunnyvale, CA, USA) according to methods previously described (Bong et al., 2016). Honey samples were diluted with distilled water to 2% w/v, and loaded as 100 µl aliquots onto a flat-bottom microplate (Optiplate™ 384, black). Measurements were obtained using endpoint scans at three sets of excitation–emission wavelength pairs: ex270–em365 nm (MM1), ex330–em470 nm (MM2), and ex275–em305 nm (KM1). All samples were analysed in duplicate, and fluorescence intensity was expressed as relative fluorescence unit (RFU).

2.5. Stability study

Ten drum honeys representing monofloral manuka and manuka blend honeys were incubated at 37 °C and subsampled at Day 0, 70, 155, and 444 for HPLC analysis.

2.6. Statistical and multivariate analysis

Statistical analysis was performed using Graphpad Prism (Version 6.01). For analyses purposes, all concentrations below the detection limit, non-detectable (n.d.), concentrations, were censored and substituted with a constant value of zero. Difference between two group means was compared by two-tailed unpaired t-test, and multiple group means by one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison post-test. The concentration change of chemical markers relative to Day 0 in the stability study was performed by one-way ANOVA followed by Dunnett’s multiple comparison post-test. Correlations were determined by regression analysis.

Multivariate analysis was performed using the Web application MetaboAnalyst 3.0 (http://www.metaboanalyst.ca). Extraction of unique chemical features was performed using the principal component analysis (PCA) algorithm in the Statistical Analysis module. The data was pre-treated with range scaling normalisation (mean-centered and division by the value range of each variable) to adjust for the fold differences in chemical concentrations. Optimal cut-offs for selected marker compounds were computed by means of receiver operating characteristic (ROC) curves in the Biomarker Analysis module.

3. Results and discussion

3.1. Nectars

Nectars collected from seven surplus nectar-producing species in New Zealand were analysed to determine the presence of a range of compounds that have been proposed as floral markers of New Zealand manuka and kanuka honeys. These include kojic acid, gallic acid, 4-hydroxyphenyllactic acid, lepteridine, syringic acid, leptosperin, 3-phenyllactic acid, 2-methoxybenzoic acid, 4-methoxyphenyllactic acid, methyl syringate, 4-methoxybenzoic acid, lumichrome, and 2-methoxyacetophenone. The concentrations are expressed as a weight ratio of the compound of interest/80 °Brix sugar solution in mg/kg and reported as mean ± standard error mean (SEM) (Supplementary Table 1). Normalisation to 80 °Brix eliminates the difference relative to sugar content and allows direct comparison with chemical concentrations in honey.

Leptosperin, lepteridine, and 2′-methoxyacetophenone were exclusive to manuka nectar, with a mean concentration of 3928 ± 697 mg/kg, 72 ± 11 mg/kg, and 161 ± 15 mg/kg, respectively. These manuka nectars were represented by L. scoparium in the Northland (n = 7), Wairarapa (n = 8), and Central North Island (n = 5) regions. Whilst there were no significant differences in lepto- sperin and 2′-methoxyacetophenone concentrations among the regions (p > 0.05), lepteridine concentration was significantly elevated in the Northland manuka compared to the Central North Island and Wairarapa populations (p < 0.001). The other major honey-producing species, kanuka, rewarewa, clover, pohutukawa, vipers bugloss, and NZ ling contained no trace of leptosperin, lepteridine, and 2′-methoxyacetophenone.

The presence of 2-methoxybenzoic acid was also confined to manuka nectar (20 ± 8 mg/kg), however this does not appear universal to all regions. We detected no measurable concentration of this compound in Wairarapa manuka nectar. Lumichrome also appears exclusive to kanuka nectar, with a mean concentration of 171 ± 11 mg/kg.

4-Hydroxyphenyllactic acid was present in both manuka and kanuka nectars but not the other nectar species examined. This compound concentration was significantly elevated in kanuka (96 ± 17 mg/kg) compared to manuka nectar (14 ± 3 mg/kg) (p < 0.05). Methyl syringate and gallic acid were present in manuka and kanuka nectars but at concentrations that were not significantly different (p > 0.05). The mean methyl syringate concentration was 79 ± 18 mg/kg and 165 ± 69 mg/kg in manuka and kanuka nectar respectively, and 9 ± 2 mg/kg and 11 ± 5 mg/kg respectively for gallic acid.

3-Phenyllactic acid was present in kanuka, manuka, and NZ ling nectar, with a mean concentration of 1556 ± 264 mg/kg and 2755 ± 995 mg/kg in manuka and NZ ling nectar, respectively. However, in kanuka nectar the concentration of 3-phenyllactic acid was not quantified due to co-elution with another compound. Analysis of the UV–Vis spectrum of the peak of interest strongly indicates 3-phenyllactic acid presence (data not shown), and this was confirmed by evidence from independent mass spectrometry (T. J. Braggins, personal communication, 21st April 2017).
4-Methoxyphenyllactic acid and 4-methoxybenzoic acid were present in all kanuka but only some manuka nectars. Both compound concentrations were significantly elevated in kanuka nectar with a mean concentration of 2365 ± 668 mg/kg and 17 ± 6 mg/kg respectively, compared to 751 ± 295 mg/kg and 0.5 ± 0.3 mg/kg respectively for manuka nectar (p < 0.05). These compounds were confined to manuka nectars collected from the Wairarapa population, indicating a possible genetic or environmental effect.

Syringic acid was present in 70% of manuka (3 ± 1 mg/kg) and 50% of kanuka (1.2 ± 0.7 mg/kg) nectars, however no regionality was present. Kojic acid was not detected in any nectar species examined despite its reported presence in manuka honey (Oelschlägel et al., 2012).

These nectar findings reinforce the use of chemical fingerprinting for authentication of New Zealand honeys. The exclusivity of leptosperin, lepteridine, and 2-methoxyacetophenone to manuka nectar confirms that these candidate compounds are appropriate markers for authentication of manuka honey. The presence of these compounds at low levels in other honey types most probably represents manuka nectar contamination during bee harvest. Similarly, the presence of lumichrome in honeys other than kanuka would indicate floral dilution by kanuka nectar. Although elevated levels of 4-methoxyphenyllactic acid, methyl syringate, and 4-methoxybenzoic acid are characteristically associated with kanuka honeys (Beitlich et al., 2014; Senanayake, 2006; Stephens et al., 2010), these compounds are not exclusive to kanuka nectar.

3.2. Honeys

The chemical profiles of New Zealand honeys have been extensively examined. However, most studies were restricted to honey crops such as manuka, kanuka, and clover. Honey produced in a mixed forage field are often sourced from a number of surplus nectar-producing species. Although floral source ascription of the honey crop is principally based on the predominant species harvested, it is extremely likely that these honeys contain traces of other floral source markers.

Here we examined a collection of 183 honeys encompassing all the major and many minor honey crops harvested throughout New Zealand. Fig. 1 shows the HPLC chromatograms of 16 New Zealand honey varieties grouped by plant family and the target marker compounds at 265 nm. 5-Hydroxymethylfurfural content was quantified as a quality index for the honeys; elevated 5-hydroxymethylfurfural is associated with prolonged storage or extended heat treatment (Stephens et al., 2015).

The chromatograms mostly demonstrate different chemical profiles between plant families, and in some cases between genera of the same family. Manuka (L. scoparium) and kanuka (K. ericoides) honeys exhibited a relatively similar chemical profile that was distinct from all the other New Zealand honey types. This may be attributed to the abundant suite of phenolic compounds characteristically associated with manuka and kanuka honeys (Stephens et al., 2010; Tan et al., 1988). By comparison, chromatographic traces of the representative Metrosideros species pohutukawa (M. excelsa) and rata (M. umbellata) were considerably less complex. Although these honey crops belong within the Myrtaceae family, phylogenetically L. scoparium and K. ericoides are more closely related. The Leptospermum and Kunzea genus are taxonomically ranked in the Leptospermum suballiance (O’Brien, Quinn, & Wilson, 2000), whilst Metrosideros is classified under a separate clade within the Metrosideros alliance (Wilson, O’Brien, Gadek, & Quinn, 2001).

Kahumi and towai honeys, harvested from two closely related Weinmannia species of the Cunoniaceae family, also showed relatively similar chromatographic traces. Whilst rewa rewa honey is known to be rich in aliphatic dicarboxylic acids (Wilkins, Lu, & Tan, 1995), we were unable to detect these compounds in the present study. A derivatisation procedure is most likely necessary for quantification of these compounds.

In comparison to all the other honey varieties, tawari (L. brexioides) honey exhibited the least complexity in chromatographic profile. It is probable that this honey species inherently carries a low concentration of phenolic compounds and organic acids often found in the other honey types. In addition, tawari nectar is recognised anecdotally as a rich sugary secretion favoured by the honey bees, therefore tawari honey is often harvested with minimal nectar contribution from the other floral sources. The chromatogram of clover honey also reflects a lack of phenolic acids as reported in literature (Stephens et al., 2010; Tan et al., 1988).

NZ ling (C. vulgaris) honey exhibited a distinct cluster of peaks in the chromatographic trace. This particular profile may be a reflection of the diverse array of aromatic compounds and phenolic acids reported to be present in Ericaceae honeys (Guyot, Scheirman, & Collin, 1998; Tan, Wilkons, Holland, & McGhie, 1989). Whilst both beech and willow honeydew honeys are Hemiptera insect-derived, these honeys appear to have a distinct chemical profile, most likely derived from the plant species fed upon.

Fig. 2 shows the concentration range of the 13 targeted marker compounds in manuka and kanuka honeys. By comparison to the other New Zealand honeys, manuka honey contained significantly elevated concentrations of leptosperin (398 ± 34 mg/kg), lepteridine (14 ± 1 mg/kg), 2-methoxybenzoic acid (5.1 ± 0.5 mg/kg), and 2-methoxyacetophenone (7.0 ± 0.4 mg/kg) (p < 0.0001). 2-Methoxybenzoic acid was detected in 98% of the manuka honeys analysed irrespective of regions despite this compound presence only in Northland and Central North Island manuka nectars. We also recorded significantly higher concentration of kojic acid in manuka honey (18 ± 0.8 mg/kg) compared to kanuka (10 ± 1 mg/kg) and the other honeys collectively (5.6 ± 0.7 mg/kg) (p < 0.0001).

Whilst leptosperin, lepteridine, 2-methoxybenzoic acid, and 2-methoxyacetophenone in manuka honey are nectar-derived, our nectar analyses did not detect kojic acid in manuka or the other major surplus nectar-producing species. Kojic acid is a carbohydrate derivative and forms principally from enzymatic degradation of sugar molecules such as glucose and sucrose (Zirak & Eftekhar-Sis, 2015). There is also evidence demonstrating kojic acid formation from other carbohydrate derivative precursors such as DHA (Arstein & Bentley, 1953; Challenger, Klein, & Walker, 1931). It is possible that this compound forms during the honey ripening process as a result of sugar catabolism through the action of bee enzymes. The inherently abundant DHA reservoir in manuka honey may alternatively explain its elevated concentration compared to the other honey types.

Kanuka honey, on the other hand, carried significantly elevated concentrations of lumichrome (8 ± 1 mg/kg), 4-methoxyphenyllactic acid (402 ± 64 mg/kg), methyl syringate (140 ± 24 mg/kg), and 4-methoxybenzoic acid (4 ± 1 mg/kg) compared to manuka and the other New Zealand honey types (p < 0.0001). The elevated concentration of lumichrome in kanuka honey and its exclusivity to kanuka nectar strongly reinforce the use of this compound as a floral marker for kanuka honey. We detected low concentrations of lumichrome in 16% of the manuka honeys examined, which is most likely a reflection of kanuka nectar contamination as lumichrome was not detected in manuka nectar. The other proposed kanuka markers 4-methoxyphenyllactic acid, methyl syringate, and 4-methoxybenzoic acid are present at low concentrations in some manuka nectars, making it impossible to distinguish the proportion of manuka and kanuka nectar contribution in a honey using these markers.

4-Hydroxyphenyllactic acid was present in manuka (5 ± 2 mg/kg), kanuka (4 ± 3 mg/kg), and NZ ling (6.5 ± 0.3 mg/kg) honeys but not the other honey types. The origin of this compound in manu-
uka and kanuka honeys is most likely nectar-derived as this compound is present in both nectars. However, we found no detectable concentration of 4-hydroxyphenyllactic acid in NZ ling nectar at the concentration examined. 3-Phenyllactic acid was also present in these honeys at elevated concentrations, with a mean of 740 ± 43 mg/kg, 660 ± 199 mg/kg, and 940 ± 64 mg/kg in manuka, kanuka, and NZ ling honeys, respectively. These concentrations reflect the nectar data but were not significantly different (p > 0.05), thus limiting the use of 3-phenyllactic acid for distinguishing between these honeys.

The April 2017 MPI recommendations for 4-hydroxyphenyllactic acid and 3-phenyllactic acid levels in monofloral manuka honey are >1 mg/kg and >400 mg/kg respectively for monofloral manuka honey, and >1 mg/kg and >20 mg/kg but <400 mg/kg respectively for multifloral manuka honey (MPI, 2017b). Consequently, the levels of 4-hydroxyphenyllactic acid and 3-phenyllactic acid we report in the kanuka and NZ ling honeys examined would incorrectly classify these honeys as "monofloral manuka honey" according to the current MPI classification scheme.

Gallic acid was present in most manuka and kanuka honeys with a mean concentration of 0.6 ± 0.0 mg/kg and 0.7 ± 0.2 mg/kg, respectively. These concentration range corresponds with Oelschlaegel et al. (2012). Previously, Yao et al. (2003) proposed gallic acid as a characteristic manuka marker reporting 71 mg/kg as mean concentration, however this was not encountered in this study. Gallic acid was also detected in pohutukawa (1.2 ± 0.1 mg/kg), koromiko (4 mg/kg; n = 1), beech honeydew (1.6 ± 0.2 mg/kg), and willow honeydew (1.2 ± 0.0 mg/kg) honeys. Statistical analysis demonstrated significantly higher concentration of gallic acid in beech honeydew compared to manuka and kanuka honeys (p < 0.05) but not between manuka and kanuka as well as the other honey pairings (p > 0.05). Accordingly, gallic acid is unlikely to be an appropriate marker for New Zealand honeys.

Syringic acid, on the other hand, was present at significantly elevated concentration in thyme honey with a mean concentration of 7.0 ± 0.4 mg/kg (p < 0.05). This attribute may be nectar-derived, however analysis of thyme nectar would be necessary to confirm this. Previously, Oelschlaegel et al. (2012) noted higher levels of syringic acid in manuka blend honeys with substantial kanuka nectar contribution. We found variable occurrence of this compound in manuka honey, with a mean concentration of 0.3 ± 0.1 mg/kg and detectable levels recorded in only 19% of the honeys examined. In comparison, kanuka honey carried this compound at a mean concentration of 1.3 ± 0.2 mg/kg. As syringic acid is present in both manuka and kanuka nectars, it is not possible to determine the botanical origin of these compounds in honey.

The findings of our chemical analysis show that the effect of floral dilution is apparent in honeys harvested in a natural environment. For instance, leptosperm, lepteridine, 2-methoxybenzoic acid, and 2’-methoxysyrcine are exclusively derived from manuka nectar. However these compounds are often present in the other honey types, in particular kanuka honey, a reflection of...
bee foraging behaviour. In New Zealand, L. scoparium and K. erioides often flower simultaneously in the same environments, and honey bees will exploit any nectar source in proximity to the hive so long as the harvest is worthwhile (Stephens, 2006). The use of chemical fingerprinting would allow identification of such floral source contamination. For example, a monofloral manuka honey contains elevated levels of leptosperin, lepteridine, 2-methoxybenzoic acid, and 2'-methoxycacetophenone. A honey labelled as manuka but containing a significantly reduced concentration of these compounds and elevated concentration of other honey markers such as lumichrome should be classified as non-monofloral as it would contain kanuka nectar contribution.

Comparisons of nectar and honey indicate that the mean normalised nectar concentrations of a target chemical is often higher than its concentration in honey. For instance, the mean leptosperin and lepteridine concentrations were ten- and fivefold higher, respectively, in manuka nectar compared to honey, and is consistent with previous findings (Bong et al., 2017; Lin et al., 2017). Although both these compounds are chemically stable in ripened honey (Bong et al., 2017; Lin et al., 2017), their stability during the nectar-to-honey conversion process has not been examined previously. It is possible that chemical degradation occurs such as hydrolysis which was recently reported for leptosperin by β-glucosidase (Ishisaka et al., 2017).

3.3. Stability of manuka markers

To examine the reliability of potential markers as a measure of manuka honey authenticity, we examined a set of ten independent manuka and blend honeys subjected to prolonged storage at 37 °C for 444 days. Fig. 3 shows the percentage change of leptosperin, lepteridine, 2-methoxybenzoic acid, 2'-methoxycacetophenone, 4-hydroxyphenyllactic acid, 3-phenyllactic acid, kojic acid, and 5-hydroxymethylfurfural relative to Day 0. 5-Hydroxymethylfurfural is an age marker for honey (Stephens et al., 2015), and was selected as a control in present study.

One-way ANOVA with Dunnett’s multiple comparisons test showed no significant change in 4-hydroxyphenyllactic acid and 3-phenyllactic acid concentrations in honey following storage (p > 0.05), indicating that these compounds are stable. These findings align with the April 2017 MPI report (MPI, 2017b). Similarly, leptosperin, lepteridine, and kojic acid concentrations did not vary significantly from Day 0 throughout the treatment period (p > 0.05). Previously, Kato et al. (2014) also found leptosperin to be chemically stable during elevated temperature storage. These findings for leptosperin contrast with the findings presented by MPI in the April 2017 report (MPI, 2017b). Lepteridine was not examined in the April 2017 MPI report, nevertheless our findings shows that this compound deserves further consideration as a chemical marker in view of its uniqueness to Leptospermum honeys, nectar origin, and stability.

On the other hand, 2-methoxybenzoic acid and 2'-methoxycacetophenone concentrations appear to be unstable in honey, with a significant increase of 9% (p < 0.05) and a decrease of 23% (p < 0.01), respectively, at Day 444. Again, this is in contrast to the April 2017 stability data reported by MPI with similar storage length and temperature (MPI, 2017b). By comparison, the concentration of 5-hydroxymethylfurfural increased exponentially up to sixfold over the same length of time.

3.4. Fluorescence markers

Among the chemical examinations, leptosperin and lepteridine have been reported as fluorophores responsible for the unique fluorescence characteristics of manuka honey (Beitlich et al., 2016; Bong et al., 2017; Lin et al., 2017). To reinforce the application of these compounds as fluorescence markers for rapid screening of manuka honey, we examined a total of 113 manuka honeys at a concentration of 2% w/v honey solution encompassing 94 field-collected and 19 commercial samples. This testing concentration provides the optimal balance between minimising the effect of quenching and preventing coordinate shift of the honey fluorescence (Aitkenhead, Rosendale, Schlothauer, & Stephens, 2014).

Our fluorescence analyses based on leptosperin and lepteridine provides a promising approach for robust and high throughput screening of New Zealand manuka honeys. Fig. 4A shows the correlation between leptosperin and lepteridine concentration with MM1 (ex270–em365 nm) and MM2 (ex330–em470 nm) fluorescence, respectively. Although MM2 fluorescence is not exclusively derived from lepteridine (Beitlich et al., 2016; Lin et al., 2017), the strong correlation indicates an equally distributed additive effect from other compounds that fluoresce at these wavelengths. It is possible to resolve the recently elucidated 6,7-dimethyl-2,4-(1H,3H)-pteridinedione from lepteridine at the MM2 marker wavelength by a thin-layer chromatography (TLC) method described by Beitlich et al. (2018). Non-manuka honey will not display these characteristic fluorescence signatures and is most likely to be identified as mislabelled. Furthermore, this fluorescence-based technique could be easily transferred to a handheld device for routine application in the field by beekeepers as well as in the market by retailers and regulatory authorities.

Conceivably, the MM1 and MM2 fluorescence signatures could be introduced artificially into a non-manuka honey by adding either these fluorophores or other substances with similar fluorescence characteristics. However, such adulteration is unlikely to be successful as neither leptosperin nor lepteridine are commercially available, and chemical synthesis is difficult and costly. The purified fluorophores could be extracted in principle from other Leptospermum honeys and used to spike non-manuka honey, nevertheless this approach would likely be counter-productive on an industrial scale as significant quantities of both these compounds would have to be spiked even at the minimum threshold levels for authentic manuka honey. The use of other compounds with similar fluorescence profiles would be detectable by any chromatographic technique.

Elevated concentration of 4-methoxyphenyllactic acid is characteristically associated with a monofloral kanuka honey (Beitlich et al., 2014; Senanayake, 2006; Stephens et al., 2010). Previously, we demonstrated that 4-methoxyphenyllactic acid is responsible for honey fluorescence at KM1 (ex275–em305 nm) and KM2 (ex330–em470 nm) fluorescence, respectively. Although KM2 fluorescence is not exclusively derived from kojic acid (Beitlich et al., 2016), Fig. 4B shows the linear positive correlation between 4-methoxyphenyllactic acid concentration and KM1 fluorescence for 135 honeys with detectable levels of this compound (R²=0.9002). All kanuka honeys examined carried 4-methoxyphenyllactic acid, and the higher end of the correlation appeared to be dominated by this honey type. Hones labelled as manuka generally exhibited low to moderate fluorescence at this marker wavelength with the exception of three samples (circled) which contained substantial amount of 4-methoxyphenyllactic acid, suggesting that they may be predominantly kanuka honey. All other honeys exhibited less than 2000 RFU at KM1, with a mean 4-methoxyphenyllactic acid concentration of 36 ± 4 mg/kg.

As 4-methoxyphenyllactic acid is also inherently present in manuka nectars from certain geographical regions, the use of the KM1 fluorescence signature for estimating kanuka nectar contribution in a honey should be applied with caution. Whilst it cannot be used as a stand-alone marker for kanuka honey, elevated fluorescence at this marker wavelength could be useful as an adjunct indicator for the presence of significant kanuka nectar contribution. Although lumichrome is a fluorescent compound, we did not identify any fluorescence marker wavelength that associates with
Fig. 3. Temporal stability of potential chemical markers in manuka honey at 37 °C. Data shows mean percentage change relative to Day 0 ± SEM.
lumichrome in the 2% w/v honey solutions analysed, possibly due to the low concentration of this compound in honey.

3.5. PCA and ROC analysis

To extract chemical features that best define New Zealand honeys, a PCA was performed on the honey data set comprising 13 quantified marker compounds. Towai, koromiko, willow honey-dew, and multifloral honeys were excluded due to a limited sample size. The data was normalised by range scaling to adjust for fold differences in compound concentrations so all variables were assigned equal importance. Fig. 5A shows the fraction of total variance explained by the top five PCs and the accumulative variance. PC1 explained 43% of the variance accounted by the 13 variables followed by PC2 and PC3 which captured 26% and 6% of the variance, respectively. Altogether, 85% of the total variance exhibited by the data set was accounted for by the top five PCs.

Fig. 5B illustrates a pairwise score plot for PC1, PC2, and PC3. Manuka honey clusters along the PC1 axis in the positive direction, and kanuka honeys along PC2 axis in the negative direction. Analysis of the component loading matrix (Supplementary Table 2) revealed leptosperin, lepteridine, 2-methoxycetophenone, and 4-hydroxyphenyllactic acid as the principal contributors to PC1 with a loading score of 0.402, 0.453, 0.424, and 0.429, respectively. Lumichrome (−0.537) and 4-methoxyphenyllactic acid (−0.534) appeared to be the greatest contributors to PC2, followed by methyl syringate (−0.448) and 4-methoxybenzoic acid (−0.315). PC3 appeared to separate out thyme from the other honeys, with syringic acid being the highest contributor in the negative direction with a loading score of −0.857.

Whilst the score plot did not allow complete separation of all New Zealand honeys due to a lack of chemical features in non-manuka and non-kanuka honeys, the data showed a good distinction between manuka, kanuka, and thyme honeys (Fig. 5B inset). There was some overlap between manuka and kanuka clusters, possibly due to shared chemical characteristics and to some degree manuka/kanuka blends. Honeys harvested in the natural environment often contain nectar contributions from sources other than the predominant floral source, and chemical fingerprinting may provide a robust and reliable method for determining the extent of the honey florality status. The chemical approach described herein could be used in conjunction with the recently proposed HAHSUS (Honey Authentication by HS-SPME-GC/MS and UHPLC-PDA-MS/MS combined with Statistics) classification system for authentication of manuka honey (Beitlich & Koelling-Speer, 2016). The HAHSUS method distinguishes honeys based on both

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Fig. 5. (A) Scree plot illustrating the fraction of total variance explained by the top five PCs and the accumulative variance. (B) Pairwise score plot for PC1, PC2, and PC3. Inset: Three-dimensional score plot of PC1, PC2, and PC3.
Fig. 6. (A) Univariate ROC curves and box plots showing concentration distribution of leptosperin, lepteridine, 2'-methoxyacetophenone, and lumichrome. Values in parenthesis (x, y): x = sensitivity; y = specificity. (B) Multivarite ROC model for leptosperin, lepteridine, 2'-methoxyacetophenone, and lumichrome. Inset: Predictive accuracies of each model using two, three, and four features.

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volatile and non-volatile components. Further identification of unique chemical markers for the other honey types in the future would help establish a more objective classification of New Zealand honeys, thus contributing to the development of a robust definition for manuka and other honey types.

To establish a guideline for using chemical markers to define New Zealand manuka and kanuka honeys, we computed the optimal cut-off concentration for the manuka and kanuka-specific marker compounds based on ROC analysis using the same dataset. A promising diagnostic marker must have high sensitivity (true positive rate) and high specificity (true negative rate), and ROC analysis provides a means of describing the trade-off between these two criteria with regard to the marker performance. Fig. 6A shows the area under curve (AUC) and optimal cut-off concentration for leptosperin, lepteridine, and 2′-methoxyacetophenone as manuka honey markers, and lumichrome as a kanuka honey marker. All three manuka-specific markers, leptosperin, lepteridine, and 2′-methoxyacetophenone had an AUC greater than 0.9, suggesting a strong discriminatory power for manuka honey using these compounds. The optimal cut-off concentration for these compounds was established to be 94 mg/kg for leptosperin, and 2.1 mg/kg for lepteridine, and 2.0 mg/kg for 2′-methoxyacetophenone. These threshold values translate into the minimal concentrations all of these compounds should be present in honey in order for the honey to be classified as a manuka honey. At these cut-off concentrations, both sensitivity and specificity were approximately 0.9. Accordingly, classification of manuka honey based on these threshold values is expected to give a true positive rate of 90% and a false negative rate of 10%. Although increasing the concentration threshold of leptosperin, lepteridine, and 2′-methoxyacetophenone increases the true positive identification proportion for manuka honey, this would also result in a higher proportion of genuine manuka honey misidentified as non-manuka honey.

Considering kanuka honey, lumichrome exhibited a favourable degree of discrimination for kanuka and non-kanuka honeys with an AUC greater than 0.8. The optimal cut-off concentration computed for lumichrome was 4.5 mg/kg, with sensitivity and specificity of approximately 0.8. In order for a honey to be classified as a kanuka honey, the honey should therefore contain at least 4.5 mg/kg lumichrome.

A multivariate ROC analysis for the four selected biomarkers was generated using the partial least squares-discriminant analysis (PLS-DA) algorithm (Fig. 6B). The software evaluates model performance using the Monte-Carlo cross validation. In each validation, two-thirds of the samples were used to evaluate importance of the markers. The ranking order of markers based on frequency of selection was leptosperin > lepteridine > 2′-methoxyacetophenone > lumichrome (data not shown). Three models were generated based on the top-ranking two, three, and four features, which were validated against the remaining one-third of samples excluded from model generation. All models demonstrated an AUC above 0.9, and the model featuring four markers appears to have the greatest predictive accuracy of 79% compared to 77% and 74% for the three- and two-feature models, respectively (Fig. 6B inset).

4. Conclusion

In this study, we investigated for the first time the presence of a range of proposed honey marker compounds in nectars representing the major New Zealand honey crops, and the honeys themselves. Some compounds are uniquely present in nectars and the matching honey type, whereas other compounds are shared between nectars of different species and different honey types. We confirmed that the manuka honey marker compounds leptosperin, lepteridine, and 2′-methoxyacetophenone are exclusive to manuka nectar. 2-Methoxybenzoic acid, which has been proposed as a marker, whilst most likely nectar-derived and present in manuka honey, may be confined to some manuka populations.

4-Hydroxyphenyllactic acid was present in both manuka and kanuka nectars and honeys. 3-Phenyllactic acid was present in manuka, kanuka, and NZ ling nectars, and its concentration was elevated in these corresponding honey types compared to the other New Zealand honeys. Whilst 3-phenyllactic acid and 4-hydroxyphenyllactic acid readily distinguish manuka and kanuka honeys from most other New Zealand honey types, they do not differentiate manuka and kanuka honeys. Therefore, the definition of manuka honey based on the April 2017 MPI criteria may inadvertently include some non-manuka honeys such as kanuka and NZ ling as “manuka honey”.

Our stability study showed that leptosperin, lepteridine, 4-hydroxyphenyllactic acid, 3-phenyllactic acid, and kojic acid were chemically stable in honey following prolonged storage at 37 °C. By comparison, 2-methoxybenzoic acid and 2′-methoxyacetophenone were unstable with their concentrations changing significantly following storage. To include these latter apparently unstable compounds in the criteria for defining manuka honey would require further stability research.

We propose, on the basis of our findings, that the manuka-specific and chemically stable leptosperin and lepteridine are definitive chemical markers for the classification of monofloral manuka honey. Further, our fluorescence analysis strongly reinforces the application of leptosperin and lepteridine as fluorescence markers for the rapid screening and authentication of manuka honey.

We also confirmed the nectar origin of lumichrome in kanuka honey, thus supporting its potential usefulness as a floral marker for kanuka honey. The other proposed kanuka markers 4-methoxyphenyllactic, methyl syringate, and 4-methoxybenzoic acid appear to be present across manuka and kanuka honeys and nectars, thus limiting their use as chemical markers for distinguishing between these honeys. Kojic acid was not detected in manuka, kanuka, rewarewa, clover, pohutukawa, NZ ling, and vipers bugloss nectars despite its elevated concentration in manuka honey and presence in most honey types.

Finally, principal component analysis based on the selected chemical markers showed a good separation of manuka, kanuka, and thyme honeys in the current dataset. By means of ROC analysis, we established an optimal cut-off concentration for the three manuka-specific chemical markers leptosperin, lepteridine, and 2′-methoxyacetophenone at 94 mg/kg, 2.1 mg/kg, and 2.0 mg/kg, respectively. Lumichrome also showed potential discriminatory power to distinguish between kanuka and non-kanuka honeys with an optimal cut-off concentration of 4.5 mg/kg.

Disclosure statement

Jonathan Stephens is an employee of Comvita NZ Ltd., Paengaroa, PB1, Te Puke, New Zealand.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.07.065.

References


