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# The fluorescence of MM1, leptosperin in Australian and New Zealand Manuka Honeys

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ABSTRACT

The Manuka Marker 1 fluorescence of Australian and New Zealand Manuka honeys at $_{ex}270$-$_{em}365$ nm correlates to their leptosperin content. Non-Manuka honey varieties did not fluoresce at these wavelengths.

A strong correlation $R^2 = 0.96$ was found between the fluorescence response and leptosperin content by HPLC of Manuka honeys. The highest concentrations of leptosperin were observed in Australian Manuka honey with a maximum value of 2031 mg/kg in comparison to New Zealand Manuka honey 729 mg/kg. In this study, no correlation was observed between leptosperin and methylglyoxal content.

## Introduction

Bioactive Manuka honey is produced by honeybees (*Apis Mellifera*), foraging on nectar from certain species of the *Leptospermum* genus endemic across Australia and New Zealand. The honeybee was introduced to both countries in 1822 and 1839 respectively [1,2].

Australia has over 80 endemic species of *Leptospermum* [3,4], whilst New Zealand has two endemic species, *Leptospermum scoparium* (Mānuka) and *L. repo* [5], plus another seven introduced species [6].

Manuka honey is renowned for its Non-Peroxide Antibacterial activity (NPA) and wound healing properties [7-11]. The NPA of Manuka honey is predominately derived from Methylglyoxal (MGO) [12-14] which is itself formed from the three-carbon sugar Dihydroxyacetone (DHA) (Fig. 1) during honey maturation [13]. Whilst MGO is absent in the nectar of *Leptospermum* flowers, the precursor molecule DHA is found at elevated concentrations [15,16]. High levels of DHA in nectar correlate to subsequent high MGO Manuka honey [16].

The concentration of DHA in *Leptospermum* nectar is highly variable and influenced by both species and geographical region [15-17]. For instance, *L. scoparium* which is endemic to Australia and New Zealand averages 2000 mg of DHA per kg of nectar sugars, however, it can vary greatly by region [16,17]. Approximately, 40 of the 55 Australian *Leptospermum* species sampled by Williams et al. produced nectar DHA, including *L. polygalifolium* and *L. whitei* which averaged 9000 and 16,000 mg/kg respectively [16]. These floral sources produce Manuka honeys of greater NPA than *L. scoparium*.

Previous studies have shown Manuka honey contains elevated concentrations of phenolic compounds, which are responsible for the anti-inflammatory and antioxidant activities of the honey [18,19]. One compound unique to Manuka honey is leptosperin (Fig. 1) [18,20]. The molecule is the β-d-gentiobioside of methyl syringate and fluoresces at 270 nm excitation and 365 nm emission. Bong et al. [21] labelled this fluorescence as "Manuka Marker 1″ (MM1), and subsequently determined leptosperin as responsible [22,23], recommending a minimum concentration of 94 mg/kg to authenticate Manuka honey from New Zealand. Leptosperin has been previously analysed by fluorescence [21-24], by HPLC-UV [22,23] and LC-MS/MS [25]. LC-MS/MS is the gold standard for leptosperin analysis, but at an instrument turnaround time of approximately 20 min per sample, it is time and resource expensive. Hence, the development of a convenient and cost-effective method of analysis is of great importance.

According to the New Zealand UMFHA *"Leptosperin is a compound found only in mānuka flower nectar and can only be created by nature. Measuring this unique component ensures the authenticity of mānuka honey."* [26]. The New Zealand Ministry of Primary Industries (NZ MPI) in developing the standards of New Zealand Manuka honey exports excluded leptosperin on the rationale that the molecule was naturally present in Australian Manuka as well as New Zealand Manuka and therefore not a distinguishing marker of the New Zealand product [27].

The objectives of this study were to analyze Australian and New Zealand Manuka honeys at MM1 wavelengths by microplate fluorescence, and to compare this to leptosperin data obtained by HPLC-UV

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from an independent testing lab. In addition, the MGO and DHA content of the honeys was measured and compared.

## Materials and methods

### *Honey samples*

Manuka honey samples ($n = 7$) from New Zealand were purchased from Chemist Warehouse pharmacy stores in Queensland in February 2023 (Comvita®, Egmont®, Bramwell®). Additionally, the sample GP1, a *L. scoparium* sample was supplied by Global Proficiency. Manuka honeys ($n = 20$) from Australia were research samples supplied from reputable beekeepers as monofloral to species, collected from January 2019 to December 2021 and stored at 4 °C. These include *L. scoparium* ($n = 8$) from Tasmania, and *L. polygalifolium* ($n = 5$), *L. liversidgei* ($n = 2$), *L. whitei* ($n = 3$) plus *L. speciosum* ($n = 2$) all from New South Wales. Five varieties of non-Manuka honeys (each $n = 3$) were donated by Capilano Honey, Australia or by reputable beekeepers as true to type.

### *Leptosperin standards and honey samples preparation*

Natural leptosperin was extracted and purified from *L. scoparium* honey from Tasmania, Australia, and confirmed by $^{1}$H NMR (Bruker Avance 400 MHz) using data reported by Kato et al. (2012) [18]. A leptosperin stock solution at 500 ppm was prepared in 50/50 aqueous ethanol. An artificial honey of Fructose 4.3 g/Glucose 3.7 g/Sucrose 0.3 g/Water 1.7 mL was spiked with leptosperin standard stock to give a leptosperin in honey range of 0–2000 mg/kg based on the artificial honey mass.

Honey samples (~1.0 g) were weighed accurately, then 4.25 volume (g/mL) of distilled water was added to dissolve the honey. This afforded honey stock concentration at 1.0 g/5 mL, (20% m/v). Each sample (0.5 mL) was further diluted with 100% ethanol (9.5 mL) to yield a 0.01 g/mL (1% m/v) final solution in 95% ethanol.

### *Fluorescence instrumentation and analysis*

Fluorescence analysis was performed on a Perkin Elmer Enspire Multimode Plate Reader with a xenon light source. Honey samples and standards were made to 1% solutions in 95% ethanol and 300 uL loaded in quadruplet into 96 well Invitrogen microplates for fluorescence. The measurements were taken at $_{ex}$270nm - $_{em}$365nm. Each plate was read in triplicate. The Relative Fluorescence Units (RFU) were obtained using the Enspire Manager software.

The leptosperin content of the Manuka honeys were tested independently at Analytica Laboratories, Hamilton, New Zealand [28]. The non-Manuka honeys were analysed in-house to confirm zero leptosperin content [29].

### *HPLC analysis of DHA and MGO*

DHA and MGO of all honeys were analysed by derivatisation with O-2,3,4,5,6-(pentafluorobenzyl)hydroxyl amine.HCl (PFBHA) followed by HPLC-DAD array via the procedures of Pappalardo et al. [30]. Briefly, honey (0.3–0.4 g) was weighed accurately into a test-tube. PFBHA reagent in citrate buffer was added. The mixture was vortexed, then was incubated at room temperature for 75 min. Next, an internal standards solution of anisole in acetonitrile/water was added and mixed well. A 1.0 mL aliquot was taken and analysed by HPLC-DAD at 263 nm using a Phenomenex Synergy Fusion RP 75 × 4.6 mm column and a gradient program of 75:25 ACN: $H_2O$ to 100% ACN.

## Results and discussion

A calibration curve was prepared using the leptosperin standards with concentrations ranging from 0 to 2000 mg/kg of artificial honey. The standard solutions were analysed by fluorescence at $_{ex}$270-$_{em}$365nm, and the Relative Fluorescence Units (RFU) of leptosperin standards were recorded. After blank correction for the artificial honey with zero added leptosperin, a non-linear calibration curve was obtained with a correlation coefficient of $R^2 > 0.99$, as shown in Table 1 and Fig. 2a. This non-linear relationship is due to fluorescence quenching at higher concentrations [23], which is consistent with previous fluorescence studies that utilised 200 µL of 2% honey concentration in water [23]. To mitigate this effect, trials of manuka honey at differing concentrations (1%, 2%), solvents (water, acetonitrile, 95% ethanol) and volumes (200µL, 300 µL) were undertaken. The greatest fluorescence (least quenching) was observed using 300 µL 1% honey solutions in 95% ethanol.

The Excel calibration of fluorescence to leptosperin spike isolated from Tasmanian L. *scoparium* honey in an artificial honey (Fig. 2a) gave a quadratic equation of power 2 (Eq (1)), of $R^2 > 0.99$.

$$RFU^{*}/1000 = -4.74 \times 10^{-5}X^2 + 0.1851X \quad R^2 = 0.9905 \tag{1}$$

Where RFU* is the standard's fluorescence minus artificial honey blank and X is the leptosperin concentration in mg/kg.

**Table 1**
Relative fluorescence units* of calibration standards.

| Leptosperin standards | RFU* |
|---|---|
| 2000 mg/kg | 186,949 |
| 1500 mg/kg | 160,088 |
| 1000 mg/kg | 135,232 |
| 500 mg/kg | 90,931 |
| 200 mg/kg | 44,106 |
| 100 mg/kg | 17,837 |
| 0 mg/kg | 0 |

* After subtraction of blank (after correction).

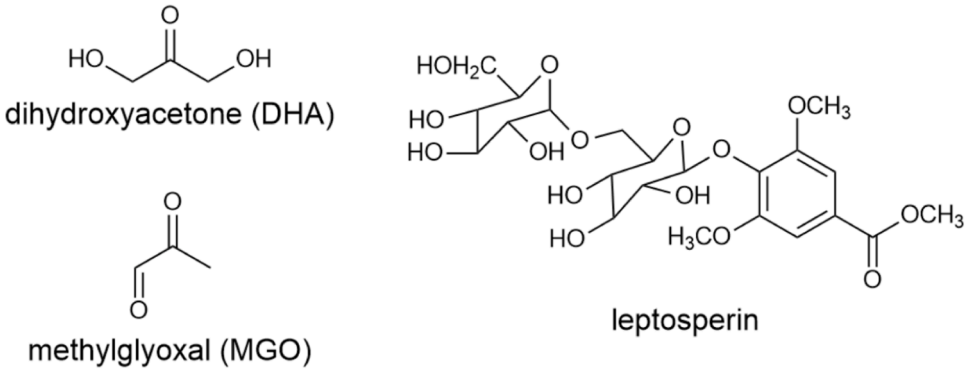


**Fig. 1.** Chemical structures of DHA, MGO and Leptosperin.

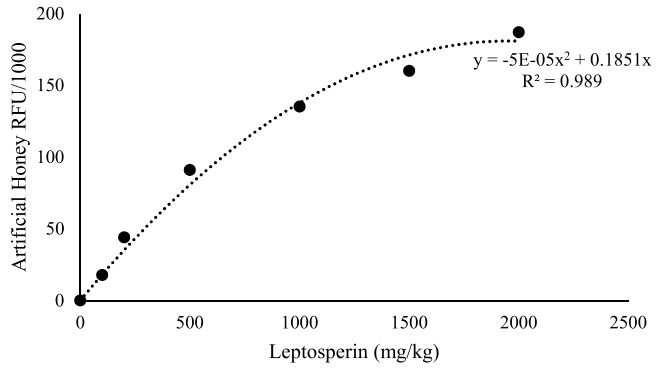


**Fig. 2a.** Calibration of Relative Fluorescence Units (RFU*) vs Leptosperin spike. Average ($n = 3$).

In order to facilitate the calculation of leptosperin concentration within Manuka honey, the axes were rearranged (Fig. 2b) providing another quadratic equation (Fig. 2b, Eq (2)) of $R^2 = 0.9965$.

$$\text{Leptosperin (mg/kg)} = 0.0512X^2 + 1.0174X \quad R^2 = 0.9965 \qquad (2)$$

Where X equals RFU*/1000

Using Eq. (2), the leptosperin concentrations in the Manuka honeys were established, and results are shown in Table 2.

While no leptosperin, nor DHA and MGO were found in any non-Manuka honey varieties, all Manuka honeys analysed in this study showed the presence of leptosperin, MGO and DHA, with the highest concentrations observed for Tasmanian *L. scoparium* 3, New South Wales *L. polygalifolium* 1 and New South Wales *L. polygalifolium* 2 respectively.

HPLC-UV and $_{ex}270$—$_{em}365$ fluorescence confirmed leptosperin was present in all honeys derived from the *Leptospermum* genus tested and not confined only to *L. scoparium* from New Zealand. This is consistent with previous studies that identified leptosperin in *L. polygalifolium* honeys [22]. Thus, the compound can be taken as a *Leptospermum* marker. The levels display what appears to be a species dependency, in that New Zealand *L. scoparium* (89–729 mg/kg), Australian *L. scoparium* (434–2031 mg/kg), *L. polygalifolium* (300–1702 mg/kg), *L. liversidgei* (58–71 mg/kg), *L. whitei* (261–471 mg/kg) and *L. speciosum* (120–147 mg/kg), although more sample numbers are required to confirm the concentration ranges. Even so, higher levels of leptosperin were found in nine of the Australia samples than the highest New Zealand sample. The highest concentrations observed for Tasmanian *L. scoparium* 3.

Of all Manuka honeys tested from Australia and New Zealand, a L. *liversidgei* displayed the lowest MM1 RFU* of 25,086 (leptosperin, 58 mg/kg) while lowest MGO was observed for the Bramwell MGO 50+ sample with a concentration of 85 mg/kg and the lowest DHA was recorded for Comvita 5+ sample of 115 mg/kg. The highest values were: Australian *L. scoparium* (RFU* 189,465; leptosperin 2031 mg/kg), an *L. polygalifolium* MGO 1252 mg/kg and another *L. polygalifolium* of DHA 4660 mg/kg respectively.

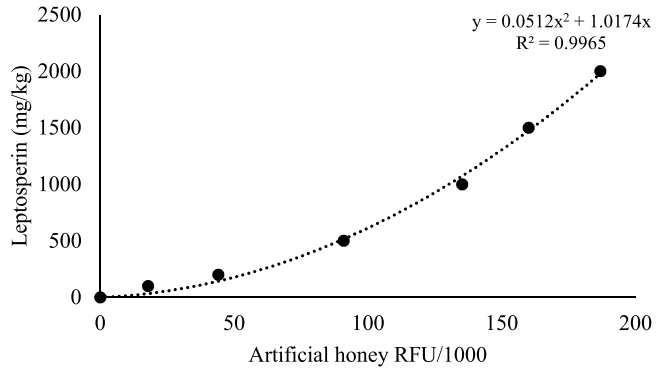


**Fig. 2b.** Calibration of Leptosperin levels vs Relative Fluorescence Units (RFU*) average ($n = 3$).

**Table 2**
Leptosperin, DHA and MGO data for Australian and New Zealand Honeys.

| Honey Identifier | RFU* | Leptosperin by fluorescence (mg/kg) | Leptosperin by HPLC (mg/kg) | MGO (mg/kg) | DHA (mg/kg) |
|---|---|---|---|---|---|
| NZ Comvita 5+ | 61,875 | 259 | 201 | 148 | 115 |
| NZ Comvita 10+ | 81,281 | 421 | 340 | 403 | 483 |
| NZ Comvita 15+ | 87,029 | 476 | 511 | 658 | 655 |
| NZ Comvita 20+ | 109,828 | 729 | 811 | 1145 | 1039 |
| NZ Egmont MGO 100+ | 47,578 | 164 | 159 | 166 | 302 |
| NZ Bramwell MGO 50+ | 32,815 | 89 | 159 | 85 | 118 |
| NZ GP-1 | 74,279 | 358 | 303 | 331 | 489 |
| *L. scoparium* 1 | 145,992 | 1240 | 1310 | 537 | 669 |
| *L. scoparium* 2 | 82,627 | 434 | 492 | 184 | 529 |
| *L. scoparium* 3 | 189,465 | 2031 | 2450 | 181 | 767 |
| *L. scoparium* 4 | 91,630 | 523 | 492 | 590 | 1391 |
| *L. scoparium* 5 | 139,841 | 1143 | 1230 | 415 | 3680 |
| *L. scoparium* 6 | 148,077 | 1273 | 1380 | 435 | 3468 |
| *L. scoparium* 7 | 96,962 | 580 | 561 | 439 | 2308 |
| *L. scoparium* 8 | 124,126 | 915 | 851 | 402 | 588 |
| *L. polygalifolium* 1 | 172,666 | 1702 | 1750 | 839 | 1370 |
| *L. polygalifolium* 2 | 158,942 | 1455 | 1400 | 652 | 3313 |
| *L. polygalifolium* 3 | 67,234 | 300 | 152 | 1252 | 1602 |
| *L. polygalifolium* 4 | 155,163 | 1390 | 1130 | 1243 | 4660 |
| *L. polygalifolium* 5 | 112,999 | 767 | 620 | 1150 | 1925 |
| *L. liversidgei* 1 | 28,669 | 71 | 109 | 261 | 238 |
| *L. liversidgei* 2 | 25,086 | 58 | 83 | 218 | 248 |
| *L. whitei* 1 | 62,139 | 261 | 191 | 465 | 1498 |
| *L. whitei* 2 | 86,481 | 471 | 359 | 961 | 1373 |
| *L. whitei* 3 | 82,046 | 428 | 252 | 473 | 1795 |
| *L. speciosum* 1 | 39,418 | 120 | 157 | 442 | 1502 |
| *L. speciosum* 2 | 44,614 | 147 | 185 | 407 | 1358 |
| *C. maculata* n = 3 | 0 | 0 | NA | 0 | 0 |
| *E. coolabah* $n = 3$ | 0 | 0 | NA | 0 | 0 |
| *E. camaldulenisn*=3 | 0 | 0 | NA | 0 | 0 |
| *E. ochrophloia* $n = 3$ | 0 | 0 | NA | 0 | 0 |
| L. *confertus* $n = 3$ | 3856 | 5 | NA | 0 | 0 |

* After subtraction of blank (after correction). NA: not analysed.

Honey varieties from non-*Leptospermum* sources display no or very low fluorescence at the MM1 wavelengths. Five varieties with three individual samples of each were selected from studies conducted at the University of the Sunshine Coast [29]. These contained no Manuka markers of DHA, MGO or leptosperin. Four of the varieties (*Corymbia maculate,* (spotted gum); *Eucalyptus coolabah,* (coolabah); *Eucalyptus camaldulenis,* (river red gum); and *Eucalyptus ochrophloia*), (yapunyah) displayed an uncorrected RFU of less than the blank, while *Lophostemon confertus* (brush box) displayed a slight positive RFU*. This is possibly from trace amounts of *Leptospermum* nectar content, or from an unknown fluorescence.

The fluorescence analysis of leptosperin in Manuka honey reported here displayed a strong correlation with the results from the independent Analytical Labs, New Zealand and validates the method of analysis (Fig. 3). The equipment and time requirements to run samples was greatly reduced compared to traditional chromatography-based methods, and lends itself to higher throughput, small scale operations.

Not surprisingly, leptosperin did not correlate to MGO levels (Fig. 4). The MGO precursor, DHA in the floral nectar is known to be both species and region dependent [16,17]. Furthermore, the development of MGO is storage dependent [13], while leptosperin is relatively stable in honey [22]. Hence, leptosperin is more suited to being a Manuka floral source marker rather than an indicator of potency.

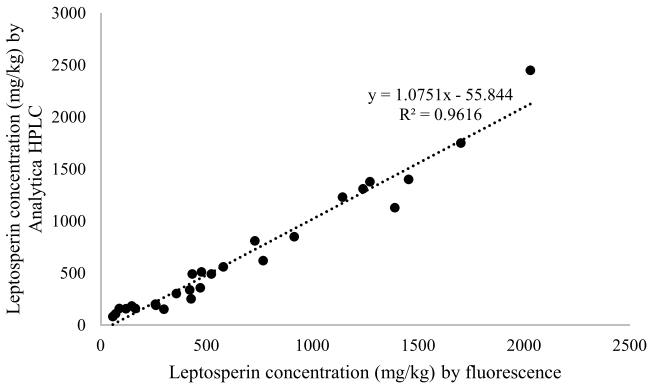


**Fig. 3.** Leptosperin by HPLC vs Leptosperin by fluorescence.

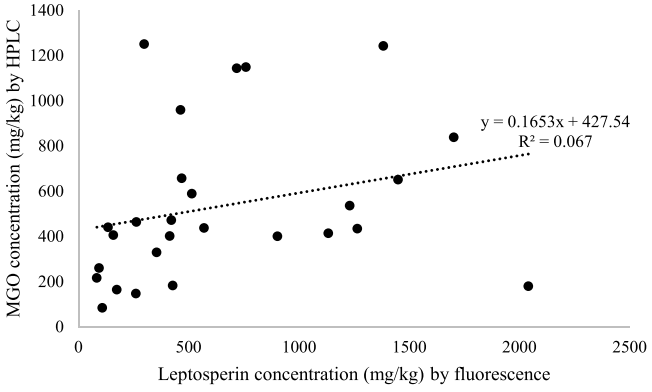


**Fig. 4.** MGO by HPLC vs Leptosperin by fluorescence.

## Conclusions

*Leptospermum* derived (Manuka) honeys from Australia and New Zealand display Manuka Marker 1 fluorescence at $_{ex}270$-$_{em}365$ nm that is absent or at trace levels in non-Manuka varieties. It is clear that leptosperin is not limited to a single species from only one Country of Origin. The compound distinguishes Manuka from non-Manuka honeys, with further geographical provenance being obtained by Country of Origin.

## CRediT authorship contribution statement

**Peter Brooks:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Asmaa Boufridi:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis, Data curation. **Georgia Moore:** Writing – review & editing, Methodology, Investigation. **Linda Pappalardo:** Writing – review & editing.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

PB is the Scientific Director on the Australian Manuka Honey Association board. All authors have no known completing financial interests.

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