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Regional, Annual, and Individual Variations in the Dihydroxyacetone Content of the Nectar of Mānuka (*Leptospermum scoparium*) in New Zealand

Simon Williams,[†] Jessica King,[†] Maria Revell,*[,†] Merilyn Manley-Harris,[†] Megan Balks,[‡] Franziska Janusch,[§] Michael Kiefer,[§] Michael Clearwater,[#] Peter Brooks,[⊥] and Murray Dawson[⊗]

[†]Chemistry, School of Science, University of Waikato, Hamilton 3216, New Zealand

[‡]Earth and Ocean Sciences, School of Science, University of Waikato, Hamilton 3216, New Zealand

[§]School of Applied Chemistry, Reutlingen University, Reutlingen 72762, Germany

[#]Biological Sciences, School of Science, University of Waikato, Hamilton 3216, New Zealand

[⊥]Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Maroochydore, Queensland 4558, Australia

[⊗]Landcare Research New Zealand, Lincoln 7640, New Zealand

Supporting Information

ABSTRACT: A method was designed and validated for the analysis of dihydroxyacetone in the floral nectar of mānuka (*Leptospermum scoparium*). The method was applied to samples collected from different regions of the North Island and the Nelson region of the upper South Island of New Zealand during the period 2009−2012 as well as to nectar samples from some Australian *Leptospermum* species. The ratio of dihydroxyacetone to total sugar (DHA/Tsugar) was classified as low (<0.001 mg/mg), moderate (0.001−0.002 mg/mg), or high (>0.002 mg/mg). Inter- and intraregional variation were observed as well as interannual variation with variation from low to high classification occurring within one region and from low to moderate between years. Australian species also demonstrated elevated levels of dihydroxyacetone in the nectar. Some garden cultivars were shown to produce very high nectar DHA/Tsugar, and a survey of cultivars was undertaken; cultivars with single-flowered red or pink flowers were the most common producers of very high nectar DHA/Tsugar.

KEYWORDS: *dihydroxyacetone, Leptospermum scoparium, cultivar, mānuka, nectar, New Zealand, honey*

INTRODUCTION

New Zealand mānuka honey commands a premium price internationally because of its nonperoxide antibacterial activity (NPA). This activity has been shown to be principally related to high levels of methylglyoxal found in the mature honey.[1,2] It has been demonstrated that the methylglyoxal derives from dihydroxyacetone found in the floral nectar of mānuka, *Leptospermum scoparium* J.R.Forst. & G.Forst. (Myrtaceae). The dihydroxyacetone is chemically converted to methylglyoxal as the honey matures;[3] specifically, a high ratio of dihydroxyacetone to nectar sugars (DHA/Tsugar) in young honey results in a high NPA honey once mature. Australian honeys derived from species in the same genus, for example, *Leptospermum polygalifolium* Salisb., exhibit similar properties.[4] Adams et al.[3] found intertree variation in the amounts of dihydroxyacetone present in floral nectar in a small sample of mānuka trees.

The NPA of mānuka honey is notoriously variable from region to region and from year to year. Currently, mānuka honey is harvested from wild mānuka populations, although there is interest in selecting or breeding mānuka cultivars or seed sources with optimum nectar and dihydroxyacetone production. It is not known why species of *Leptospermum* produce dihydroxyacetone in the nectar or why intertree variation exists, and the effects of environment and climate have not been elucidated. *L. scoparium* displays wide morphological variation throughout New Zealand,[5−9] and regional variation is manifest in leaf oil chemotypes from different regions of New Zealand.[10] The biology of *L. scoparium* in New Zealand has been reviewed.[11]

Inter- and intraregional variability of dihydroxyacetone content in the nectar of *L. scoparium* has not been assessed, although variation in NPA of honeys from different regions indicates indirectly that there is likely to be regional variation.[12] This work is a preliminary study of the DHA/Tsugar of the floral nectar of mānuka that was undertaken across a variety of locations in the North Island and in the Nelson region of the South Island of New Zealand during the period 2009−2012. Horticultural cultivars of mānuka were included after it was found that some cultivars exhibited consistently high nectar DHA/Tsugar. The aim was to increase understanding of the variations that might be expected both geographically and chronologically.

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MATERIALS AND METHODS

Flower Collection Sites. Flower collection sites were selected with the assistance of local beekeepers. The sites were spread across New Zealand between latitudes 34° and 41° S and between longitudes 171° and 179° E. The sites recommended by beekeepers were those regarded as yielding mānuka honey with measurable NPA; the Whanganui site was chosen to provide contrast as it yielded "bush" honey rather than mānuka honey with measurable NPA, and the Northland survey was undertaken because the variety of *L. scoparium* in Northland differs from the rest of New Zealand. The range of sites also afforded the opportunity for comparison with the study of honeys by Stephens.[12] Sampling at the Coromandel, East Cape, Waikato, Wairarapa, Whanganui, and Nelson sites was carried out during the flowering seasons (November−December) in 2009 and 2010 and for the East Cape and Waikato sites also in 2011. A separate collection of samples from the Northland region was made in 2010 and 2011. Most site visits in remote locations occurred once each year, and only specimens that were flowering on the day of the visit were sampled; this was an unavoidable bias. More accessible cultivated trees around the University of Waikato campus were used for testing of the effect of sampling methods, floral gender, and age on nectar quality.

Sampling Protocol. The trees sampled in 2009 were resampled in 2010 except for those that had died in the interim. In 2011 additional trees located within 50 m of the existing test trees at the East Cape sites were sampled. Further samples were collected in the Auckland and Waikato regions in 2012, both cultivars and wild accessions. The trees were sampled, whenever possible, on a fine day. Entire flowers were picked off individually or scraped off between two fingers drawn from the base toward the apex of the shoot. In addition to collection of flower samples in 2009, soil was collected (with a small trowel at 2 m from the trunk of each tree, samples approximately 15 × 10 × 20 cm in dimension), and foliage was collected for leaf oil analyses. All samples were stored in airtight bags and chilled with ice while transported, until they could be transferred to a freezer (−20 °C) for storage until analysis.

Herbarium Depository. Accessions collected from the wild were assigned to one of the two varieties recognized for *L. scoparium*: either var. *scoparium* or var. *incanum*.[13] Voucher specimens for each tree were deposited in the University of Waikato Herbarium (WAIK).

Materials. *o*-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) (derivatization grade) and methylglyoxal (43.2%) were obtained from Fluka Analytical (Buchs, Germany). Dihydroxyacetone (97%), hydroxyacetone (technical grade 90%), sucrose (99+%), D-(−)-fructose (99+%), D-mannitol (98+%), and citric acid (99.5%) were obtained from Sigma-Aldrich (Sydney, Australia). 1-(Trimethylsilyl)imidazole (TMSI) was obtained from Thermo Scientific (Scoresby, Australia). D-(+)-Glucose (AR) grade was obtained from BDH Laboratory (Murarrie, Australia). Deionized water was obtained from a Barnstead Epure water system at 17.9 MΩ. Dichloromethane (analytical grade) was obtained from Ajax Finechem (Sydney Australia) or purified using a Pure Solv solvent purification system model PS-SD-5 (Innovative Technology, Amesbury, MA, USA).

Extraction of Nectar. The methodology was based upon that of Morrant et al.[14] Frozen flowers were removed from the sample bag, giving preference whenever possible to open flowers in good condition. Either 20 (20F), 10 (10F), or 1 (1F) flower(s) was placed in 4, 2, or 1 mL of water, respectively, and kept for 20 min. After standing, the flowers were removed and the nectar/water solution was frozen if analysis was not performed immediately. Ten replicates of the 20F or 10F method were carried out for each bag/tree when there was sufficient material.

Single-Flower Test. Some of the trees, especially the Northland and East Cape samples collected in 2011, yielded insufficient flowers for the 10F or 20F test. Comparison of the 1F and 20F tests was carried out using replicates (n = 10) from three *L. scoparium* 'Martinii' (*L.* 'Martinii') trees sampled on the University of Waikato campus.

Preparation of Nectar for Dihydroxyacetone Detection. To a vial containing hydroxyacetone (internal standard, 10 μL, 0.5 mg/mL) and nectar extract (20F, 200 μL; 10F, 200 μL; 1F, 400 μL) was added and mixed PFBHA (50 μL, 20 mg/mL in a pH 4 citric buffer), and the mixture was kept for 1 h. Dichloromethane (20F, 2 mL; 10F, 2 mL; 1F, 1 mL) was added and mixed. The vial was then placed in a freezer (0 °C) until the aqueous layer froze to aid extraction. An aliquot (1 mL) of the dichloromethane layer was withdrawn into a GC vial (1.5 mL). TMSI (50 μL) was added, mixed, and allowed to stand (1 h) before analysis by GC-FID.

Preparation of Nectar for Sugar Detection. To a vial containing mannitol (internal standard, 5 μL, 5 mg/mL) was added nectar extract (20F, 20 μL; 10F, 20 μL; 1F, 50 μL), and the vial contents were freeze-dried. To the dry sample was added and mixed TMSI (50 μL), and the mixture was kept at room temperature. Heptane (1 mL) was added with mixing before analysis by GC-FID.

GC-FID Parameters. The column used was a 0.32 mm × 0.25 μm i.d., 30 m, Zebron ZB-5 capillary GC column (Agilent Technologies, Santa Clara, CA, USA). For all methods a 2 μL injection into a splitless inlet with a 2 mL/min purge flow was used, with the FID held at 300 °C. H_2 flow was 30 mL/min; air flow, 400 mL/min; and N_2 makeup flow, 10 mL/min.

For the detection of dihydroxyacetone, the inlet was set to 250 °C and used a H_2 carrier gas flow of 2 mL/min. The temperature program was 130 °C held for 5 min, increased at 10 °C/min to 250 °C, and then held at that temperature for 6 min. Dihydroxyacetone was quantitated against hydroxyacetone as an internal standard and used a calibration curve constructed over the range of 0.003−0.015 mg/mL. The limits of detection for dihydroxyacetone and methylglyoxal were 3.00×10^{-8} and 4.58×10^{-7} g/mL, respectively. The limits of quantitation for dihydroxyacetone and methylglyoxal were 1.00×10^{-7} and 1.53×10^{-6} g/mL, respectively.

For the detection of sugars, the inlet was set to 280 °C with a H_2 carrier gas flow of 4 mL/min. The temperature program was 100 °C held for 1 min, increased at 30 °C/min to 200 °C, increased at 10 °C/min to 250 °C, and finally increased at 30 °C/min to 300 °C. The sugars were quantitated against D-mannitol as an internal standard with a response factor found for each sugar. The response was measured over the range of 50−150 mg/mL and gave fructose, 0.5335; glucose, 0.8217; and sucrose, 0.7126. Fructose, glucose, and sucrose were summed to give total sugars.

Expression of Results for Dihydroxyacetone Content. Dihydroxyacetone content is expressed as a ratio of DHA/total nectar sugar (Tsugar). The assumption was made that all of the sugars in honey derive ultimately from the nectar and that the ratio for dihydroxyacetone to total sugar would be indicative of the expected ratio in the early honey before maturation because dihydroxyacetone might be expected to have volatility similar to that of the sugars. Ten replicates for each tree were assayed and after outliers were determined (single outlier and largest/smallest or two largest/smallest tests);[17] results are expressed as the mean and 95% standard error.

Measurement of Nectar DHA/Tsugar in Australian Species. The nectars of some Australian species (*L. juniperinum* Sm., *L. liversidgei* R.T.Baker & H.G.Sm., *L. laevigatum* (Gaertn.) F.Muell.) were analyzed at the University of the Sunshine Coast in southern Queensland. Sampling from wild populations occurred principally in the coastal region of northern New South Wales. Sample preparation was as above, but GC-MS was utilized.

GC-MS Parameters. The column used was a 30 m × 0.25 mm × 0.25 μm i.d. Elite 5MS gas capillary column (PerkinElmer, Melbourne, Australia). Analyses were carried out using a PerkinElmer Clarus 580 gas chromatograph coupled to a PerkinElmer Clarus SQ85 mass spectrometer (all PerkinElmer instruments were supplied by PerkinElmer, Melbourne, Australia). The carrier gas (helium) flow was set to 1 mL/min for both the sugar and dihydroxyacetone analyses. For the sugar analysis the inlet was configured to shut the split from 0.20 to 1.00 min and open with a 50:1 ratio; it was maintained at 280 °C. The oven program was the same as described for the New Zealand samples. Compound ionization was at 70 eV electron impact, analyzing m/z 70−440 over 4.50−11.00 min. For the dihydroxyacetone analysis, the inlet was configured as for the sugar analysis except the temperature was maintained at 250 °C. The oven

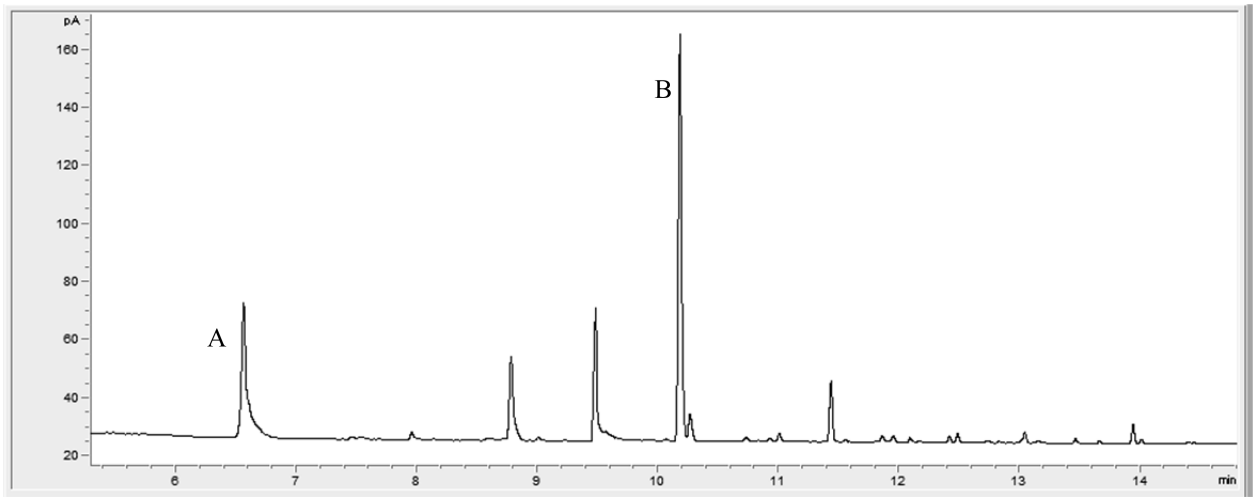

Figure 1. GC-FID chromatogram of a nectar sample used for dihydroxyacetone quantitation: (A) hydroxyacetone used as internal standard; (B) dihydroxyacetone.

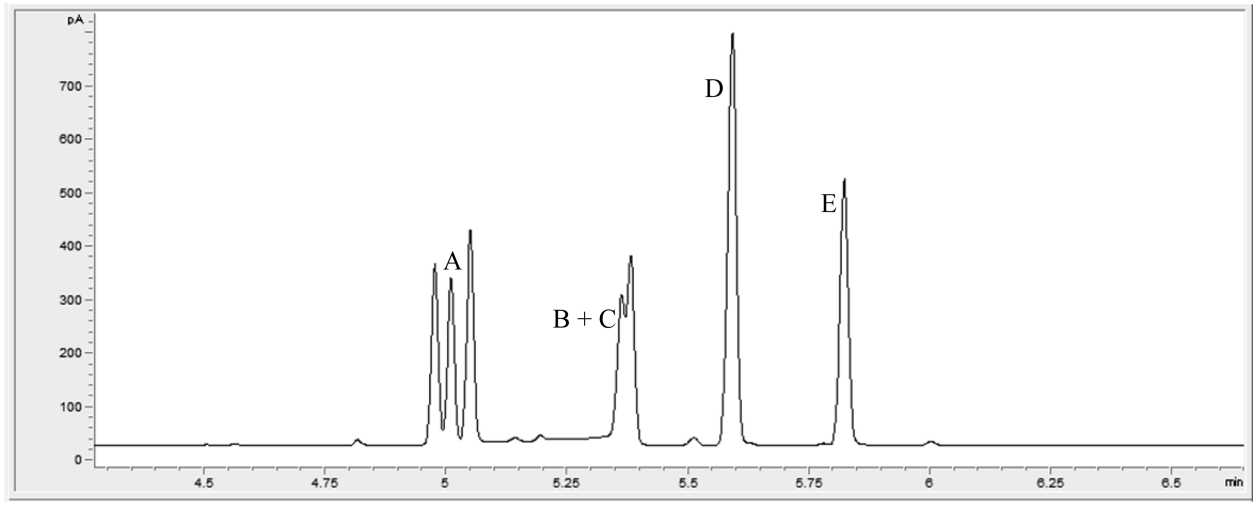

Figure 2. GC-FID chromatogram of a nectar sample used for sugar quantitation. The peaks at (A) are fructose, those at (C) and (E) are glucose, and that at (D) is mannitol used as an internal standard. (B) arises from an organic acid and was not included in the glucose peak area.

program was the same as that outlined for the New Zealand specimens. Compound ionization was at 70 eV electron impact, analyzing m/z 45−440 over 4.50−23.00 min.

Statistical Analyses. Statistical analyses were carried out using Microsoft Excel or Minitab 16.

RESULTS AND DISCUSSION

To simplify discussion, nectar DHA/Tsugar has been divided into three classifications: low (<0.001 mg/mg), moderate (0.001−0.002 mg/mg), and high (>0.002 mg/mg).

Choice of Methodology for Nectar Sampling and Extraction. Stephens et al.[15] analyzed nectar of *L. scoparium* (n = 6) for phenolics and for methylglyoxal; the methodology employed by those authors was to use direct sampling by pipet.[15] For the current study this method was considered, but it was decided that it was far too time-consuming for this broad-based preliminary survey, which was often carried out in isolated areas, accessed only with difficulty and often under inclement conditions. This view is supported by the conclusions of Morrant et al.,[14] who have reviewed methods of nectar collection.

Two methods of extracting nectar from the previously collected flowers were compared: washing (20 min) and individual dipping (three times). Each method was repeated eight times using 20 flowers per sample for flowers from the same tree, and the absolute dihydroxyacetone content was analyzed; mean and %RSD were respectively 0.0056 mg (26%) and 0.00020 mg (90%). Washing was therefore used in this survey. Morrant et al.[14] also recommended washing of cut flowers as the most practical method and, indeed, found this to be preferable to and more reproducible than the use of micropipets.[14]

Stephens et al.[15] did not assay dihydroxyacetone; methylglyoxal was assayed but only reported as a trace, so no numerical comparison between methods in that study and the current study is possible.

Method Validation. *Gas Chromatography Methodology for Analysis of Dihydroxyacetone and Methylglyoxal with PFBHA Derivatization.* PFBHA derivatization with analysis by reversed phase HPLC has been used previously to assay dihydroxyacetone and methylglyoxal simultaneously in honey;[4] it was decided that GC-FID would supply the requisite sensitivity for nectar samples. The derivatization and GC-FID

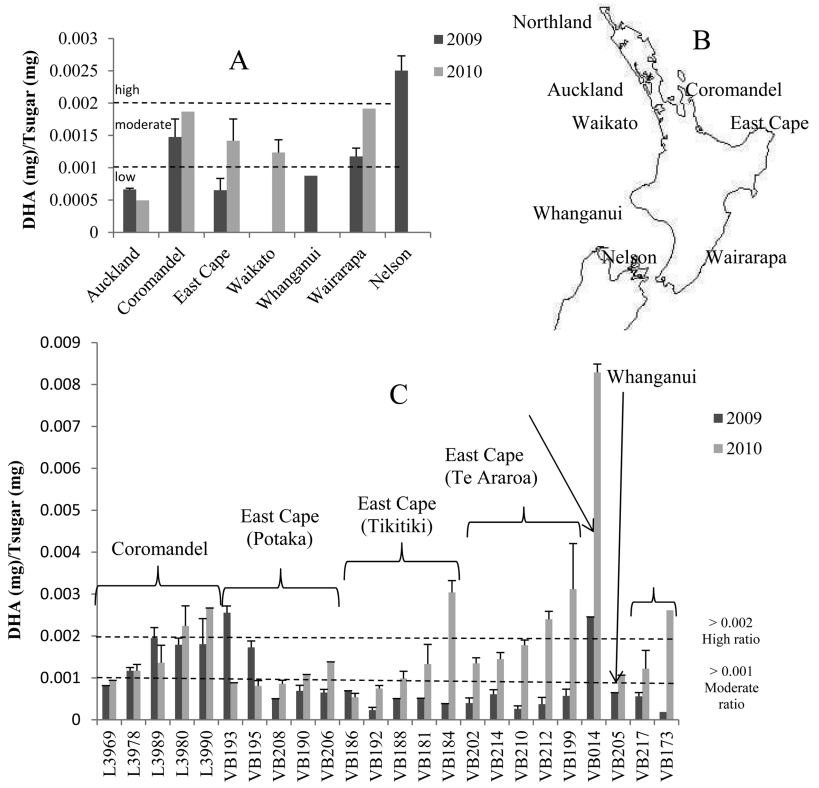

Figure 3. (A) Plot of the mean nectar DHA/Tsugar of all wild trees for each region for the two flowering seasons 2009 and 2010 (error bars are standard error values). (B) Map of locations across the North Island and upper South Island of New Zealand. (C) Plot of nectar DHA/Tsugar values for all trees that were sampled in both 2009 and 2010, grouped into regions and then ascending order of 2010 value. The only tree from Waikato that was tested in both years was a cultivar growing in a suburban garden (not included in panel A; error bars are standard error values).

analysis of samples was optimized using standards of hydroxyacetone, dihydroxyacetone, and methylglyoxal. Nectar samples spiked with these standards were used to ensure that the derivatizing agent PFBHA was present in sufficient excess. Under the conditions used, hydroxyacetone (internal standard), dihydroxyacetone, and methylglyoxal eluted at 6.6, 10.1, and 13.1 min, respectively. Responses for dihydroxyacetone and methylglyoxal (measured as analyte/internal standard versus analyte) were linear from 0 to 0.0016 mg (R^2 = 0.9998) and from 0 to 0.015 mg (R^2 = 0.9815), respectively. The LOQ for dihydroxyacetone corresponded to a minimum detectable mass of 20.5 ng and that for methylglyoxal to 313 ng. The LODs were respectively 6.16 and 93.8 ng. Methylglyoxal was never detected at a level that allowed quantitation in any nectar samples in this study (Figure 1); the presence of methylglyoxal in nectar samples assayed by others[15] might be because the methodology employed was more sensitive than that used here or might indicate that the flowers or nectar samples had been exposed to elevated temperatures or stored for prolonged periods at room temperature prior to analysis.[16]

Gas Chromatography Methodology for Analysis of Sugars with TMS Derivatization. Response factors of sucrose, glucose, and fructose relative to the internal standard mannitol were ascertained by varying weight ratios and comparing area ratios and were 0.7126, 0.8217, and 0.5335, respectively. Varying the quantity of derivatization agent from 50 to 200 μL demonstrated that 50 μL was sufficient excess. As sugars were always readily observed, LOD and LOQ were not required. Under the chromatographic conditions used for the analysis, fructose (multiple furanose and pyranose anomers) eluted at 4.9−5.1 min, glucose (pyranose anomers) at 5.6 and 5.8 min, and mannitol at 5.7 min (Figure 2).

Duration of Washing for Nectar Extraction. The duration of washing was based upon the method of Morrant et al.,[14] who found no difference between 1 and 20 min but a significant difference between 20 and 60 min. In this study extended washing of the flowers (230 min) resulted in a ∼25% decrease in DHA/Tsugar, presumably because sugar was leaching from other parts of the flower.

Reproducibility Tests. To ascertain day-to-day reproducibility of the method, samples from eight trees were assayed (20F, n = 10) on two different days. Statistically, three of the trees gave significantly different results between days (p = 0.0010, 0.0007, and 0.000), whereas five did not (p = 0.1597, 0.8899, 1.000, 1.000, and 1.000), but in only one case did a tree change its DHA/Tsugar classification. The weather conditions on each day of sampling may have had an influence on these results as rain and wind can remove nectar from the open bowl of the mānuka flower, as can visits by pollinators.

Stability of Nectar Extract. The stability of the nectar extract was ascertained for samples from three trees in which replicates (n = 10) were stored frozen and others (n = 5) were left to stand at room temperature overnight before analysis. The samples that were left to stand darkened in color overnight, but a z test for two sample means showed that there was no change in the DHA/Tsugar ratio. To test the stability of the derivatized samples, duplicates from one tree were prepared for both dihydroxyacetone and sugar assays and injected into the GC at the beginning and the end of the sequence; the time lapse for the sugar assay was ∼315 min and for the dihydroxyacetone

assay, ~702 min. The results for DHA/Tsugar were identical to two significant figures.

Comparison of 10-Flower (10F) and 20-Flower (20F) Tests. The 10F test was compared with the 20F test using replicates (n = 10) from three different trees; the difference between the two tests was insignificant at the 95% confidence level. The percentage relative standard deviation was higher for the 10F than for the 20F test but did not exceed 25%.

Comparison of the Single-Flower Test (1F) and the 20F Test. Some of the trees, especially the Northland samples and the East Cape samples collected in 2011, yielded insufficient flowers for the 20 or 10 flower test. Comparison of the 1F and 20F tests was carried out using replicates (n = 10) from three trees. The ranges of percentage difference between the two tests for the two ratios, DHA/Tsugar and fructose/glucose, were 5.52−20.21 and 1.83−17.44, respectively. The percentage relative standard deviation for the 1F test was much higher than for the 20F test and also much higher than the percentage difference between the two methods. This indicates, as might be expected, that there is variability between individual flowers on the same tree. To confirm that biological variation exceeded experimental variation, replicates (n = 5) of a single extraction (20F) were compared to multiple extractions (20F, n = 10) for three different trees. For the single extraction percentage relative standard deviations were 1.20, 2.54, and 3.13 for the three trees, and the corresponding values for the multiple extractions were 14.36, 9.06, and 25.80. This confirms that the greatest source of variation is that within a single tree, the cause of which is not yet established but may include active removal by pollinators and variation related to the different stages of flower maturity.

Transference of Nectar. The residue from an empty sample bag was rinsed out and assayed, and dihydroxyacetone equivalent to about half that found in 20 flowers was measured (a bag typically contained ~500 flowers). This confirms that transfer of nectar between flowers and the bag and presumably also between flowers is occurring; this may partially account for the variation observed in the single-flower test.

Variation of Nectar DHA/Tsugar between Regions and Years and within Regions. *Variation of Nectar DHA/Tsugar between Regions and Years.* Regional and annual variation in nectar DHA/Tsugar was observed (Figure 3). Only the Coromandel and East Cape regions had sufficient samples in both years for a statistical test of the effect of sampling year. A balanced ANOVA showed that the mean DHA/Tsugar for the East Cape region was significantly different (p = 0.02) between 2009 and 2010, whereas the Coromandel region did not differ between years (p = 0.5). The change observed in the East Cape samples was sufficient to alter the classification from low in 2009 to moderate in 2010.

Only the Coromandel, East Cape, and Wairarapa regions had sufficient samples for a test of effect of region on DHA/Tsugar. A one-way ANOVA test followed by mean separation using Tukey's method detected an overall region effect (p = 0.05). Mean nectar DHA/Tsugar did not differ between the Coromandel and Wairarapa samples, but the East Cape values were lower in both 2009 and 2010.

Variation within Regions. Significant differences in nectar DHA/Tsugar were observed between trees within the Coromandel sampling site (Figure 3). All trees were located within a radius of 100 m, but a range of classifications from low to high was observed. There were three soil orders present at this Coromandel site, but no relationship between soil order and DHA/Tsugar could be found. To further investigate variation within a region, an additional collection was made in the East Cape region in 2011. From eight sites, one or more trees of various classifications (low−high), which had been sampled previously, were selected, and other trees within a 50 m radius were also sampled. Even within a narrow radius individual trees differed significantly in their nectar DHA/Tsugar (Table 1), in some cases ranging from high to low classifications.

The Northland/Auckland region was surveyed in 2010 and 2011 (Figure 4). For this survey the region was divided into two zones: Northland, encompassing the area from Cape Reinga to 36.3° S (zone 1) and the Auckland/Coromandel regions south of 36.3° S (zone 2). In zone 1 *L. scoparium* var. *incanum* Cockayne predominates,[18] whereas in zone 2 and the rest of New Zealand most wild mānuka honeys are considered to be *L. scoparium* var. *scoparium*. The individual tree returning an exceptionally high value belonged to *L. scoparium* var. *incanum* located in a maintained area. The individual with a midmoderate result was also from *L. scoparium* var. *incanum*. The only other individual demonstrating a high classification belonged to *L. scoparium* var. *scoparium* as did the two specimens with low−moderate results. The remaining individuals consisted of a mixture of var. *scoparium* (n = 23) and var. *incanum* (n = 16), all of which scored in the low classification. No apparent trend exists relating either of these varieties to high nectar DHA/Tsugar.

Stephens[12] grouped 463 mānuka honey samples (from a single season, 2001−2002 and supplied by apiarists) by region and by mean "unique mānuka factor" (UMF), a measure of nonperoxide antibacterial activity. Waikato (n = 6) and Coromandel (n = 23) returned high values of activity (>14 UMF), although other Coromandel honeys returned intermediate levels (n = 16; 12.6 UMF) and low levels (n = 63; 10.5 UMF). Some East Coast honeys returned intermediate levels of activity (n = 22, 12.5 UMF), whereas others returned lower levels (n = 17; 10.9 UMF). Wairarapa honeys returned low levels of activity (n = 12; 9.4 UMF) as did samples from the northern South Island (n = 41; 9.1 UMF), whereas Northland demonstrated high levels of activity (n = 35; 14.8 UMF). These findings concur with the variability observed in the current study in both the Coromandel region and the East Cape and, to some extent, the finding that the East Cape values were lower than those for the Coromandel. However, the Wairarapa, Northland, and northern South Island results are in contrast. The Waikato result, with the exclusion of the garden cultivar, is somewhat lower than expected from Stephens's results, which relate to honeys originating from wild mānuka in swamplands, but in the current study the trees were sampled from drier locations, which might possibly account for the observed difference.

Caution should be exercised when these two studies are compared because variability of classification of dihydroxyacetone content between seasons was observed in the current study at one location, and further work (unpublished data) indicated that the conditions of storage by the apiarist strongly influence the efficiency of conversion of dihydroxyacetone to methylglyoxal in maturing mānuka honey and hence the final UMF value.

Nectar DHA/Tsugar in Commercial New Zealand Originated Cultivars. The specimen used to first illustrate the presence of dihydroxyacetone in nectar was a red-pink-flowered cultivar *L.* 'Martinii', which is widely available from

Table 1. Comparison of DHA/Tsugar in Trees Sampled in 2011

site	specimen ID	DHA/Tsugar (mg/mg)
1	**VB184**[a]	**0.00032**
	VB052	0.00049[b]
	VB074	NQ[c]
	VB107	0.00082[c]
2	**VB192**	**0.00086**
	VB050[b]	0.00039[c]
3	**VB197**	**0.00095**
	VB088[b]	0.00141[c]
	VB090	0.00071[c]
	VB131[b]	0.00261[c]
	VB146[b]	0.00271[c]
4	**VB193**	**0.00088**
	VB002[b]	0.00079
	VB010	0.00104[c]
	VB089[b]	0.00066
5	**VB195**	**0.00081**
	VB053	0.00080
	VB054	0.00032[c]
	VB140[b]	0.00063
6	**VB181**	**0.00133**
	VB051	0.00053[c]
	VB083	0.00064[c]
	VB099	0.00186[c]
	VB100	0.00109
	VB105[b]	0.00088[c]
	VB142	0.00107[c]
7	**VB183**	**0.00034**
	VB093	0.00242[c]
	VB114	0.00248[c]
	VB115	0.00091[c]
	VB145	0.00095[c]
8	**VB199**	**0.00312**
	VB202	**0.00130**
	VB214	**0.00145**
	VB137	0.00421[c]
	VB070	0.00200
	VB067	0.00195
	VB071	0.00168
	VB102	0.00044[c]

[a]The trees (and the corresponding DHA/Tsugar value) originally sampled in 2010 are shown in bold. [b]Tree with a significantly different DHA/Tsugar from the original tree at that site. [c]Dihydroxyacetone was below the level of quantitation.

plant nurseries and which was growing in a suburban garden in Hamilton. This cultivar consistently gave substantially higher nectar DHA/Tsugar values than any of the wild specimens tested (Figure 3).

L. 'Martinii' is believed to be a cross between the pink-flowered, *L. scoparium* 'Keatleyi' and the red-flowered *L. scoparium* 'Nichollsii' (*L.* 'Nichollsii'),[19] which is no longer found in New Zealand, but *L. scoparium* 'Red Ensign' (*L.* 'Red Ensign') is thought to be a newer name for *L.* 'Nichollsii Improved'.[19] The nectar DHA/Tsugar for several *L.* 'Martinii' and *L.* 'Red Ensign' plants from which flowers were collected in 2011 and 2012 (including VB014 from the original survey) was classified as high or very high (Table 2). When compared to regional variation between wild plants, only the Nelson region had a mean DHA/Tsugar approaching that of *L.* 'Martinii' and *L.* 'Red Ensign' (between 0.002 and 0.003 in 2009); all other regions had means <0.002 (Figure 3).

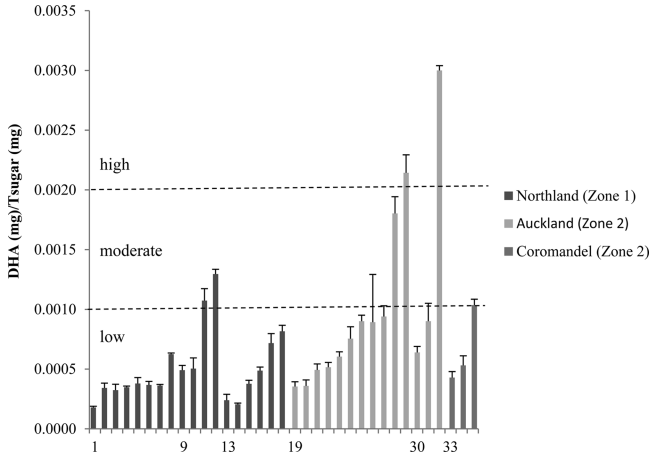

Figure 4. Nectar DHA/Tsugar values for Northland, Auckland, and Coromandel trees sampled between 2010 and 2011 (error bars are standard error values). The numbers on the *x*-axis indicate the first tree in each zone of sampling. Trees 1–8 were from upper Northland, trees 9–12 from upper East Northland, trees 13–18 from lower East Northland, trees 19–29 from North Auckland, trees 30–32 from South Auckland, and trees 33–35 from the Coromandel region.

Table 2. DHA/Tsugar Values for *L.* 'Martinii' and *L.* 'Red Ensign' Collected in 2011–2012

sample	variety	year	DHA/Tsugar (mg/mg)
VB014	*L.* 'Martinii'	2011	0.00435
1	*L.* 'Martinii'	2011	0.00353
2	*L.* 'Martinii'	2011	0.00374
3	*L.* 'Martinii'	2012	0.00287
4	*L.* 'Martinii'	2012	0.00402
5	*L.* 'Martinii'	2012	0.00379
6	*L.* 'Martinii'	2012	0.00304
7	*L.* 'Red Ensign'	2012	0.00318
8	*L.* 'Red Ensign'	2012	0.00429
9	*L.* 'Red Ensign'	2012	0.00511
10	*L.* 'Martinii'	2012	0.00171

In addition to the aforementioned pink- and red-flowered garden cultivars, >150 cultivars have been named. These have been obtained from the wild and also selected in cultivation.[5,19–21]

To further examine the possibility that some garden cultivars may exhibit unusually high nectar DHA/Tsugar, a collection of cultivars was made at the Auckland Botanic Gardens in a single day (10F, 10 replicates) (Table 3). All cultivars with levels of dihydroxyacetone below the limit of quantitation were still producing nectar sugars; that is, the Tsugar values were readily measured. From this small, preliminary survey it would appear that red or pink coloration in a cultivar is often associated with high nectar DHA/Tsugar but that this is also often suppressed to moderate or low when a double flower is present; the only

Table 3. Comparison of Nectar DHA/Tsugar Values of Mānuka Cultivars at the Auckland Botanic Gardens in 2012

cultivar[a]	description	DHA/Tsugar
***L.* 'Nanum'**	pink single-flowered dwarf	NT[b]
L. 'Tui'	pink-centered single-flowered dwarf	0.00435 mg/mg **HIGH**
L. 'Kea'	pink single-flowered dwarf	0.00105 mg/mg **HIGH**
L. 'Wiri Shelley' (*L.* 'Kea' × *L.* 'Wiri Sandra')	pink single-flowered	0.00452 mg/mg **HIGH**
***L.* 'Sherryl Lee'**	pink single-flowered	NT[a]
L. 'Wiri Sandra'	pink single-flowered	0.00312 mg/mg **HIGH**
***L.* 'Pink Pearl'** (seed parent = *L.* 'Album Flore-pleno')	white double-flowered	NT[a]
L. 'Sunraysia'	white and red double-flowered	NQ[c] **LOW**
L. 'Rose Glory'	pink double-flowered	NT[a]
L. 'Wiri Joan' (seed parent = *L.* 'Rose Glory')	red double-flowered	NQ **LOW**
L. 'Rose Queen'	pink double-flowered	0.00079 mg/mg **LOW**
L. 'Wiri Linda' (seed parent = *L.* 'Rose Queen')	white double flowered	0.00079 mg/mg **LOW**
L. 'Crimson Glory'	red double-flowered	0.00099 mg/mg **LOW**
L. 'Blossom'	pink double-flowered	0.00103 mg/mg **MODERATE**
L. 'Autumn Glory'	pink double-flowered	0.00144 mg/mg **MODERATE**
L. 'Rosy Morn'	pink double-flowered	0.00152 mg/mg **MODERATE**
L. 'Burgundy Queen'	red double-flowered	0.00208 mg/mg **HIGH**
L. 'Wiri Donna'	red single-flowered	0.00414 mg/mg **HIGH**
***L.* 'Flore-pleno'**	pink double-flowered	NT[a]
L. 'Snow Flurry'	white double-flowered	NQ[b] **LOW**
L. 'Red Damask'	red double-flowered	NQ[b] **LOW**
***L.* 'Nichollsii'**	red double-flowered	NT[a]
L. 'Red Ensign'	red single-flowered	NT[a]
L. 'Wiri Amy' (seed parent = *L.* 'Red Ensign')	red single-flowered	0.00694 mg/mg **HIGH**
L. 'Wiri Kerry' (seed parent = *L.* 'Wiri Amy')	red double-flowered dwarf	NQ[b] **LOW**
unconfirmed parentage		
L. 'Black Robin'	red single-flowered	0.00598 mg/mg **HIGH**
L. 'Elizabeth Jane'	red single-flowered dwarf	0.00742 mg/mg **HIGH**

[a]Cultivars are presented in groups, with the putative original seed parents of each group in bold.[4,19] [b]Cultivars not available for testing in this survey, but known to belong within the same group. [c]Dihydroxyacetone was below the level of quantitation.

exception to this is *L. scoparium* 'Burgundy Queen', a red double that was classified as high. High nectar DHA/Tsugar values are observed in *L. scoparium* 'Nanum Tui' (*L.* 'Nanum Tui'), a single-flowered dwarf ,and in pink single-flowered cultivars also derived from *L. scoparium* 'Nanum'.

Nectar DHA/Tsugar in Australian Species of *Leptospermum*. Australia has 87 species of *Leptospermum* inhabiting various ranges.[21] Honey from *L. polygalifolium* has also been shown to contain dihydroxyacetone and methylglyoxal,[4] indicating that production of dihydroxyacetone in the nectar is found elsewhere in the genus. To confirm this, a limited survey of Australian species was carried out in southern Queensland at the University of the Sunshine Coast. Samples were obtained from wild populations in southern Queensland or northern New South Wales, which abuts southern Queensland. *Leptospermum scoparium* 'Merinda' (*L.* 'Merinda'), which is a complex cross between two interspecific hybrids of Australian species, was also included in this survey, but the plant sampled was growing in New Zealand. Some of the Australian *Leptospermum* species contained dihydroxyacetone (in two cases being nonquantatible), and DHA/Tsugar differed both between and within species (Table 4). This confirms that

Table 4. DHA/Tsugar Ratios in Australian Species of *Leptospermum*

species	sample	DHA/Tsugar (mg/mg)
L. liversidgei	1	0.00273
	2	0.00084
L. juniperinum	1	NQ[a]
	2	0.00386
	3	0.00159
L. laevigatum	1	NQ[a]
L. 'Merinda'	1	0.00069

[a]Dihydroxyacetone was below the level of quantitation.

production of high levels of dihydroxyacetone in the nectar is a characteristic of the genus *Leptospermum* and that the same degree of variation in DHA/Tsugar seen in *L. scoparium* probably occurs in other *Leptospermum* species as well.

Other Factors That Might Relate to DHA/Tsugar. *Composition of Leaf Oil.* Some degree of correlation occurred between leaf oil and nectar DHA/Tsugar in some regions (East Cape and Wairarapa regions; adjusted R^2 values of 80.7 and 88.9%, respectively) with multivariate analysis of different leaf oils in different areas. This agrees with recently published findings[22] that FT-Raman spectroscopy of leaf material can be used as a predictive model for dihydroxyacetone levels in floral nectar of mānuka.

Sex of the Flower. *L. scoparium* is andromonecious, that is, it bears both hermaphrodite and male-only flowers.[23] A significant difference was observed between flowers of different sexes with male flowers having higher DHA/Tsugar due to elevated levels of dihydroxyacetone.

Age of the Flower. The hypanthium is initially green in new flowers and as a general rule turns dark red with age. Both dihydroxyacetone and Tsugar values were higher in flowers with red hypanthia.

Soil Composition. No correlation could be found with soil order or soil quantifiable components. This is similar to the observation that soil properties had no discernible effect upon leaf oil[10] or the activity of honey derived from a certain area.[12]

Coverage with Sooty Mold. Sooty mold indicates a scale insect infestation, which is a potential stressor for the plant and which might affect dihydroxyacetone production, but no correlation was found between DHA/Tsugar and coverage with sooty mold.

Nectar Sugars. Dihydroxyacetone in nectar could be produced by the plant or by microbes present in the flower.[24,25] Sucrose was not detected in any of the *L. scoparium* nectars

assayed, indicating a hexose-dominant nectar of the type associated with pollination by small, unspecialized insects.[26] This concurs with classification of the dish-shaped mānuka flower as open-access and visited by a variety of insect feeders/pollinators.[27] The ratio of fructose to glucose in the 2009 and 2010 North Island nectar samples was 1.65:1 (R^2 = 0.9164), clearly differing from the 1:1 ratio expected if the nectar was solely derived from the hydrolysis of sucrose. The predominance of fructose found in the nectars of *L. scoparium* in the field may indicate the effect of a microorganism, specifically yeast, transported by visiting insects such as ants.[24,25]

An ongoing study of fructose/glucose ratios in mānuka honeys of various NPA found a mean of 1.29 and a range from 0.98 to 1.67 (n = 1483, s = 0.10) (P. Bray, personal communication). There is no obvious explanation for the difference between the honeys and the nectar as it seems unlikely that the nectar extraction procedure utilized here is selective for fructose. Given that visitation by insects and colonization by microbes are essentially random, it is possible that this particular set of trees simply fell to the high end of the range expressed by the honeys.

This method was developed and validated for assaying DHA/Tsugar in floral nectar from cut flowers and was applied to a survey of wild *L. scoparium* and also for a survey of cultivars and a limited survey of Australian species. Within the wild populations, variation in DHA/Tsugar within and between regions and, in some cases, between seasons was observed although no relationship to soil composition could be elucidated. Results were partially aligned with a previous survey of honeys from these regions[12] with the reservation that levels of dihydroxyacetone in nectar do not always accurately reflect methylglyoxal in honey because of the effects of storage conditions. In some regions leaf oil composition could be correlated to DHA/Tsugar, and this is corroborated by a study demonstrating that spectroscopy of leaf material can be used to predict dihydroxyacetone content of nectar.[22] Nectar sugars indicate that the flower is pollinated by small nonspecialized insects and that sugar composition is likely to be affected by microbial species transferred from a variety of pollinators. Horticultural cultivars frequently exhibited very high nectar DHA/Tsugar, and this is likely to indicate a genetic predisposition. Australian species also exhibited elevated nectar DHA/Tsugar, and variation within and between species was observed; this confirms that the phenomenon operates at the genus level. This was, however, a limited survey, and a larger survey needs to be undertaken of further species and cultivars in Australia and extended to other wild populations in New Zealand. There were indications that the age and the sex of the flower may influence DHA/Tsugar, and more detailed studies under controlled conditions will be reported separately.

■ ASSOCIATED CONTENT

Supporting Information

Complete information on location and type of trees sampled, details of experimental procedures, and results for investigation of other factors that might influence DHA/Tsugar (Tables S1 and S2 and Figures S1-S6 as well as additional text and experimental details). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(M.R.) E-mail: mtr9@students.waikato.ac.nz.

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■ ABBREVIATIONS USED

PFBHA, *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride; TMSI, 1-(trimethylsilyl)imidazole; Tsugar, total sugar in the nectar sample; 20F, 20-flower extraction; 10F, 10-flower extraction; 1F, single-flower extraction

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