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Quantitation of Dihydroxyacetone in Australian *Leptospermum* Nectar via High-Performance Liquid Chromatography

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ABSTRACT: The nonperoxide antibacterial activity of New Zealand mānuka honey originates from dihydroxyacetone (DHA) within *Leptospermum scoparium* nectar. This study determined if DHA was present within the nectar of four Australian *Leptospermum* species: *L. laevigatum*, *L. polygalifolium*, *L. trinervium*, and *L. whitei*. A rapid and convenient new method was developed, which quantitated DHA/sugar ratios (ppm). The DHA and sugars were derivatized with *o*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride and analyzed via RP-HPLC with diode array detection at two wavelengths (200 and 243 nm). DHA was detected in all *L. whitei* and *L. polygalifolium* samples, where DHA/sugar ratios ranged from 10169 to 24199 ppm and from 9321 to 20174 ppm, respectively. DHA was undetected in any of the *L. laevigatum* and *L. trinervium* samples, and nectar activity was <100 ppm. The results of this study have implications for the Australian beekeeping industry, as the findings indicated that not all species of *Leptospermum* will produce active honey.

KEYWORDS: *dihydroxyacetone, Leptospermum, nectar, Australia, high-performance liquid chromatography, honey*

INTRODUCTION

New Zealand mānuka honey, derived from *Leptospermum scoparium* nectar, has premium commercial value worldwide due to its high antibacterial activity. Mānuka honey has been shown to have an inhibitory effect against a broad range of bacterial species.[1−4] All honeys create conditions unsuitable for bacterial growth due to high sugar concentrations, acidic pH (3.2−4.5), and the formation of hydrogen peroxide upon dilution.[5−7] Unlike non-*Leptospermum* honeys, mānuka honey has non-peroxide antibacterial properties, which are associated with the 1,2-dicarbonyl compound methylglyoxal (MGO) present within the honey.[3,8−12] Studies have shown that MGO is generated from the spontaneous chemical conversion of precursor molecule dihydroxyacetone (DHA).[13,14] The mechanism is not fully understood; however, DHA is believed to be nonenzymatically dehydrated to MGO in honey. DHA has been shown to be present, in various concentrations, within the nectar of *L. scoparium* flowers.[13,15] The reason behind DHA occurrence or concentration variation within nectar is currently unknown.

L. scoparium is indigenous to New Zealand but also occurs naturally in southeastern Australia.[16−18] Australia holds the greatest diversity of *Leptospermum* with 81 endemic species.[19] Windsor et al.[20] reported that DHA and MGO were present within six Australian *Leptospermum* honeys: *L. polygalifolium*, *L. liversidgei*, *L. semibaccatum*, *L. laevigatum*, *L. trinervium*, and *L. speciosum*. The honeys with the highest MGO content were derived from *L. polygalifolium*. Windsor et al.[20] suggested that DHA may originate in the nectar of several *Leptospermum* species, which warrants further research into the nectar activity of species found within Australia.

To date, analysis of the presence of DHA within *Leptospermum* nectar has focused on *L. scoparium*.[13,15] However, Williams et al.[15] analyzed a small number of nectar samples obtained from Australian *Leptospermum* species, located in southeastern Queensland (SEQ) and northern New South Wales (NNSW) by GC-MS. The sampled species included *L. liversidgei*, *L. juniperinum*, *L. laevigatum*, and *Leptospermum* 'Merinda'. It is of scientific and commercial interest to determine whether DHA occurs within the nectar of other Australian *Leptospermum* species, particularly *L. polygalifolium* nectar, which produces non-peroxide active honey.[20] This study investigated the presence of DHA within the nectar of Australian *L. laevigatum*, *L. polygalifolium*, *L. trinervium*, and *L. whitei* samples. The aim was to quantitate the DHA/sugar ratios within the nectar through the application of a rapid and convenient new method.

MATERIALS AND METHODS

Nectar Collection Sites. Nectar was collected from *Leptospermum* trees between September and November 2014. Collection sites were located in the Sunshine Coast (SC) region of SEQ, the Northern Rivers (NR) region of NNSW, northwestern Tasmania (NWT), and Port Stephens (PS) in NSW. Nectar samples were collected in replicate from individual trees (n).

Sampling Protocol. The criteria for flower selection was open flowers, which displayed the presence of nectar and were situated <2 m from ground level. Trees were sampled on a fine day, where no precipitation had occurred for at least 2 days. Flowers were removed from the trees after samples were collected to ensure extractions were not duplicated.

Nectar was obtained in situ. Milli-Q water (10 μL) was dispensed into the disk of a selected flower and repeatedly mixed via autopipet. The collected nectar solution was deposited into a high-performance liquid chromatography (HPLC) vial. Each vial contained nectar from 10 flowers. Replicate vials from each tree were collected to account for natural variation between flowers. Vials were stored on ice until arrival at the laboratory and transferred to a freezer (−18 °C) until analysis was performed.

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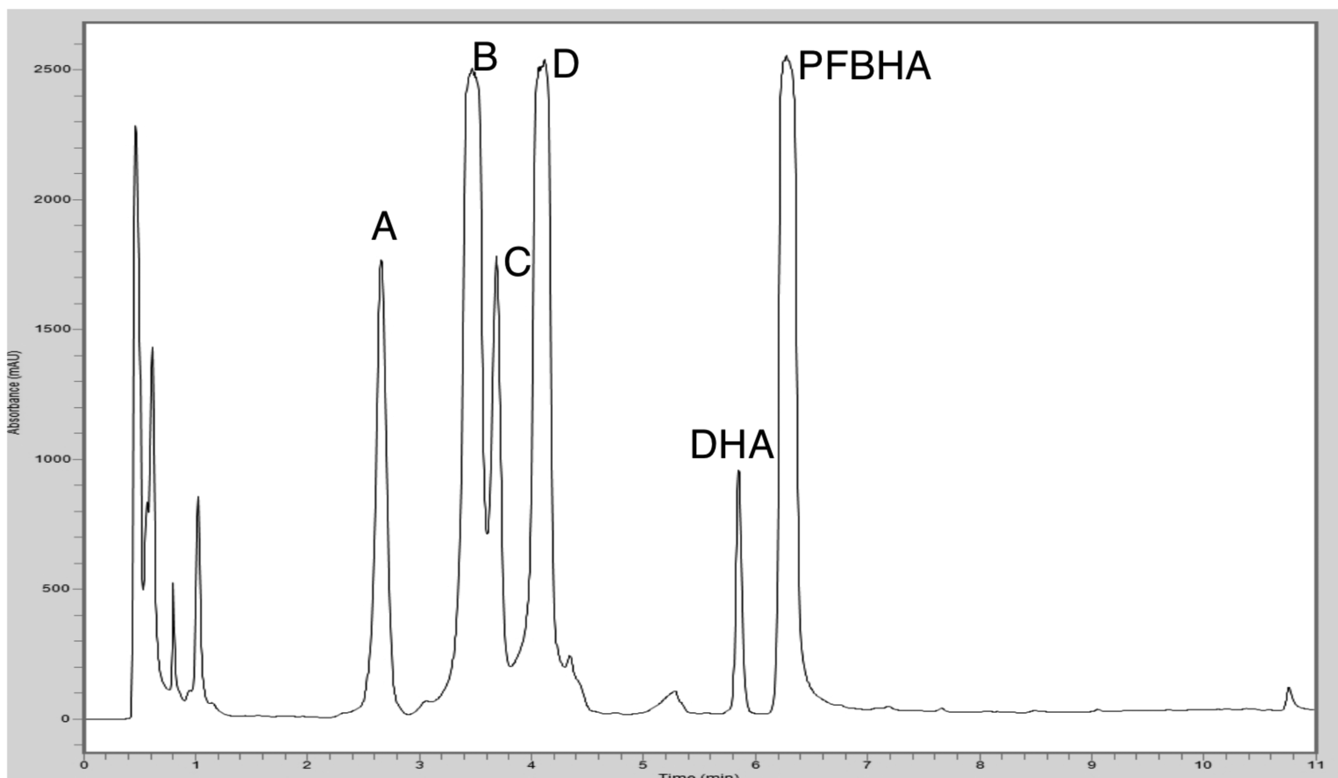

Figure 1. RP-HPLC chromatogram at λ = 200 nm of a *Leptospermum polygalifolium* sample obtained from the Sunshine Coast. The chromatogram shows a sharp peak for DHA derivative at 5.8 min. In contrast, sugar derivatives (A−D) showed high sensitivity and were unable to be accurately quantitated.

Herbarium Depository. Flower and shoot morphology were used to identify wild specimens to species level. Specimens were deposited in the Queensland Herbarium, Toowong, and identified by a chief botanist.

Chemicals. HPLC grade Milli-Q water was used in all analyses. HPLC Chromasolv gradient grade acetonitrile (ACN) was purchased from Merck (Kilsyth, Australia). *o*-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA) was purchased from Bio-Scientific (Gymea, Australia). Reagent grade citric acid, 1,3-dihydroxyacetone (DHA) (dimer, 97%), D-fructose 99+%, and *β*-D(+)-glucose 99+% were purchased from Sigma-Aldrich (Ryde, Australia).

HPLC Conditions. Analysis was conducted from an adapted Windsor et al.[20] method, via reverse-phase high-performance liquid chromatography (RP-HPLC) on a PerkinElmer series 200 HPLC with a Phenomenex Synergi Fusion column (75 mm × 4.6 mm, 4 μm particle size) and a Flexar photodiode array. Detection was set for 200 and 243 nm. Mobile phase A (MPA) was water 90%/ACN 10% (v/v), and mobile phase B (MPB) was ACN 100%. The pump program had a flow rate of 1.5 mL/min, with an injection volume of 20 μL. A gradient elution was employed: MPA/MPB = 95:5 (isocratic 0.5 min), 60:40 (gradient 4.0 min), 50:50 (gradient 2.0 min), 10:90 (gradient 2.0 min), 10:90 (isocratic 1.0 min), 95:5 (gradient 0.5 min), and 95:5 (isocratic over 1.0 min).

Preparation of Standards. Standard aqueous solutions of sugars or DHA were prepared with D-fructose (0.500 g) and D-glucose (0.500 g) dissolved in 50.0 mL of water and 0.050 g of DHA in 50.0 mL of water. DHA standards (0, 1, 2, 5, 10, 20, and 40 μL) were added to seven sugar standard solutions (200 μL), which gave DHA/total sugar mass ratios of 0, 250, 500, 1000, 2000, 5000, and 10000 ppm, respectively.

A PFBHA buffer solution was made with 0.20 g of PFBHA dissolved in 10. mL of 0.10 M citric acid buffer (pH 4). The DHA and sugar standards were derivatized quantitatively with 600 μL of PFBHA buffer solution at 65 °C for 2 h, prior to analysis via RP-HPLC as above. A calibration curve was constructed, whereby the known mass ratios (DHA/sugar ppm) were plotted against the peak area ratios of the DHA derivative (λ = 200 nm) to total sugar derivatives (λ = 243 nm).

Sample Preparation. Prior to analysis, thawed nectar samples were derivatized with 400 μL of PFBHA buffer solution and heated at 65 °C for 2 h. RP-HPLC analysis was performed as above, and peak area ratios of DHA (λ = 200 nm) to total sugars (λ = 243 nm) were obtained. The DHA/sugar ppm of individual samples was determined against the calibration curve by linear regression.

Statistical Analysis. The DHA/sugar ratio (ppm) for an individual tree (n) was the calculated mean of the replicated vials per tree. Differences in DHA/sugar concentration between species were analyzed with a Mann−Whitney *U* test with IBM SPSS (v.22).

■ RESULTS

Under the derivatization conditions, DHA formed a single compound, eluting at 5.8 min in Figure 1. The fructose and glucose formed four partially resolved derivatives by HPLC, 2.4−4.4 min in Figure 1. The multiple peaks were likely due to cis/trans isomers of the derivative oximes. Hence, total peak area measurements of sugars were used. Initial analysis of PFBHA derivatized sugars and DHA within nectar was performed at 259 nm. At this wavelength neither DHA nor sugars could be accurately quantitated as DHA had low sensitivity and sugars had high sensitivity. Peak sensitivity of DHA was increased 10-fold at 200 nm; however, the sugar peaks were saturated and unable to be accurately quantitated as shown by peaks A−D in Figure 1. In contrast, optimal sensitivity of the sugars was obtained at 243 nm, yet DHA showed poor sensitivity. Optimal sensitivity for both components was achieved when the peak area values were obtained at 200 nm for DHA and at 243 nm for sugars. The

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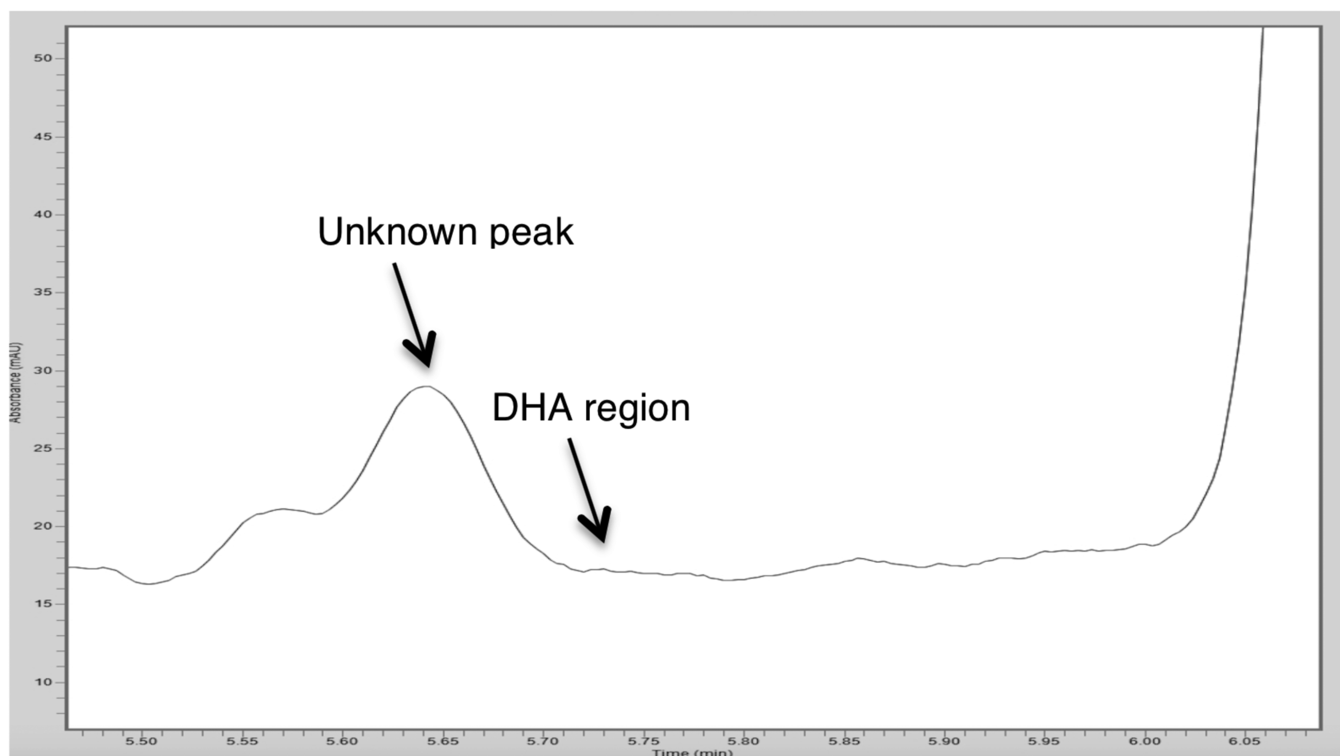

Figure 2. RP-HPLC chromatogram at λ = 200 nm of a *Leptospermum laevigatum* sample obtained from Northern Rivers. The chromatogram shows a broad peak of unknown 5.64 min, which eluted earlier than expected for DHA in relation to the sugars and derivative. No peak was observed in the DHA region.

Table 1. *Leptospermum* Nectar Samples Collected in Replicate from Individual Trees (n) between September and November 2014

	L. laevigatum		*L. polygalifolium*			*L. trinervium*		*L. whitei*	
	NR region	NWT region	NR region	SC region	NR region	SC region	PS region	NR region	SC region
trees (n)	37	1	22	4	22	2	1	10	3

seven standards showed a linear relationship between peak area ratio of DHA/sugar, at 200 and 243 nm, respectively against the known mass ratios of DHA/sugar (R^2 = 0.9982). Therefore, the mass ratio of DHA/sugar in individual samples was determined against the calibration curve by linear regression, as displayed by eq 1:

$$\text{DHA/sugar (ppm)} = \text{DHA/sugar (peak area ratio)} \times 11021 \quad (1)$$

DHA was present in all *L. polygalifolium* and *L. whitei* samples (Figure 1). In contrast, NR *L. laevigatum* samples showed a broad unknown peak with low absorbance at approximately 5.64 min, which eluted earlier than expected for DHA (Figure 2). To confirm whether the unknown peak was DHA, four *L. laevigatum* samples were spiked with 1 μL of DHA standard and analyzed again by RP-HPLC. A sharp DHA peak at 5.72 min was observed in the spiked runs, which verified that the unknown peak was not DHA (data not shown). To determine the identity of the source of the unknown peak, 400 μL of PFBHA buffer solution was analyzed via RP-HPLC alone. The unknown was observed on the chromatogram at 5.62 min, which suggested the unknown was a component within the derivatizing reagent (data not shown). DHA was not detected in any of the *L. laevigatum* samples collected from NR or NWT, nor was DHA detected in any of the *L. trinervium* samples collected from NR, SC, or PS (Table 1).

The DHA/sugar ratio in *L. polygalifolium* and *L. whitei* nectar samples varied between species (Table 2; Figure 3). The highest nectar activity levels were observed in the *L. whitei* samples, where the DHA/sugar ratio ranged from 10169 to 24199 ppm. In comparison, the nectar activity of *L. polygalifolium* ranged from 9321 to 20174 ppm. The data were not normally distributed; therefore, statistical analysis was performed with a nonparametric test. Differences in nectar activity between species were analyzed with a Mann−Whitney U test, which showed that there was a statistically significant difference between the DHA/sugar ratio of *L. polygalifolium* and that of *L. whitei* (U = 270; n_1 = 26, n_2 = 13; $P < 0.05$).

Table 2. Comparison of DHA/Sugar Concentration (ppm) between *Leptospermum polygalifolium* and *Leptospermum whitei* Nectar Collected at Sunshine Coast and Northern Rivers Sites

species	n	min	max	median	IQR
L. polygalifolium	26	9321	20174	12182	4741
L. whitei	13	10169	24199	15625	6909

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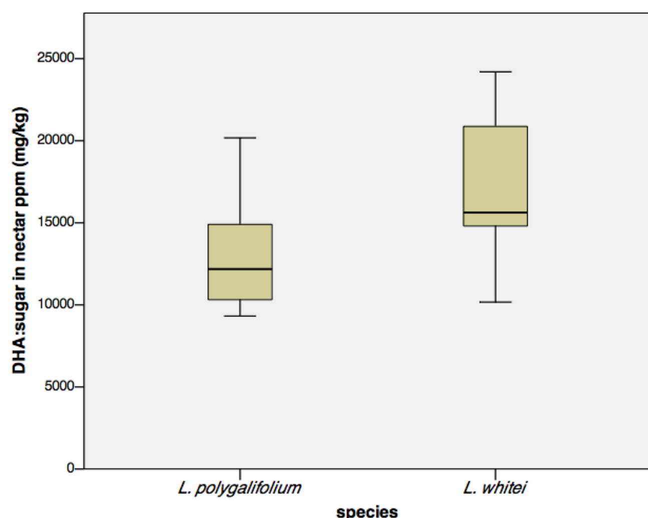

Figure 3. Box-plot comparison of the range of DHA/sugar in nectar from individual *Leptospermum polygalifolium* (n = 26) and *Leptospermum whitei* (n = 13) trees sampled at both Sunshine Coast and Northern Rivers sites.

■ DISCUSSION

The novel nectar extraction technique developed in this study has not to our knowledge been previously reported. Windsor et al.[20] reported successful analysis of PFBHA derivatized DHA and MGO in Australian *Leptospermum* honeys via RP-HPLC; hence, a modified version of this procedure was used to quantitatively derivatize the sugars and DHA in nectar. The PFBHA derivatization of DHA and sugars improved the chromatographic separation and detection of compounds. The method developed in this study was both rapid and convenient. The nectar solution extracted in situ was derivatized with PFBHA buffer solution upon analysis, and after 2 h at 65 °C, samples were analyzed directly with RP-HPLC and photodiode array detection. In addition, total sugars and DHA were analyzed within the one sample, via detection at two wavelengths. In contrast, the method described by Williams et al.,[15] which analyzed DHA/sugar in New Zealand *L. scoparium* by GC-FID and in Australian *Leptospermum* samples by GC-MS, had increased complexity and involved two separate procedures to detect DHA and sugars. Furthermore, Williams et al.[15] found that approximately 4% of DHA was transferred to the sample bag during flower collection. The method described in this study eliminates such loss, as nectar is deposited directly to the HPLC vial at the time of collection.

The results of this study indicated that perhaps not all species within the *Leptospermum* genus produce DHA within their nectar and, consequently, will not produce non-peroxide active honeys. The results showed that DHA was not detected in any of the *L. laevigatum* nectar samples, as illustrated by the sharp DHA peak when samples were spiked with 1 μL of DHA (data not shown). It was calculated that 1 μL of DHA standard was equivalent to approximately 300 ppm of DHA/sugar in nectar; therefore, it was conservatively estimated that all of the *L. laevigatum* nectar samples had a DHA activity level <100 ppm. No prior studies within the available literature have analyzed *L. laevigatum* nectar, apart from one sample analyzed by Williams et al.,[15] who were also unable to detect DHA. As DHA was also undetectable in the replicated NWT *L. laevigatum* samples, it appeared unlikely that lack of nectar activity was a result of environmental factors, as the NR and NWT samples were separated by approximately 1500 km. Moreover, this suggested that lack of bioactivity in *L. laevigatum* nectar is species wide. Similarly, DHA was not detected in any of the *L. trinervium* samples collected from the SC, NR, and PS regions. As a result, it was concluded that *L. trinervium* samples also had an activity level <100 ppm. No prior studies were found to have previously analyzed monofloral *L. trinervium* honey.

As *L. laevigatum*, *L. polygalifolium*, *L. trinervium*, and *L. whitei* flower at similar times of the year, beekeepers in NNSW target all four species to produce active honey. The results indicated that honey produced from *L. laevigatum* and *L. trinervium* nectar has a high probability of being inactive. Therefore, the findings suggested that beekeepers should avoid both species if they intend to produce active honey. Windsor et al.[20] detected DHA and MGO in *L. leavigatum* and blended *L. trinervium*/*Guioa semiglauca* honey, at low activity levels of <750 and <150 ppm, respectively. However, the presence of DHA may have been attributed to bees collecting nectar from other active *Leptospemum* species in flower at the same time, such as *L. polygalifolium* or *L. whitei*.

DHA was present in all *L. polygalifolium* and *L. whitei* nectar samples, yet nectar activity significantly varied between species. The *L. whitei* samples had the highest nectar activity (Table 2). No previous studies within the available literature have analyzed the DHA/MGO concentration or antibacterial activity of monofloral *L. whitei* honey. The environmental conditions at the sample sites were not quantitatively measured within this study. However, the *L. polygalifolium* and *L. whitei* trees sampled at SC and NR were believed to be wild populations in sandy, low-nutrient soils. It is possible that environmental factors, such as nutrient availability, may have contributed to the variation in nectar activity observed between species. Williams et al.[15] measured soil type, yet no correlation was found between soil type and nectar activity.

Williams et al.[15] classified nectar activity into three categories: low (<1000 ppm), moderate (1000–2000 ppm), and high (>2000 ppm). The study reported that the majority of nectar samples collected from *L. scoparium* trees over a two-year period had a moderate to low activity level. The highest observation was <9000 ppm from a garden cultivar from the Waikato region. In contrast, the lowest observations for *L. polygalifolium* and *L. whitei* in this study were >9000 ppm (Table 2). The high DHA/sugar ratios found in this study suggested that perhaps honey derived purely from *L. polygalifolium* or *L. whitei* may have higher bioactivity than mānuka honey. Research into the antibacterial activity of *L. polygalifolium* and *L. whitei* honey is recommended. In addition, it is possible that the results of this study may not be comparable to the values reported by Williams et al.,[15] due to method-related differences. Overall, the findings of the study have implications for beekeepers and the honey industry. Active honey is of high commercial value; therefore, the findings of this study indicated that Australian beekeepers should target *L. polygalifolium* and *L. whitei* if they intend to produce active honeys.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ACN, acetonitrile; DHA, dihydroxyacetone; GC-MS, gas chromatography−mass spectrometry; IQR, interquartile range; MGO, methylglyoxal; NNSW, northern New South Wales; NR, Northern Rivers; NWT, northwestern Tasmania; PFBHA, *o*-(2,3,4,5,6-pentafluorobenzyl) hydrochloride; PS, Port Stephens, NSW; RP-HPLC, reverse-phase high-performance liquid chromatography; SC, Sunshine Coast; SEQ, southeastern Queensland

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on July 17, 2015, with an error to equation 1. The corrected version reposted on July 21, 2015.