Chapter 5

Fluorescence: A Novel Method for Determining Manuka Honey Floral Purity

Jonathan M. Stephens, Kerry M. Loomes, Terry J. Braggins, Jessie Bong, Bin Lin and Gordana Prijic

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66313

Abstract

Manuka honey, harvested from *Leptospermum scoparium*, is New Zealand's most recognised honey type and commands a premium due to health-related benefits. However, the plant's distribution, relative to other species flowering simultaneously, allows honeybees to incorporate alternative nectars into the honey. Melissopalynological analysis in New Zealand is often unrepresentative due to the presence of many pollen-bearing sources; consequently, alternative means of categorising manuka honey were examined. RP-HPLC revealed that manuka honey contains distinct compounds, of which were relatively enriched and not present in the other New Zealand monofloral honeys. These main candidate compounds were isolated and have been described by mass spectrometry and nuclear magnetic resonance, synthesised to confirm structure, and as standards. These compounds, Leptosperin and Lepteridine, are a methyl syringate glycoside and pteridine derivative, respectively. Examination of these compounds revealed unique fluorescence signatures, this fluorescence could be detected in manuka honey samples the signal used to confirm that a honey was solely or predominantly consisted of *L. scoparium* nectar. Commercial manuka honeys were assessed by traditional analytical techniques, and comparisons were made with fluorescence signature; the fluorescence technique determined the authenticity of the honeys accurately.

Keywords: Honey, Floral, Fluorescence, Manuka, *Leptospermum scoparium*, Authenticity, purity

1. Introduction

New Zealand manuka honey is harvested from *Leptospermum scoparium* (Myrtaceae) throughout the country. Internationally, this honey has received considerable attention and value due to its unique health-related benefits. Major destination markets include Hong Kong and China, Japan, the European Union, United Kingdom, the United States and Australia. Over 80% of the total honey exported from New Zealand is now pre-packaged, hive numbers in the country have almost doubled in the last 10 years [1] and the value of the manuka honey industry is now estimated in the vicinity of NZ$150 million.

Codex Alimentarius [2] defines that a honey must be derived wholly or predominantly from a particular floral source and display the corresponding organoleptic, physico-chemical and microscopic properties for a floral attribution to be made. Within New Zealand, a number of surplus nectar-producing common plant species exist with similar distributions and flowering times as *L. scoparium*. Consequently, manuka honey may contain different levels of dilution by other floral types, as honey produced in a natural environment containing a range of plant species is unlikely to be monofloral because of bee behaviour in the forage field [3].

Historically, New Zealand honeys have been classified by physico-chemical analysis and melissopalynology. Melissopalynology is a common technique internationally for describing honeys; however, in New Zealand *Kunzea ericoides* often flowers simultaneously with *L. scoparium*, and the pollen grains of these species are virtually indistinguishable in a honey medium [4]. To overcome this, a classification structure was built upon the unique non-peroxide antibacterial activity that manuka honey exhibits, yet this system did not take into the account of honey's floral composition.

Honey is a complex supersaturated sugar solution containing approximately 80% sugars and a unique combination of other compounds suspended in water. The sugar proportion is principally the monosaccharide fructose and glucose, and the non-sugar proportion includes a range of bee- and plant-derived compounds such as organic acids, proteins, amino acids, phenolic acids, flavonoids, pollen and waxes [5]. This chemical composition varies between honey types, geographical origin and climate may additionally alter the constituents [5], and furthermore honey processing techniques and age may also be influential [6].

Manuka honey contains a diverse array of compounds that range from unique carbohydrate metabolites to phenolics, flavonoids and volatiles. Many of these have received attention [7–11], and clearly, this honey carries a number of distinct compounds that may be diagnostic for classification. For example, 2-methoxyacetophenone (**Figure 1**) [7, 11] and 2-methoxybenzoic acid [10, 11] have been proposed as floral markers for manuka honey.

In addition, dihydroxyacetone (DHA) and methylglyoxal (MGO) (**Figure 1**) are solely derived from *L. scoparium* nectar in New Zealand honeys [12, 13]. Dihydroxyacetone is present in *L. scoparium* nectar, converting non-enzymatically and irreversibly to MGO in the acidic environment of a ripened honey solution [13]. This conversion is non-stoichiometric [14, 15] due to the presence of side reaction pathways in the honey. However, the concentrations of these compounds are not stable throughout a manuka honey's shelf-life and therefore neither are suitable as reliable chemical markers [6].

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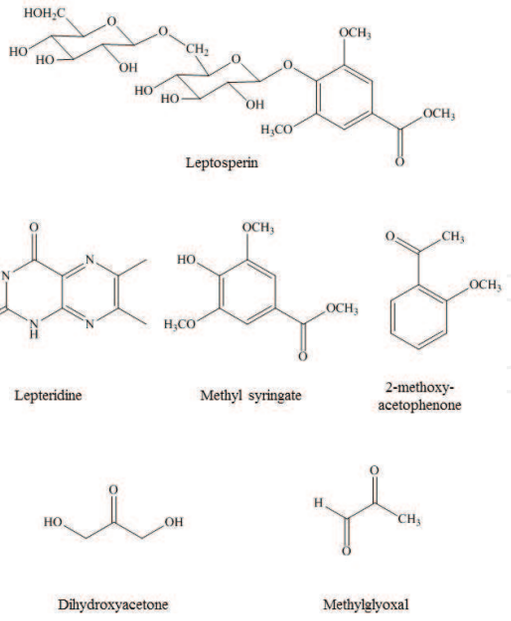

Figure 1. Chemical structure of Leptosperin, Lepteridine, 2-methoxyacetophenone, methyl syringate, dihydroxyacetone and methylglyoxal.

Internationally, classifying honeys by chemical signature or key components has received increasing attention over the last 20 years. European honeys have been thoroughly investigated [16] confirming earlier work on, for example, rosemary [17] and heather [18, 19] honeys.

Further investigation on the phenolic and flavonoid profile of manuka honey has revealed two unique compounds. First, a nectar-derived glycoside of methyl syringate has been described [20, 21]. Whilst this compound is present in the wider *Leptospermum* genus throughout Australasia, it is restricted to *L. scoparium* in New Zealand and therefore is potentially a suitable floral marker. Consequently, methyl syringate 4-O-*β*-D-gentiobiose in manuka honey, named Leptosperin[1] (**Figure 1**), has been analysed by high-performance liquid chromatography (HPLC), mass spectrometry, immunochemistry and immunochromatography [21–24].

More recently, analysis showed the presence of another unique compound in *L. scoparium* nectar and honey. In this case, the compound was described as a pteridine derivative 3,6,7-trimethyllumazine, and named Lepteridine (**Figure 1**) [25]. This compound has also been quantified by HPLC in manuka honey [26]. Both Leptosperin and Lepteridine have been reported to be chemically stable over prolonged storage in honey [21, 22, 26].

Methyl syringate (**Figure 1**) has also been shown to be present at elevated concentrations in manuka honey. However, previous studies indicate that this compound does not correlate with non-peroxide activity [20, 21]. Additionally, methyl syringate concentration has also been reported as elevated in kanuka honeys (*K. ericoides*) and is higher than that reported in manuka honeys [7, 10]. Accordingly, methyl syringate may not be a suitable chemical marker for manuka honey.

[1]Leptosperin was initially named 'leptosin' [20] but was later renamed to avoid confusion with the marine fungus-derived leptosins [21].

Beyond traditional analytical techniques, fluorescence spectroscopy has demonstrated use in analysing a range of food products including honeys [27–30]. Fluorometric methods are reported to be up to 1000 times more sensitive than absorption-based techniques [31]. Fluorescence spectroscopy provides improved specificity by examining distinct excitation and emission wavelengths and is a rapid, cost-effective and efficient non-destructive method [32, 33].

Fluorescence in honeys has been attributed to phenolic and polyphenolic compounds [27–30], amino acids [28–30] and Maillard reaction products [28, 29]. As phenolic and polyphenolic compounds have been described as reliable indicators of botanical and geographical origin of honeys [10, 16, 34, 35]; the fluorescence properties of these intrinsic and unique fluorophores may inform identification of floral source reliably.

Recent examination of the fluorescence profiles of the main New Zealand honey types demonstrated that manuka honey exhibited unique fluorescence characteristics that distinguish it from the other honey types [36]. Manuka honey contained two unique fluorescence signatures, $_{ex}270–_{em}365$ nm and $_{ex}330–_{em}470$ nm, named MM1 and MM2, respectively [36]. Dilution of manuka honey with other New Zealand honey types, which did not fluoresce at the diagnostic wavelengths, resulted in a reduction of the fluorescence signal in the manuka honey that was proportional to the dilution.

Further work confirmed that Leptosperin was responsible for the MM1 fluorescence signature ($_{ex}270–_{em}365$ nm) [22] and Lepteridine was the principal compound associated with MM2 fluorescence ($_{ex}330–_{em}470$ nm) in manuka honey [26]. For these compounds, standards were synthesised for Leptosperin [37] and Lepteridine [25], and seeding of honeys experimentally confirmed that these compounds are the primary fluorophores.

Consequently, these findings demonstrate manuka honey contains unique fluorophores that may be quantified to establish floral authenticity. As this technique is fluorescence-based, it provides the opportunity for rapid screening of honey samples to confirm honey labelling is appropriate and complies with the wholly or predominantly ruling in Codex Alimentarius [2]. In this chapter, the fluorescence technique is applied to sets of field-collected manuka honeys and a set of manuka honeys purchased commercially in 2016. Other compounds of interest in manuka honey, such as 2-methoxyacetphenone, methyl syringate, MGO and DHA, are additionally quantified.

2. Fluorescence markers in manuka honey

The fluorescence markers in manuka honey were assessed in a number of honey collections, first, field honeys harvested from *L. scoparium* hive sites in New Zealand, and second, a commercial set purchased from retail distributors in Singapore. The honeys in the purchased set were labelled as manuka honey and therefore should be wholly or predominantly sourced from *L. scoparium*.

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2.1. Leptosperin

Leptosperin has been shown to be uniquely derived from the *Leptospermum* genus in New Zealand and is present in manuka nectar and honey. This compound is readily quantified by liquid chromatography and mass spectrometry techniques and has been recently shown to be primarily responsible for the fluorescence exhibited by manuka honey at MM1 wavelengths [22]. As Leptosperin has been demonstrated to be chemically stable during extended storage experiments [22], this compound is an ideal candidate as a chemical and fluorescence marker for manuka honey.

Leptosperin is present in manuka honey at a concentration up to approximately 1700 mg/kg, with a minimum reported concentration of 93 [22] to 126 mg/kg [20], and therefore, it is probable that manuka honey can be expected to carry a minimum of 100 mg/kg. Fluorescence of Leptosperin is readily detected in manuka honey using the reported technique with lower detection limit of 10 ppm.

The field collected manuka honeys (n = 28) and the commercial honeys (n = 17) exhibited fluorescence that strongly correlated (R^2 = 0.9530) with the quantified concentration of Leptosperin (**Figure 2A**), confirming the previous research of this compound. The concentration of Leptosperin in the commercial samples fell in the lower half of the range recorded for the field samples. The mean concentration of Leptosperin was 423 and 192 mg/kg in the field and commercial samples, respectively ($p < 0.0001$). This is consistent with the previously reported comparison of field and commercial manuka samples [22]. However, two of the commercial samples contained less than 100 mg/kg Leptosperin which is considered to be the lower than acceptable minimum concentration.

Leptosperin and MM1 analysis of an additional field honey collection (n = 71) throughout New Zealand (**Figure 2B**) demonstrated that each region contained honeys that were distrib-

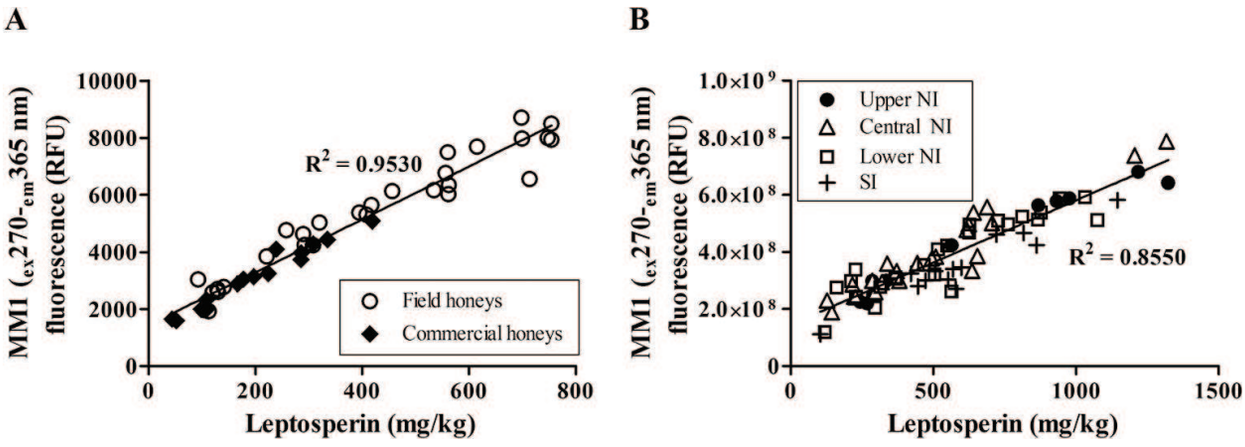

Figure 2. (A) Correlation between Leptosperin concentration and MM1 fluorescence in field and commercial manuka honeys. (B) Regional distributions of Leptosperin concentration and MM1 fluorescence, all data correlation shown.

uted throughout the range of recorded concentrations and signal. These observations reinforce the earlier findings that Leptosperin is a reliable fluorescence marker in manuka honey and can be used to categorise the national crop.

2.2. Lepteridine

Lepteridine is also uniquely derived from the *Leptospermum* nectar in New Zealand and appears to be present in all manuka honeys. This compound has been quantified by liquid chromatography, and is principally responsible for the fluorescence exhibited by manuka honey at MM2 wavelengths [26]. Lepteridine has also been shown to be chemically stable in elevated storage temperature [26] and has been proposed as an additional chemical and fluorescence marker for manuka honey.

The concentration of Lepteridine in field collected manuka honey is reported to be in the range between 5 and 50 mg/kg [26] and has not been examined in commercial samples previously. Concentrations as low as 1 ppm were detected by using the reported fluorescence method.

Again the field collected manuka honeys (n = 27) and the commercial samples (n = 17) displayed similar characteristics. The concentration of Lepteridine was correlated linearly (R^2 = 0.9433) with the fluorescence signal at MM2 (**Figure 3**). Nonetheless, the commercial samples contained significantly lower concentrations of Lepteridine than the field samples, being a mean value of 28 and 6 mg/kg, respectively ($p < 0.0001$) which is a fourfold difference. Furthermore, seven of the commercial samples contained less than the reported lower concentration of 5 mg/kg; four of which contained 4 mg/kg Lepteridine.

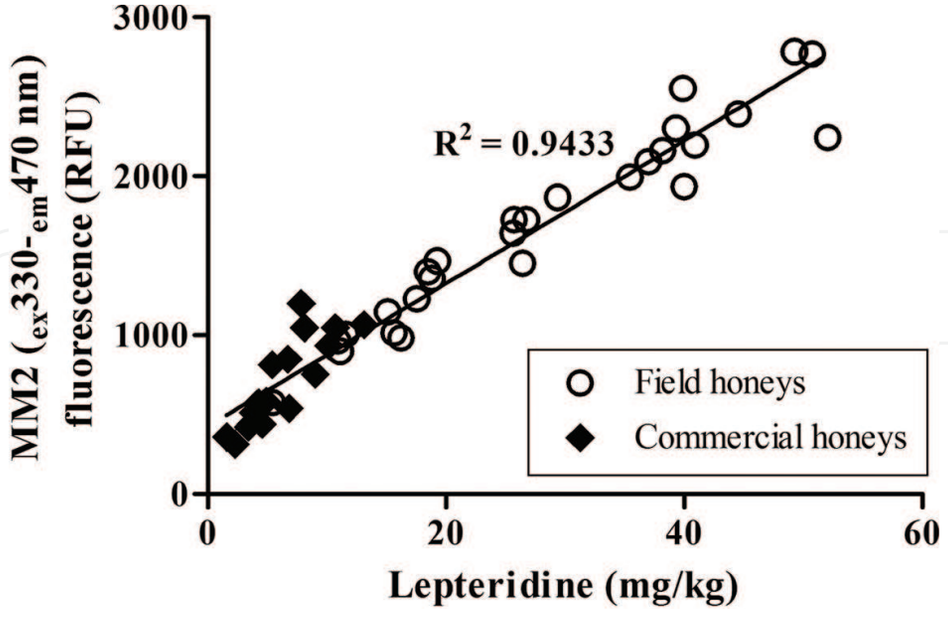

Figure 3. Correlation between Lepteridine concentration and MM2 fluorescence in field and commercial manuka honeys.

2.3. Correlation of MM1 and MM2 fluorescence signal

The fluorescence generated by Leptosperin and Lepteridine at MM1 and MM2 display a relatively strong linear correlation in manuka honeys (R^2 = 0.8620). This indicates a degree of colinearity between these compounds. Whilst this may limit the use of both compounds in model development using data from more traditional techniques such as liquid chromatography or mass spectrometry, the use of two independent wavelength pairs in fluoro-spectroscopy is expected to considerably strengthen this technique.

The fluorescence signal at both marker wavelengths in manuka field honeys (n = 27) is illustrated along with the commercial samples (n = 17) (**Figure 4**). The commercial sample mean fluorescence was significantly lower than the field samples for both MM1 ($p < 0.0001$) and MM2 ($p < 0.001$), and this reflects the concentration of Leptosperin and Lepteridine that are present in these commercial honeys.

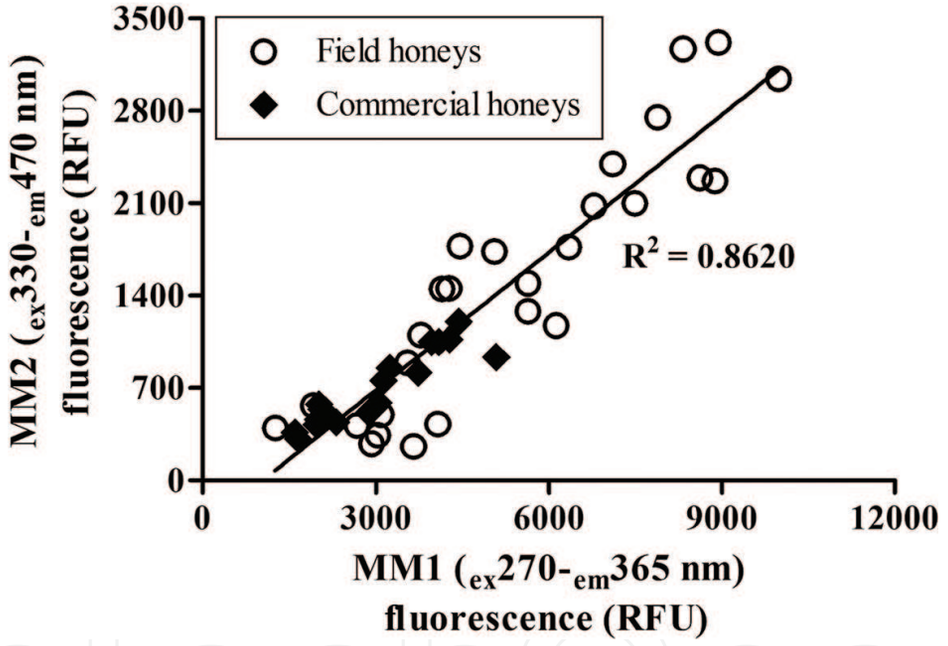

Figure 4. Correlation between MM1 and MM2 fluorescence signal in field and commercial manuka honeys.

3. Examination of non-fluorescent markers in commercial honeys

3.1. 2-Methoxyacetophenone and methyl syringate

2-Methoxyacetophenone has been described as a marker compound for manuka honey previously using HS-SPME-GC/MS [7, 11]. For the first time, this chapter reports the analysis of this compound in honey solutions using HPLC-DAD. The concentration of Leptosperin correlated strongly (R^2 = 0.8722) with 2-methoxyacetophenone concentration (**Figure 5A**) rein-

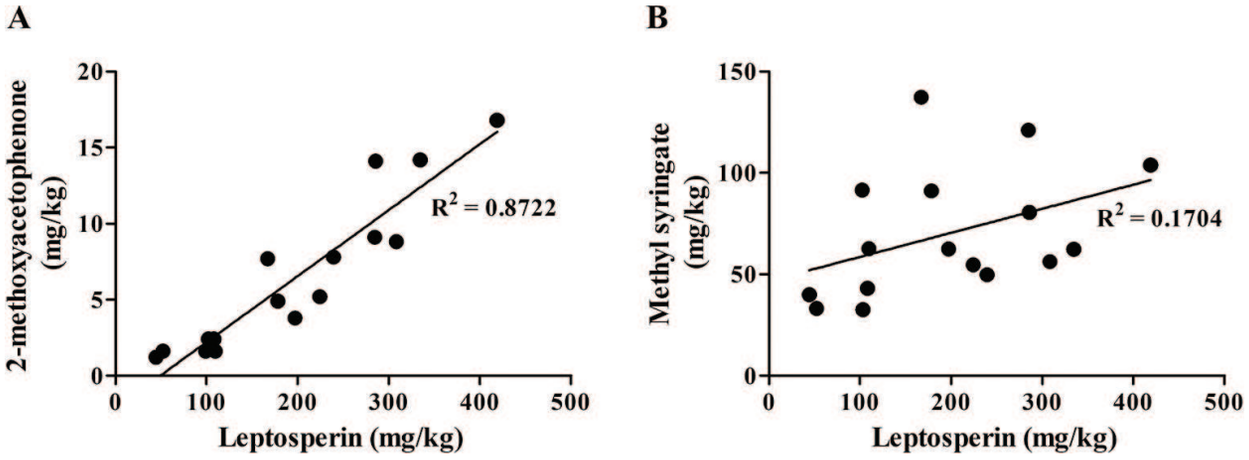

Figure 5. Correlation between Leptosperin and (A) 2-methoxyacetophenone and (B) methyl syringate in commercial manuka honey samples.

forcing the previous publications regarding the use of this compound as a potential marker for manuka honey.

Methyl syringate concentration is elevated in manuka and kanuka honeys in New Zealand [7, 10]. The linear correlation (**Figure 5B**) of Leptosperin and methyl syringate concentrations is poor (R^2 = 0.1704). This may be a reflection of kanuka content in manuka honeys; the kanuka honey is expected to contribute additional elevated levels of methyl syringate. Therefore, methyl syringate is not a reliable marker for manuka honey.

3.2. Dihydroxyacetone and methylglyoxal

Dihydroxyacetone is the precursor compound for MGO in ripening and maturing manuka honey. Dihydroxyacetone concentration in manuka honey can vary for three reasons: first, the DHA concentration in nectar harvested from the varieties of *L. scoparium* is significantly different, and this may vary as much as twofold [38], second, floral dilution will reduce the amount of DHA being incorporated into a honey during ripening and third, this precursor undergoes chemical reactions in the maturing honey solution [6, 15].

Conversely, MGO is absent in nectar. The MGO concentration increases rapidly during a manuka honey's first couple of years as the chemical conversion of DHA to MGO proceeds. However, the rate of conversion declines as the DHA pool is exhausted. Furthermore, in honeys that are reaching the end of their five-year shelf-life, it has been demonstrated that the MGO concentration begins to decline as insufficient DHA remains to sustain the MGO concentration [6].

These changes in DHA and MGO concentration are best demonstrated in elevated temperature storage experiments which promote the chemical reactions. Thirteen honeys stored for a little over 3 months at 37°C demonstrate these effects (**Figure 6A**). In this time, DHA decreased by about 18% and MGO increased by 46%, and these concentration shifts continue as the honey matures. Conversely, decreased storage temperature will significantly reduce

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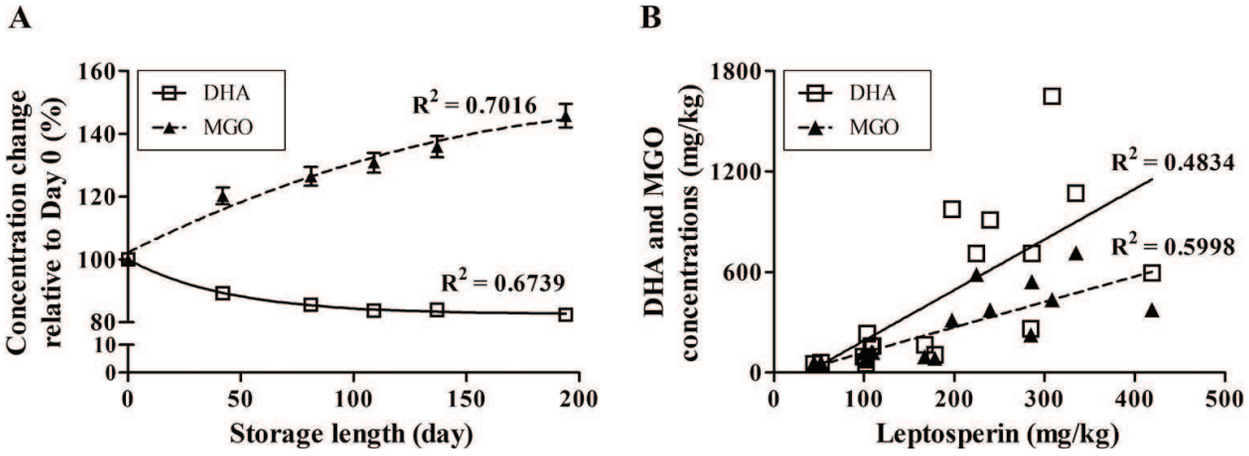

Figure 6. (A) The relative concentrations of DHA and MGO expressed as a percentage of the initial concentration in 10 manuka honeys stored at 37Â°C and (B) the relationship of DHA and MGO to the fluorescent marker Leptosperin in commercial manuka honeys.

the rate of chemical reactions in the honey; therefore, the concentration of DHA and MGO in a manuka honey can be considerably influenced by processing and storage conditions.

Leptosperin and Lepteridine have also been quantified in honeys stored at elevated temperatures for over 400 days and neither compound demonstrated any significant deviation from the initial concentration [22, 26].

For manuka honey harvested from hives in a particular well-defined region where the DHA concentrations in the nectar is relatively constant, there is a relatively strong correlation between MM1 fluorescence and DHA concentration [39]. This is because the honeys are of a uniform age and therefore exclude ageing differences, the DHA potential of the nectar is relatively similar as the harvested *L. scoparium* population is very discrete and genetically linked, and any reduction of DHA in the honey can be attributed to floral dilution from other nectar sources. Consequently, the floral dilution alone acts upon the fluorophores such as Leptosperin, resulting in a relatively stronger correlation between DHA and Leptosperin in the honey.

However, when commercial manuka honey samples are considered the effect of both different initial DHA concentrations in nectar, and ageing-driven chemical reactions, which may occur at different rates due to temperature influences, are very difficult to separate from floral dilution. The concentrations of Leptosperin, DHA and MGO (**Figure 6B**) for commercial manuka honeys demonstrate the relatively poor correlation that exists in commercial honeys of different provincial provenance, unknown age and storage conditions.

The concentrations of DHA and MGO relative to Leptosperin (**Figure 7**) in the 71 field honeys shown in **Figure 2B** reinforce the insignificant relationship between these compounds and a consistent floral marker. The regional data groups are scattered and there is no significant linear correlation as it is likely that the genetic linkage within these large regional areas is insufficient to overcome variability between DHA potential in *L. scoparium* nectar. Similarly,

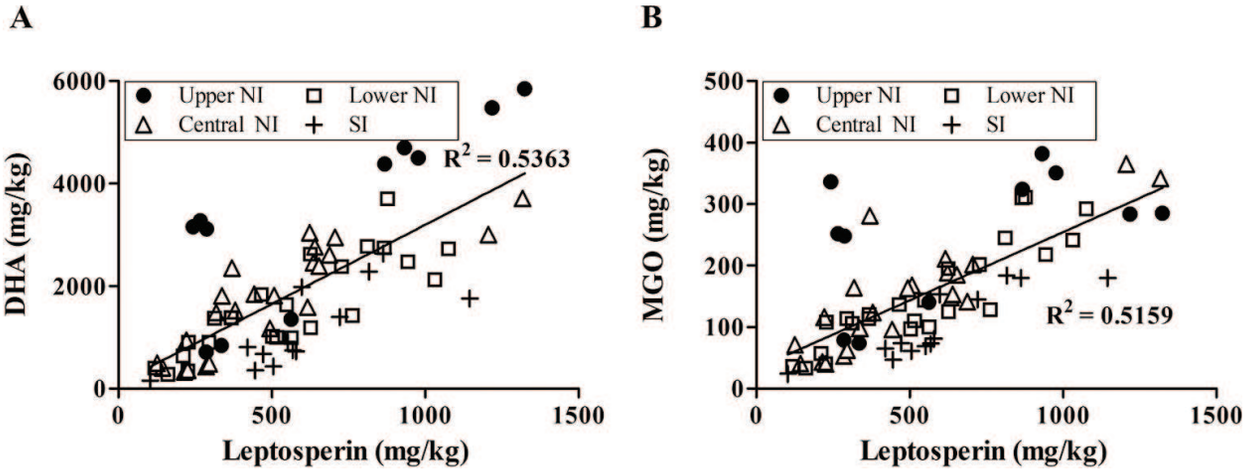

Figure 7. The relationship of (A) DHA and (B) MGO to the fluorescent marker Leptosperin in field honeys throughout New Zealand.

MGO displays a poor correlation with Leptosperin, where differences in ageing profile as well as initial DHA potential are magnified.

Therefore, despite both DHA and MGO being unique to manuka honey in New Zealand, neither of these compounds are reliable predictors of floral authenticity in commercial manuka honey samples. Both DHA and MGO should be present in genuine manuka honey; however, the concentration of these compounds does not correlate with stable nectar-derived chemical markers.

3.3. Commercial manuka honey samples summary

The concentrations of Leptosperin, Lepteridine, 2-methoxyacetophenone, methyl syringate, DHA and MGO, along with MM1 and MM2 fluorescence and Unique Manuka Factor (UMF) are presented in **Table 1**. UMF is the non-peroxide antibacterial grading system devised 25 years ago that measured bioactivity [40] and did not take into the account of floral authenticity.

The significant manuka chemical markers vary in concentration up to more than 1 order of magnitude in the examined honey samples. The harvested *L. scoparium* variety and floral dilution by other plant species contribute to this range. However, three of the analysed honeys, Samples #1, #2 and #3, demonstrated chemically low or marginal results for Leptosperin and Lepteridine, and likewise had particularly low concentrations of DHA and MGO.

Box- and whisker-plots and histograms (**Figure 8**) demonstrate the statistical distribution of Leptosperin and Lepteridine in the commercial honey set. Both datasets are positively skewed, with mean values greater than the median, and this confirms that more manuka honey in this set has greater concentrations than the proposed lower limits for Leptosperin and Lepteridine.

Sample	Concentration (mg/kg)						Fluorescence (RFU)		UMF
	Leptosperin	Lepteridine	2-MAP	MSYR	DHA	MGO	MM1	MM2	
1	44	2.28	1.18	40	52	59	1649	314	4.1
2	52	1.57	1.59	33	57	59	1594	359	4.1
3	102	3.37	2.44	91	50	69	1973	422	4.5
4	99	4.28	1.61	205	94	117	1998	458	6.1
5	103	6.82	1.87	33	231	103	2058	539	5.7
6	108	4.54	2.37	43	157	124	2319	438	6.4
7	110	4.25	1.56	63	160	121	2014	574	6.3
8	167	3.82	7.72	137	165	94	2885	511	5.4
9	178	4.89	4.88	91	104	84	3052	584	5
10	197	9.00	3.82	62	976	313	3135	754	11.1
11	224	6.73	5.20	55	712	585	3245	849	16.2
12	239	8.13	7.82	50	911	372	4097	1047	12.3
13	285	5.40	9.09	121	262	224	3735	814	9.1
14	286	10.73	14.14	81	711	542	3970	1046	15.5
15	308	13.13	8.75	56	1650	435	4277	1066	13.6
16	335	7.85	14.15	62	1070	715	4438	1199	18.3
17	419	10.15	16.83	104	594	375	5087	933	12.4

Note: 2-MAP, 2-methoxyacetophenone; MSYR, methyl syringate; DHA, dihydroxyacetone; MGO, methylglyoxal.

Table 1. Chemical composition, fluorescence, and non-peroxide antibacterial activity (UMF) of 17 commercial manuka honey samples.

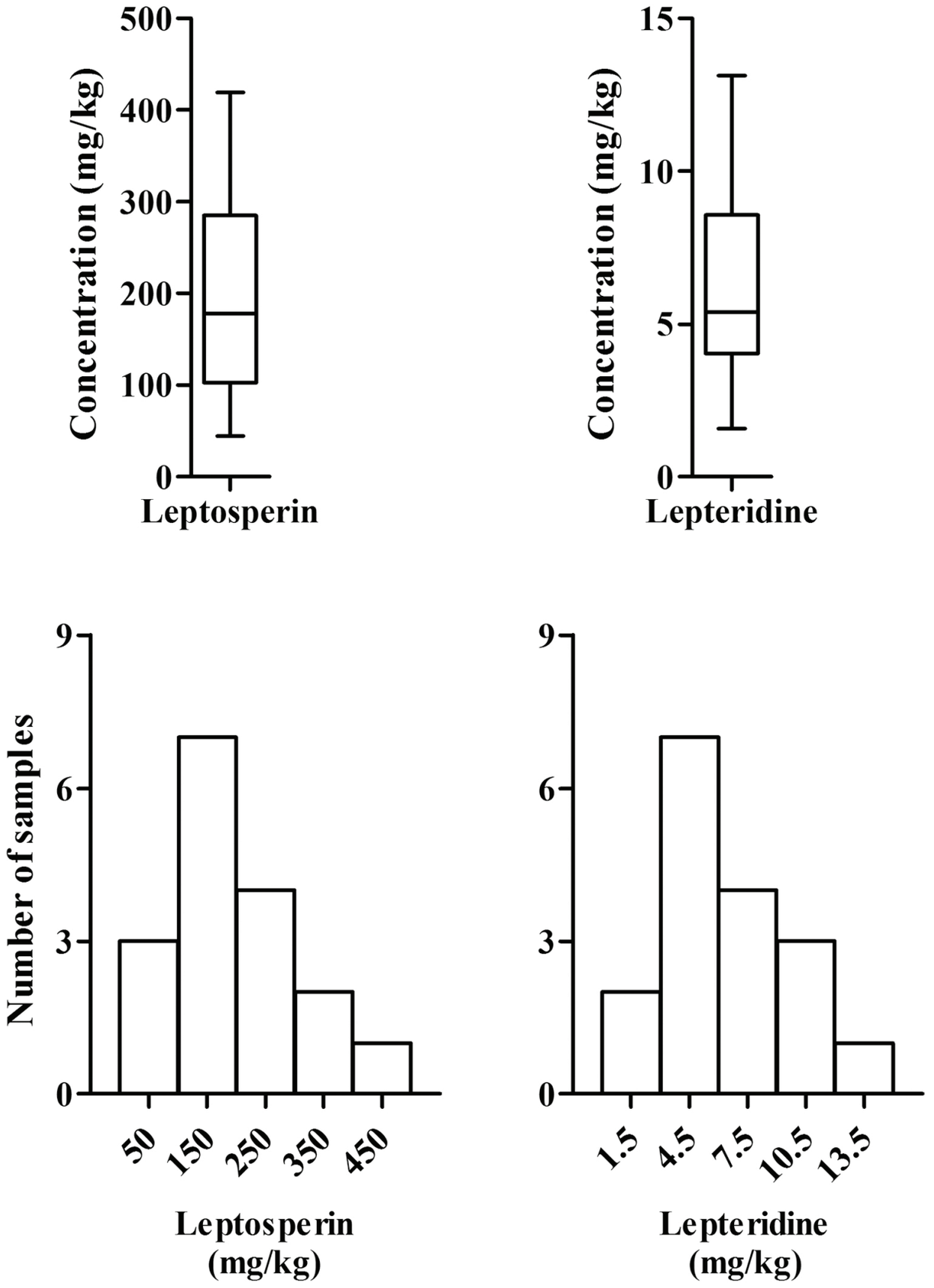

Figure 8. Statistical distribution of Leptosperin and Lepteridine in commercial manuka honeys.

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The relationship of Leptosperin and Lepteridine with the associated fluorescence for the commercial honeys is illustrated (**Figure 9**). For the purposes of this discussion, the minimum accepted concentration of Leptosperin is 100 mg/kg, and Lepteridine 4 mg/kg, these concentrations relate to MM1 (2000 RFU) and MM2 (500 RFU) in a honey matrix. RFU is an arbitrary unit and varies between fluorometers. These lower acceptable levels of these four parameters are illustrated in **Figure 9**.

Honey samples 1 and 2 do not meet the criteria for chemical concentration of either Leptosperin or Lepteridine, and fluorescence profile of both honeys did not meet the lower threshold. Honey sample 3 Leptosperin and Lepteridine concentration was 102 and 3.37 mg/kg, respectively. The fluorescence signature of this sample was slightly less than the threshold, and most probably was not wholly or predominantly harvested from *L. scoparium*.

When assessing honeys that are close to the lower threshold, it is appropriate to consider multiple characteristics and accordingly honey samples 4 and 6 are considered accepted despite the MM2 fluorescence being in the order of 450 RFU rather than 500 RFU.

Therefore, in this commercial set of manuka honeys, three out of 17 samples did not display the fluorescence characteristics or contain the concentrations of the key markers that would be expected to be encountered in an authentic manuka honey. Rapid assessment by fluorescence would have identified these three samples as requiring a full analytical workup, and allowing the balance of samples to be retailed as manuka honey.

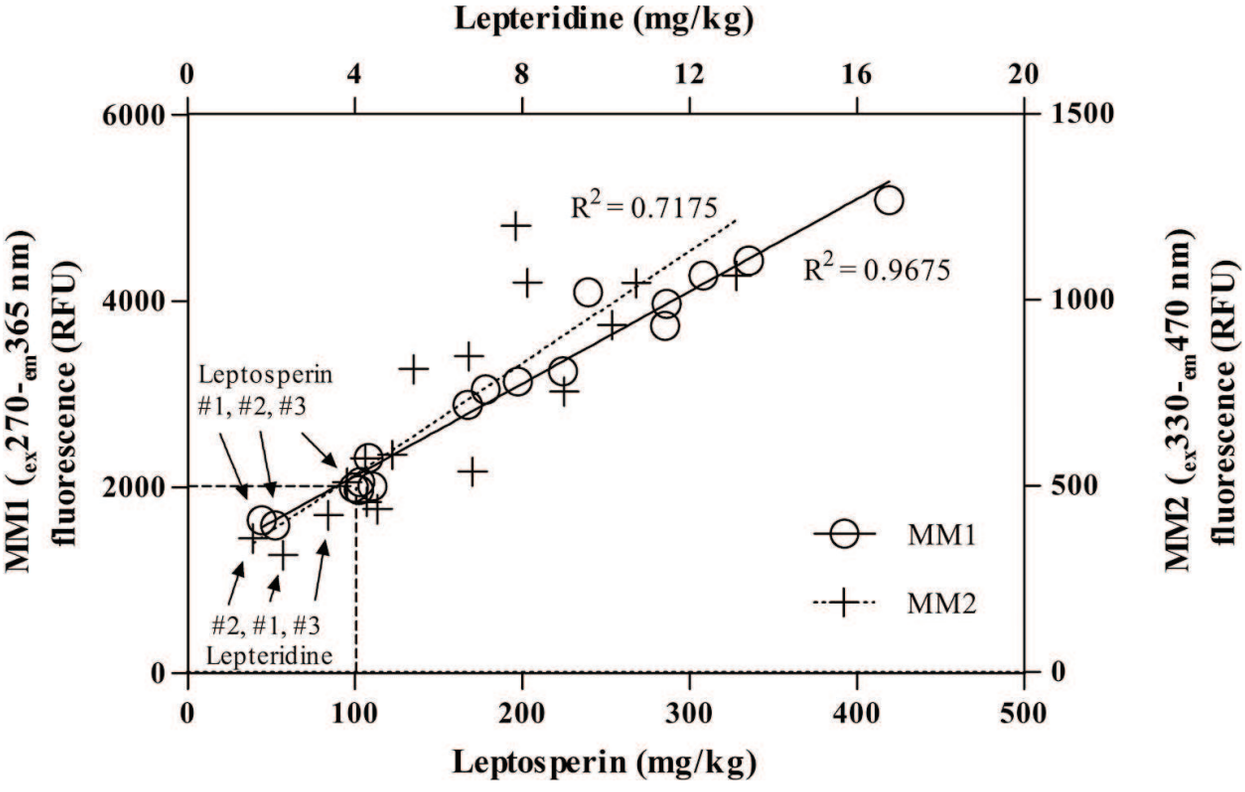

Figure 9. Commercial manuka honey samples Leptosperin and Lepteridine concentrations in relation to MM1 and MM2 fluorescence.

4. Conclusions and applications

Fluorescence analysis is a novel technique to determine manuka honey authenticity. Two unique compounds have been found in manuka honey, Leptosperin and Lepteridine, and these compounds are responsible for the MM1 and MM2 fluorescence described in the honey. In New Zealand, Leptosperin and Lepteridine are present only in *L. scoparium* nectar and therefore, these compounds are reliable chemical markers for manuka honey. However, the concentrations of these compounds do not predict DHA and MGO concentrations in a honey.

Fluorescence spectroscopy is a rapid technique with high throughput, and relatively simple fluorescence screening assessments are gaining increasing attention in food processing systems. Technology for assessing fluorescence is developing rapidly and handheld fluorometers are available. A handheld fluorometer could be used in the field by beekeepers, alternatively in market by retailers and may be of use to regulatory authorities.

Fluorescence assessment of manuka honey is an independent method separate from liquid chromatography coupled to detectors such as DAD or mass spectrometry. The use of two sets of wavelengths in combination, which can be screened simultaneously, adds robustness to this analysis.

Therefore, analysis of the MM1 and MM2 wavelengths is an efficient way of screening New Zealand honeys to ensure that attribution of floral source is appropriate and manuka honeys are wholly or predominantly sourced from *L. scoparium*, and these honeys that do not display characteristics of manuka honey are not inappropriately labelled.

5. Analytical techniques

5.1. Fluorescence spectroscopy

Honey fluorescence was analysed by scanning fluorescence spectroscopy according to methods described previously [36]. Honey samples were diluted with distilled water to 2% w/v, and loaded as 100 μL aliquots into a flat-bottom microplate (Optiplate™-384, black). Fluorescence measurements were carried out on a Gemini EM Dual-Scanning Microplate Spectrofluorometer (Molecular Devices Inc., Sunnyvale, CA, USA) operated with the SoftMax® Pro software. A fluorescence top read setting with automatic calibration and sensitivity at an ambient temperature was adopted for analysis at both MM1 and MM2 marker wavelengths. Fluorescence intensity was expressed as arbitrary units, in this case relative fluorescence units (RFU).

5.2. HPLC

Leptosperin, Lepteridine, methyl syringate and 2-methoxyacetophenone concentrations were quantified on a Dionex Ultimate™ 3000 reverse-phase HPLC system (Thermo Fisher Scientific, New Zealand) with diode-array detection (DAD).

Honey samples were diluted 1 in 5 with 0.1% v/v formic acid. The injection volume was 3 μL. Separation was carried out on a Hypersil GOLD column (150 × 2.1 mm, 3μm particle size) by gradient elution at a constant flow rate of 0.200 mL>:in. The binary mobile phase consisted of 0.1% formic acid (Solvent A) and 80:20 acetonitrile:Solvent A (Solvent B). The gradient elution programme was as follow: initial (5% B, held 2 min), 7 min (25% B), 14 min (50% B), 16 min (100% B, held 3 min), 19 min (5% B, held 1 min) and 20 min (5% B, held 10 min). The column was thermostatically controlled at 25°C. Leptosperin, Lepteridine, methyl syringate and 2-methoxyacetophenone were monitored at 262, 320, 280 and 250 nm, respectively.

Data acquisition and peak integration were performed with Thermo Fisher Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS) software. The compounds of interests were quantified using external calibration curves of respective chemical standards based on integrated measurement of peak area.

5.3. UPLC and fluorescence methods

Honey samples (0.5 g) were weighed into a polypropylene extraction tube and solubilised in 9.5 mL of 10% acetonitrile containing 0.1% formic acid in Type 1 water by shaking and ultrasonic agitation. After centrifugation to remove particulates, an aliquot was diluted a further fivefold for analysis by ThermoFisher Ultimate-3000 UPLC with an RS fluorescence detector ($_{ex}$264nm–$_{em}$365nm), using a Waters XSelect HSS T3 C18 column (2.1 × 30 mm, 2.5 μm particle size). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in methanol. The elution gradient started at 8% B (92% A) and increased to 100% B over 10 min before equilibration in 92% A for 3 min. Leptosperin was quantified against a synthetic standard.

For gross honey fluorescence analysis, 250 μL of the 20-fold diluted extract used for the Leptosperin analysis was added to the well of a fluorescence-grade 96-well plate and the gross fluorescence of each sample measured at MM1 using a SpectraMax i3 (Molecular Devices LLC, Sunnyvale CA, USA).

5.4. DHA and MGO

Concurrent analysis of DHA and MGO was carried out on a Dionex UltimateTM 3000 reverse-phase UPLC-DAD system (Thermo Fisher Scientific, New Zealand) following derivatisation with *O*-(pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA). Honey samples were prepared in distilled water at a 1:20 w/v ratio. The samples were thoroughly mixed and incubated at 50°C for 1 hour to allow complete dissolution of any sugar crystals.

The derivatisation procedure was carried out according to methods developed by Kato et al. [41] with some modifications. A 2% stock solution of PFBHA was prepared in 0.1 M citrate buffer adjusted to pH 4 with NaOH (1 M). A working solution of PFBHA derivatising reagent was prepared consisting of 7:2:1 LC-MS grade acetonitrile:distilled water:PFBHA stock solution, and added to the honey samples at a 5:1 v/v ratio. The PFBHA:honey mixture was incubated at 50°C for 1 hour and cooled to room temperature.

A 5 μL aliquot of the derivatised sample or standard was injected into the UPLC-DAD system. Separation was carried out by gradient elution on a Hypersil GOLD column (100 × 2.1 mm, 1.9μμm particle size) at a constant flow rate of 0.700 mL/min. The mobile phase consisted of 0.1% v/v aqueous formic acid (Solvent A) and LC-MS grade acetonitrile (Solvent B), and the gradient elution programme was as follows: initial (B 20%, held 0.6 min), 1.3 min (B 70%), 3 min (B 100%, held 0.5 min) and 4 min (B 20%). The column was thermostatically controlled at 50°C. Dihydroxyacetone was monitored at 214 nm and MGO at 246 nm.

Data acquisition and peak integration were performed with Thermo Fisher ScientificTM Dionex™ Chromeleon™ 7.2 CDS software. Honey DHA and MGO were quantified using external calibration curves generated from the DHA and MGO working standards by linear regression of peak area against concentration.

Acknowledgements and disclosure

The authors acknowledge the Unique Manuka Factor Honey Association (UMFHA), Analytica Laboratories Limited, and Comvita New Zealand Limited for samples and general support of this work. Part of this work was supported by Comvita Claude Stratford doctoral scholarship from Comvita NZ Limited and various New Zealand government science funding grants. Jonathan Stephens and Gordana Prijic are employed by Comvita New Zealand Limited. Terry Braggins is the co-founder of Analytica Laboratories Limited.

Author details

Jonathan M. Stephens[1]*, Kerry M. Loomes[1], Terry J. Braggins[2], Jessie Bong[1], Bin Lin[1] and Gordana Prijic[1]

*Address all correspondence to: Jonathan.Stephens@comvita.com

1 Comvita New Zealand Limited, Institute for Innovation in Biotechnology, School of Biological Sciences, University of Auckland, Auckland, New Zealand

2 Analytica Laboratories Limited, Ruakura Research Centre, Hamilton, New Zealand

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