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1 # Regional, Annual and Individual Variation in the Dihydroxyacetone Content
2 of the Nectar of Mānuka (*Leptospermum scoparium*) in New Zealand

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1 **ABSTRACT**
2 A method was designed and validated for analysis of dihydroxyacetone in the floral nectar of
3 mānuka (*Leptospermum scoparium*). The method was applied to samples collected from
4 different regions of the North Island and the Nelson region of the upper South Island of New
5 Zealand during the period 2009-2012 as well as to nectar samples from some Australian
6 *Leptospermum* species. The ratio of dihydroxyacetone to total sugar (DHA/Tsugar) was
7 classified as low (<0.001 mg/mg), moderate (0.001-0.002 mg/mg) and high (>0.002 mg/mg).
8 Inter- and intra-regional variation were observed as well as inter-annual variation with variation
9 from low to high classification occurring within one region and from low to moderate between
10 years. Australian species also demonstrated elevated levels of dihydroxyacetone in the nectar.
11 Some garden cultivars were shown to produce very high levels of nectar DHA/Tsugar and a

12 survey of cultivars was undertaken; cultivars with single flowered red or pink flowers were the

13 most common producers of very high levels of nectar DHA/Tsugar.

14 **KEYWORDS**

15 *Dihydroxyacetone, Leptospermum scoparium, cultivar, manuka, nectar, New Zealand, honey*

16

17 **INTRODUCTION**

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

28 The NPA of mānuka honey is notoriously variable from region to region and from year to year.
29 Currently mānuka honey is harvested from wild mānuka populations, although there is interest in
30 selecting or breeding mānuka cultivars or seed sources with optimum nectar and
31 dihydroxyacetone production. It is not known why species of *Leptospermum* produce
32 dihydroxyacetone in the nectar nor why inter-tree variation exists and the effect of environment
33 and climate has not been elucidated. *L. scoparium* displays wide morphological variation
34 throughout New Zealand[5-9] and regional variation is manifest in leaf oil chemotypes from
35 different regions of New Zealand.[10] The biology of *L. scoparium* in New Zealand has been
36 reviewed.[11]

37 Inter- and intra-regional variability of dihydroxyacetone content in the nectar of *L. scoparium*
38 has not been assessed, although variation in NPA of honeys from different regions indicates
39 indirectly that there is likely to be regional variation.[12] This work is a preliminary study of the

40 DHA/Tsugar of the floral nectar of mānuka that was undertaken across a variety of locations in
41 the North Island and in the Nelson region of the South Island of New Zealand, during the period
42 2009-2012. Horticultural cultivars of mānuka were included after it was found that some
43 cultivars exhibited consistently high nectar DHA/Tsugar. The aim was to increase understanding
44 of the variations that might be expected both geographically and chronologically.

45 **MATERIALS AND METHODS**

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

61 **Sampling protocol.** The trees sampled in 2009 were re-sampled in 2010 except for those that
62 had died in the interim. In 2011 additional trees located within 50 m of the existing test trees at

63 the East Cape sites were sampled. Further samples were collected in the Auckland and Waikato
64 regions in 2012; both cultivars and wild accessions. The trees were sampled, where possible, on
65 a fine day. Entire flowers were picked off individually, or scraped off between two fingers drawn
66 from the base towards the apex of the shoot. In addition to collection of flower samples in 2009,
67 soil was collected (with a small trowel at 2 m from the trunk of each tree, samples approximately
68 $15\times 10\times 20$ cm in dimension) and foliage was collected for leaf oil analyses. All samples were
69 stored in airtight bags and chilled with ice while transported, until they could be transferred to a
70 freezer (-20 °C) for storage until analysis.

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

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

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

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

95 **Preparation of nectar for dihydroxyacetone detection.** To a vial containing hydroxyacetone
96 (internal standard, 10 μL, 0.5 mg/mL) and nectar extract (20F: 200 μL, 10F: 200 μL, 1F:
97 400 μL), PFBHA (50 μL, 20 mg/mL in a pH 4 citric buffer) was added, mixed and kept (1 h).
98 DCM (20F: 2 mL, 10F: 2 mL, 1F: 1 mL) was added and mixed. The vial was then placed in a
99 freezer (- 0 °C) until the aqueous layer froze to aid extraction. An aliquot (1 mL) of the DCM
100 layer was withdrawn into a GC vial (1.5 mL). TMSI (50 μL) was added, mixed and let stand
101 (1 h) before analysis by GC-FID.

102 **Preparation of nectar for sugar detection.** To a vial containing mannitol (internal standard,
103 5 μL, 5 mg/mL) was added nectar extract (20F: 20 μL, 10F: 20 μL, 1F: 50 μL) and the vial
104 contents freeze dried. To the dry sample TMSI (50 μL) was added, mixed and kept at room
105 temperature. Heptane (1 mL) was added with mixing before analysis by GC-FID.

106 **GC-FID Parameters** The column used was a 0.32mm x 0.25 μm i.d., 30 m, Zebron ZB-5,
107 capillary GC column (Agilent Technologies, Santa Clara, CA). For all methods a 2 μL injection

108 into a splitless inlet with a 2 mL/min purge flow was used, with the FID held at 300 °C. H_2 flow
109 was 30 mL/min, air flow 400 mL/min and N_2 make up flow 10 mL/min.
110 For detection of dihydroxyacetone, the inlet was set to 250 °C and used a H_2 carrier gas flow of
111 2 mL/min. The temperature program was 130 °C held for 5 min, increased at 10 °C/min to
112 250 °C then held at that temperature for 6 min. Dihydroxyacetone was quantitated against
113 hydroxyacetone as an internal standard and used a calibration curve constructed over the range of
114 0.003 to 0.015 mg/mL. The limits of detection for dihydroxyacetone and methylglyoxal were
115 3.00×10^{-8} g/mL and 4.58×10^{-7} g/mL respectively. The limits of quantitation for
116 dihydroxyacetone and methylglyoxal were 1.00×10^{-7} g/mL and 1.53×10^{-6} g/mL respectively.
117 For detection of sugars, the inlet was set to 280 °C with a H_2 carrier gas flow of 4 mL/min. The
118 temperature program was 100 °C held for 1 min, increased at 30 °C/min to 200 °C, increased at
119 10 °C/min to 250 °C and finally increased at 30 °C/min to 300 °C. The sugars were quantitated
120 against D-mannitol as an internal standard with a response factor found for each sugar. The
121 response was measured over the range of 50 to 150 mg/mL and gave fructose: 0.5335;
122 glucose: 0.8217 and sucrose 0.7126. Fructose, glucose and sucrose were summed to give total
123 sugars.
124 **Expression of results for dihydroxyacetone content.** Dihydroxyacetone content is expressed as
125 a ratio of DHA/total nectar sugar (Tsugar). The assumption was made that all of the sugars in
126 honey derive ultimately from the nectar and that the ratio for dihydroxyacetone to total sugar
127 would be indicative of the expected ratio in the early honey before maturation because
128 dihydroxyacetone might be expected to have similar volatility to the sugars. Ten replicates for
129 each tree were assayed and after outliers were determined (single outlier and largest/smallest or
130 two largest/smallest tests)[17] results are expressed as mean and 95% standard error.

131 **Measurement of nectar DHA/Tsugar in Australian species.** The nectars of some Australian
132 species (*L. juniperinum* Sm., *L, liversidgei* R.T.Baker & H.G.Sm., *L. laevigatum* (Gaertn.)
133 F.Muell.) were analyzed at the University of the Sunshine Coast in Southern Queensland.
134 Sampling from wild populations occurred principally in the coastal region of northern New
135 South Wales. Sample preparation was as above but GC-MS was utilised.
136 **GC-MS parameters.** The column used was a 30m x 0.25 mm x 0.25 µm i.d. Elite 5MS gas
137 capillary column (Perkin Elmer, Melbourne, Australia). Analyses were carried out using a
138 Perkin-Elmer Clarus 580 gas chromatograph coupled to a Perkin-Elmer Clarus SQ85 mass
139 spectrometer (All Perkin Elmer instrument supplied by Perkin Elmer, Melbourne, Australia). The
140 carrier gas (helium) flow was set to 1 mL/min for both the sugar and dihydroxyacetone analyses.
141 For the sugar analysis the inlet was configured to shut the split from 0.20-1.00 min and open
142 with a 50:1 ratio; it was maintained at 280 °C. The oven program was the same as described for
143 the New Zealand samples. Compound ionisation was at 70 eV electron impact, analyzing
144 *m/z* +70-440 over 4.50-11.00 min. For the dihydroxyacetone analysis, the inlet was configured as
145 for the sugar analysis except the temperature was maintained at 250 °C. The oven program was
146 the same as that outlined for the New Zealand specimens. Compound ionisation was at 70 eV
147 electron impact, analyzing *m/z* +45-440 over 4.50-23.00 min.
148 **Statistical analyses.** Statistical analyses were carried out using Microsoft Excel or Minitab 16™.
149 **RESULTS AND DISCUSSION**
150 In order to simplify discussion nectar DHA/Tsugar has been divided into three classifications;
151 low (<0.001 mg/mg), moderate (0.001-0.002 mg/mg) and high (>0.002 mg/mg).
152 **Choice of methodology for nectar sampling and extraction.** Stephens et al.[15] analyzed nectar
153 of *L. scoparium* (n=6) for phenolics and for methylglyoxal; the methodology employed by those

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154 authors was to use direct sampling by pipette.[15] For the current study this method was considered
155 but it was decided that it was far too time consuming for this broad-based preliminary survey
156 which was often carried out in isolated areas, only accessed with difficulty and often under
157 inclement conditions. This view is supported by the conclusions of Morrant *et al*.[14] who have
158 reviewed methods of nectar collection.
159 Two methods of extracting nectar from the previously collected flowers were compared: washing
160 (20 min) and individually dipping (×3). Each method was repeated eight times using twenty
161 flowers per sample for flowers from the same tree and the absolute dihydroxyacetone content
162 was analyzed; mean and %RSD were respectively 0.0056 mg (26%) and 0.00020 mg (90%).
163 Washing was therefore used in this survey. Morrant *et al*.[14] also recommended washing of cut
164 flowers as the most practical method and indeed found this to be preferable to and more
165 reproducible than the use of micropipettes.[14]
166 Stephens *et al*.[15] did not assay dihydroxyacetone; methylglyoxal was assayed but only reported
167 as a trace so no numerical comparison between methods in that study and the current study is
168 possible.
169 **Method validation.**
170 *Gas chromatography methodology for analysis of dihydroxyacetone and methylglyoxal with*
171 *PFBHA derivatization*. PFBHA derivatization with analysis by reversed phase HPLC has been
172 used previously to assay dihydroxyacetone and methylglyoxal simultaneously in honey[4]; it was
173 decided that GC-FID would supply the requisite sensitivity for nectar samples. The
174 derivatization and GC-FID analysis of samples was optimized using standards of
175 hydroxyacetone, dihydroxyacetone and methylglyoxal. Nectar samples spiked with these
176 standards were used to ensure that the derivatising agent PFBHA was present in sufficient

177 excess. Under the conditions used hydroxyacetone (internal standard), dihydroxyacetone and
178 methylglyoxal eluted at 6.6, 10.1 and 13.1 min respectively. Responses for dihydroxyacetone
179 and methylglyoxal (measured as analyte/internal standard *versus* analyte) were linear from 0-
180 0.0016 mg (R^2=0.9998) and from 0-0.015 mg (R^2=0.9815) respectively. The LOQ for
181 dihydroxyacetone corresponded to a minimum detectable mass of 20.5 ng and for methylglyoxal
182 313 ng. The LOD were respectively 6.16 ng and 93.8 ng. methylglyoxal was never detected at a
183 level that allowed quantitation, in any nectar samples in this study (Figure 1); the presence of
184 methylglyoxal in nectar samples assayed by others[15] might be because the methodology
185 employed was more sensitive than that used here or might indicate that the flowers or nectar
186 samples had been exposed to elevated temperatures or stored for prolonged periods at room
187 temperature prior to analysis.[16]

188 *Gas chromatography methodology for analysis of sugars with TMS derivatization*. Response
189 factors of sucrose, glucose and fructose relative to the internal standard mannitol were
190 ascertained by varying weight ratios and comparing area ratios and were 0.7126, 0.8217 and
191 0.5335 respectively. Varying the quantity of derivatization agent from 50-200 μL demonstrated
192 that 50 μL was sufficient excess. As sugars were always readily observed LOD and LOQ were
193 not required. Under the chromatographic conditions used for the analysis fructose (multiple
194 furanose and pyranose anomers) eluted at 4.9-5.1 min, glucose (pyranose anomers) at 5.6 and 5.8
195 min and mannitol at 5.7 min (Figure 2).

196 *Duration of washing for nectar extraction.* The duration of washing was based upon Morrant *et*
197 *al.*[14] who found no difference between 1 minute and 20 min but a significant difference between
198 20 and 60 min. In this study extended washing of the flowers (230 min) resulted in an ~25%
199 decrease in DHA/Tsugar presumably because sugar was leaching from other parts of the flower.

200 *Reproducibility tests.* To ascertain day to day reproducibility of the method, samples from eight
201 trees were assayed (20F, n = 10) on two different days. Statistically three of the trees gave
202 significantly different results between days ($p = 0.0010, 0.0007, 0.000$) whereas five did not ($p =$
203 $0.1597, 0.8899, 3 \times 1.000$) but in only one case did a tree change its classification. The weather
204 conditions on each day of sampling may have had an influence on these results as rain and wind
205 can remove nectar from the open bowl of the mānuka flower as can visits by pollinators.
206 *Stability of nectar extract.* The stability of the nectar extract was ascertained for samples from
207 three trees in which replicates (n = 10) were stored frozen and others (n = 5) were left to stand at
208 room temperature overnight before analysis. The samples that were left to stand darkened in
209 color overnight but a z-test for two sample means showed that there was no change to the
210 DHA/Tsugar ratio. To test the stability of the derivatized samples, duplicates from one tree were
211 prepared for both dihydroxyacetone and sugar assays and injected into the GC at the beginning
212 and the end of the sequence; the time lapse for the sugar assay being ~315 min and for the
213 dihydroxyacetone assay ~702 min. The results for DHA/Tsugar were identical to two significant
214 figures.
215 *Comparison of ten flower (10F) and twenty flower (20F) tests.* The ten flower (10F) test was
216 compared with the 20F test using replicates (n = 10) from three different trees; the difference
217 between the two tests was insignificant at the 95% confidence level. The percentage relative
218 standard deviation was higher for the 10F than the 20F test but did not exceed 25%.
219 *Comparison of the single flower test (1F) and the 20F test.* Some of the trees, especially the
220 Northland samples and the East Cape samples collected in 2011, yielded insufficient flowers for
221 the twenty or ten flower test. Comparison of the single flower test (1F) and the 20F test was
222 carried out using replicates (n = 10) from three trees. The range of percentage difference between

223 the two tests for the two ratios, DHA/Tsugar and fructose/glucose was 5.52-20.21 and 1.83-17.44
224 respectively. The percentage relative standard deviation for the 1F test was much higher than for
225 the 20F test and also much higher than the percentage difference between the two methods. This
226 indicates, as might be expected, that there is variability between individual flowers on the same
227 tree. To confirm that biological variation exceeded experimental variation, replicates ($n = 5$) of a
228 single extraction (20F) were compared to multiple extractions (20F, $n = 10$) for 3 different trees.
229 For the single extraction percentage relative standard deviation was 1.20, 2.54 and 3.13 for the
230 three trees and the corresponding values for the multiple extractions were 14.36, 9.06 and 25.80.
231 This confirms that the greatest source of variation is variation is that within a single tree the
232 cause of which is not yet established but may include active removal by pollinators and variation
233 related to the different stages of flower maturity.
234 *Transference of nectar.* The residue from an empty sample bag was rinsed out and assayed and
235 dihydroxyacetone equivalent to about half that found in 20 flowers was measured (a bag
236 typically contained ~500 flowers). This confirms that transfer of nectar between flowers and the
237 bag and presumably also between flowers is occurring; this may partially account for the
238 variation observed in the single flower test.
239 **Variation of nectar DHA/Tsugar between regions and years and within regions.**
240 *Variation of nectar DHA/Tsugar between regions and years.* Regional and annual variation in
241 nectar DHA/Tsugar was observed (Figure 3). Only the Coromandel and East Cape regions had
242 sufficient samples in both years for a statistical test of the effect of sampling year. A balanced
243 ANOVA showed that the mean DHA/Tsugar for the East Cape region was significantly different
244 ($p = 0.02$) between 2009 and 2010, whereas the Coromandel region did not differ between years

245 (p = 0.5). The change observed in the East Cape samples was sufficient to alter the classification
246 from low in 2009 to moderate in 2010.
247 Only the Coromandel, East Cape and Wairarapa regions had sufficient samples for a test of
248 effect of region on DHA/Tsugar. A one-way ANOVA test followed by mean separation using
249 Tukey's method detected an overall region effect (p = 0.05). Mean nectar DHA/Tsugar did not
250 differ between the Coromandel and Wairarapa samples but the East Cape values were lower in
251 both the 2009 and the 2010 years.
252 *Variation within regions.* Significant differences in nectar DHA/Tsugar were observed between
253 trees within the Coromandel sampling site (Figure 3). All trees were located within a radius of
254 100 m but a range of classifications from low to high was observed. There were three soil orders
255 present at this Coromandel site but no relationship between soil order and DHA/Tsugar could be
256 found. In order to further investigate variation within a region an additional collection was made
257 in the East Cape region in 2011. From eight sites, one or more trees of varying classification
258 (low-high), that had been sampled previously, were selected and other trees within a 50 m radius
259 were also sampled. Even within a narrow radius individual trees differed significantly in their
260 nectar DHA/Tsugar (Table 1), in some cases ranging from high to low classifications.
261 The Northland/Auckland region was surveyed in 2010 and 2011 (Figure 4). For this survey the
262 region was divided into two zones: Northland, encompassing the area from Cape Reinga to 36.3
263 ^{o}S (Zone 1) and the Auckland/Coromandel regions south of 36.3 ^{o}S (Zone 2). In Zone 1
264 *L. scoparium* var. *incanum* Cockayne predominates[18] whereas in Zone 2 and the rest of New
265 Zealand most wild mānuka are considered to be *L. scoparium* var. *scoparium*. The individual
266 tree returning an exceptionally high value belonged to *L. scoparium* var. *incanum* located in a
267 maintained area. The individual with a mid-moderate result was also from *L. scoparium* var.

268 *incanum*. The only other individual demonstrating a high classification belonged to *L.*
269 *scoparium* var. *scoparium* as did the two specimens with low-moderate results. The remaining
270 individuals consisted of a mixture of var. *scoparium* (n = 23) and var. *incanum* (n = 16) varieties
271 all of which scored in the low classification. No apparent trend exists relating either of these
272 varieties to high levels of DHA/Tsugar.

273 Stephens[12] grouped 463 mānuka honey samples (from a single season, 2001-2, and supplied by
274 apiarists) by region and by mean "Unique Manuka Factor" ® (UMF); a measure of non-peroxide
275 antibacterial activity; Waikato (n=6) and Coromandel (n=23) returned high values of activity
276 (>14 UMF) although other Coromandel honeys returned mid-levels (n=16; 12.6 UMF) and low
277 levels (n=63; 10.5 UMF). Some East Coast honeys returned mid-levels of activity (n=22, 12.5
278 UMF) whilst others returned lower levels (n=17; 10.9 UMF). Wairarapa honeys returned low
279 levels of activity (n=12; 9.4 UMF) as did samples from the Northern South Island (n=41; 9.1
280 UMF) whereas Northland demonstrated high levels of activity (n=35; 14.8 UMF). These
281 findings concur with the variability observed in the current study in both the Coromandel region
282 and the East Cape and to some extent the finding that the East Cape values were lower than those
283 for the Coromandel. However the Wairarapa, Northland and Northern South Island results are in
284 contrast. The Waikato result, with the exclusion of the garden cultivar, is somewhat lower than
285 expected from Stephens' results, which relate to honeys originating from wild manuka in
286 swamplands, but in the current study the trees were sampled from drier locations, which might
287 possibly account for the observed difference.

288 Caution should be exercised when comparing these two studies since variability of classification
289 of dihydroxyacetone content between seasons was observed in the current study at one location
290 and further work (unpublished) indicate that the conditions of storage by the apiarist strongly

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291 influence the efficiency of conversion of dihydroxyacetone to methylglyoxal in maturing
292 mānuka honey and hence the final UMF value.

293 **Nectar DHA/Tsugar in commercial New Zealand originated cultivars.** The specimen used to
294 first illustrate the presence of dihydroxyacetone in nectar was a red-pink-flowered cultivar
295 *L.* 'Martinii', which is widely available from plant nurseries and which was growing in a
296 suburban garden in Hamilton. This cultivar consistently gave substantially higher nectar
297 DHA/Tsugar values than any of the wild specimens tested (Figure 3).

298 *L*. 'Martinii' is believed to be a cross between the pink-flowered, *L. scoparium* 'Keatleyi' and the
299 red-flowered *L. scoparium* 'Nichollsii' (*L.* 'Nichollsii'),[19] which is no longer found in New
300 Zealand but *L. scoparium* 'Red Ensign' (*L.* 'Red Ensign') is thought to be a newer name for *L.*
301 'Nichollsii Improved'.[19] The nectar DHA/Tsugar for several *L.* 'Martinii' and *L.* 'Red Ensign'
302 plants from which flowers were collected in 2011 and 2012 (including VB014 from the original
303 survey) was classified as high or very high (Table 2). When compared to regional variation
304 between wild plants, only the Nelson region had a mean DHA/Tsugar approaching that of *L.*
305 'Martinii' and *L.* 'Red Ensign' (between 0.002 and 0.003 in 2009); all other regions had means
306 <0.002 (Figure 3).

307 In addition to the aforementioned pink and red-flowered garden cultivars more than 150 cultivars
308 have been named. These have been obtained from the wild and also selected in cultivation.[5, 19-21]
309 To further examine the possibility that some garden cultivars may exhibit unusually high nectar
310 DHA/Tsugar a collection of cultivars was made at the Auckland Botanic Gardens in a single day
311 (10F, 10 replicates) (Table 3). All cultivars with levels of dihydroxyacetone below the limit of
312 quantitation were still producing nectar sugars i.e. the Tsugar values were readily measured.
313 From this small, preliminary survey it would appear that red or pink coloration in a cultivar is

314 often associated with high nectar DHA/Tsugar but that this is also often suppressed to moderate
315 or low where a double flower is present; the only exception to this is *L. scoparium* 'Burgundy
316 Queen' a red double which was classified as high. High nectar DHA/Tsugar are observed in *L.*
317 *scoparium* 'Nanum Tui' (*L.* 'Nanum Tui') a single-flowered dwarf and in pink single-flowered
318 cultivars also derived from *L. scoparium* 'Nanum'.

319 **Nectar DHA/Tsugar in Australian species of *Leptospermum*.** Australia has 87 species of
320 *Leptospermum* inhabiting various ranges.[21] Honey from *L. polygalifolium* has also been shown to
321 contain dihydroxyacetone and methylglyoxal[4] indicating that production of dihydroxyacetone in
322 the nectar is found elsewhere in the genus. To confirm this a limited survey of Australian species
323 was carried out in Southern Queensland at the University of the Sunshine Coast. Samples were
324 obtained from wild populations in Southern Queensland or Northern New South Wales, which
325 abuts Southern Queensland. *Leptospermum scoparium* 'Merinda' (*L.* 'Merinda'), which is a
326 complex cross between two inter-specific hybrids of Australian species, was also included in this
327 survey, but the plant sampled was growing in New Zealand. Some of the Australian
328 *Leptospermum* species contained dihydroxyacetone (in two cases being non-quantatible), and
329 DHA/Tsugar differed both between and within species (Table 4). This confirms that production
330 of high levels of dihydroxyacetone in the nectar is a characteristic of the genus *Leptospermum*
331 and that the same degree of variation in DHA/Tsugar seen in *L. scoparium* probably occurs in
332 other *Leptospermum* species as well.

333 **Other factors which might relate to DHA/Tsugar.**

334 *Composition of leaf oil.* Some degree of correlation occured between leaf oil and nectar
335 DHA/Tsugar in some regions (East Cape and Wairarapa regions; adjusted R^2 of 80.7 and 88.9%
336 respectively) with multivariate analysis of different leaf oils in different areas. This agrees with

337 recently published findings[22] that FT-Raman spectroscopy of leaf material can be used as a
338 predictive model for dihydroxyacetone levels in floral nectar of mānuka.

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

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

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

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

351 **Nectar sugars.** dihydroxyacetone in nectar could be produced by the plant, or by microbes
352 present in the flower.[24-25] Sucrose was not detected in any of the *L. scoparium* nectars assayed,
353 indicating a hexose-dominant nectar of the type associated with pollination by small,
354 unspecialized insects.[26] This concurs with classification of the dish-shaped mānuka flower as
355 open-access and visited by a variety of insect feeders/pollinators.[27] The ratio of fructose to
356 glucose in the 2009 and 2010 North Island nectar samples was 1.65:1 (R^2 = 0.9164), clearly
357 differing from the 1:1 ratio expected if the nectar was solely derived from the hydrolysis of
358 sucrose. The predominance of fructose found in the nectars of *L. scoparium* in the field may

359 indicate the effect of a microorganism, specifically yeast, transported by visiting insects such as
360 ants.[24-25]
361 An on-going study of fructose:glucose ratios in mānuka honeys of varying NPA found a mean of
362 1.29 and a range from 0.98 to 1.67 (n = 1483, s = 0.10) (P. Bray, personal communication).[28]
363 There is no obvious explanation for the difference between the honeys and the nectar as it seems
364 unlikely that the nectar extraction procedure utilized here is selective for fructose. Given that
365 visitation by insects and colonisation by microbes is essentially random it is possible that this
366 particular set of trees simply fell to the high end of the range expressed by the honeys.
367 This method was developed and validated for assaying DHA/Tsugar in floral nectar from cut
368 flowers and this method was applied to a survey of wild *L. scoparium* and also for a survey of
369 cultivars and a limited survey of Australian species. Within the wild populations variation in
370 DHA/Tsugar within and between regions and, in some cases, between seasons was observed
371 although no relationship to soil composition could be elucidated. Results were partially aligned
372 with a previous survey of honeys from these regions[12] with the reservation that levels of
373 dihydroxyacetone in nectar do not always accurately reflect methylglyoxal in honey because of
374 the effects of storage conditions. In some regions leaf oil composition could be correlated to
375 DHA/Tsugar and this is corroborated by a study demonstrating that spectroscopy of leaf material
376 can be used to predict dihydroxyacetone content of nectar. [22] Nectar sugars indicate that the
377 flower is pollinated by small non-specialized insects and that sugar composition is likely to be
378 affected by microbial species transferred from a variety of pollinators. Horticultural cultivars
379 frequently exhibited very high levels of DHA/Tsugar and this is likely to indicate a genetic
380 predisposition. Australian species also exhibited elevated levels of DHA/Tsugar and variation
381 within and between species was observed and this confirms that the phenomenon operates at the

382 genus level. This was however a limited survey and a larger survey needs to be undertaken of
383 further species and cultivars in Australia and extended to other wild populations in New Zealand.
384 There were indications that the age and the sex of the flower may influence DHA/Tsugar and
385 more detailed studies under controlled conditions will be reported separately.

386 **Abbreviations used:**

387 PFBHA: *O*-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride; TMSI: 1-
388 (Trimethylsilyl)imidazole; Tsugar: total sugar in the nectar sample; 20F : twenty flower
389 extraction; 10F: ten flower extraction; 1F: single flower extraction;

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394 samples in Northland/Auckland, the Nelson region and Northern New South Wales respectively
395 and Jack Hobbs and the staff of Auckland Botanic Gardens for allowing access to their collection
396 of cultivars and for assistance with identification of *L*. 'Martinii' and other cultivars. SW and JK
397 were supported by TechNZ MSc Scholarships during the course of this work.

398 **Supporting Information Available:** complete information on location and type of trees
399 sampled, details of experimental procedures and results for investigation of other factors that
400 might influence DHA/Tsugar are given in Supporting Information. This information is available
401 free of charge via the Internet at http://pubs.acs.org.

402 **List of Tables/Figures in Supporting Information**

403 **Table S1.** Sample name, variety or cultivar and location of mānuka trees included in the survey

404 **Table S2.** Mean and range of the four triketone components in the leaf oil for the different
405 regions studied

406 **Figure S1.** Concentration of fructose versus the concentration of glucose (per 20 μL of
407 nectar/water extraction)
408 of *L. scoparium* nectar samples collected in 2009-2010.
409 **Figure S2.** Percentage of triketone in leaf oil vs nectar DHA/Tsugar.
410 **Figure S3.** The andromonoecious nature of mānuka: hermaphrodite (upper) and male flowers
411 (lower). The hermaphrodite flower is easily identified by a prominent stigma. Photo credit: John
412 Tyrell 2013
413 **Figure S4.** *L.* 'Red Ensign' flowers at different flowering stages exhibiting a green hypanthium
414 (left) which deepens to red (right). Photo credit: Megan Grainger 2014

415 **Figure S5.** The DHA/Tsugar of mānuka flowers with either a green or red hypanthium. VB001
416 is *L.* 'Martinii' whereas VB001 and VB015 are *L.* 'Nanum Tui' (Error bars are standard error
417 vales). The dotted line represents the high DHA/Tsugar (0.00200 mg/mg) classification.

418 **Figure S6.** Nectar DHA/Tsugar related to sooty mold coverage of tree.

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1 **Figure Captions :**

2 **Figure 1.** GC-FID chromatogram of a nectar sample used for dihydroxyacetone quantitation. A
3 is hydroxyacetone used as internal standard; B is dihydroxyacetone.

4 **Figure 2.** GC-FID chromatogram of a nectar sample used for sugar quantitation. The peaks at A
5 are fructose, B and D are glucose and C is mannitol used as an internal standard.

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

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

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] Trees with a significantly different DHA/Tsugar from the original tree at that site
[c] Dihydroxyacetone was below the level of quantitation

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

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

Cultivar Name[a]	Description	DHA/Tsugar
***L.* 'Nanum'**	Pink single-flowered dwarf	NT[b]
L. 'Tui'	Pink-centred 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

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

Species	Sample Number	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

Figure Graphics:

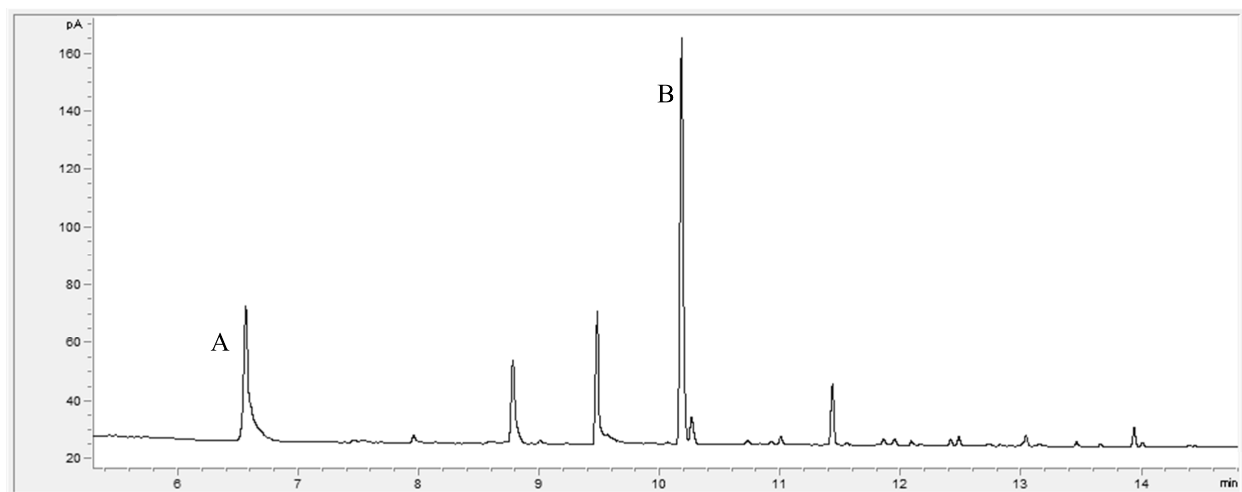

Figure 1.

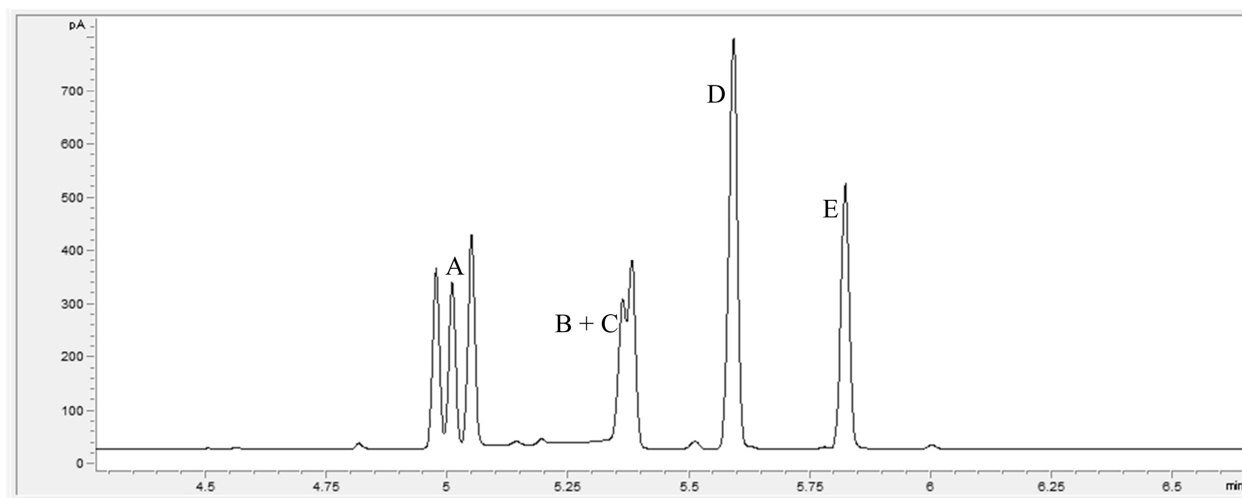

Figure 2.

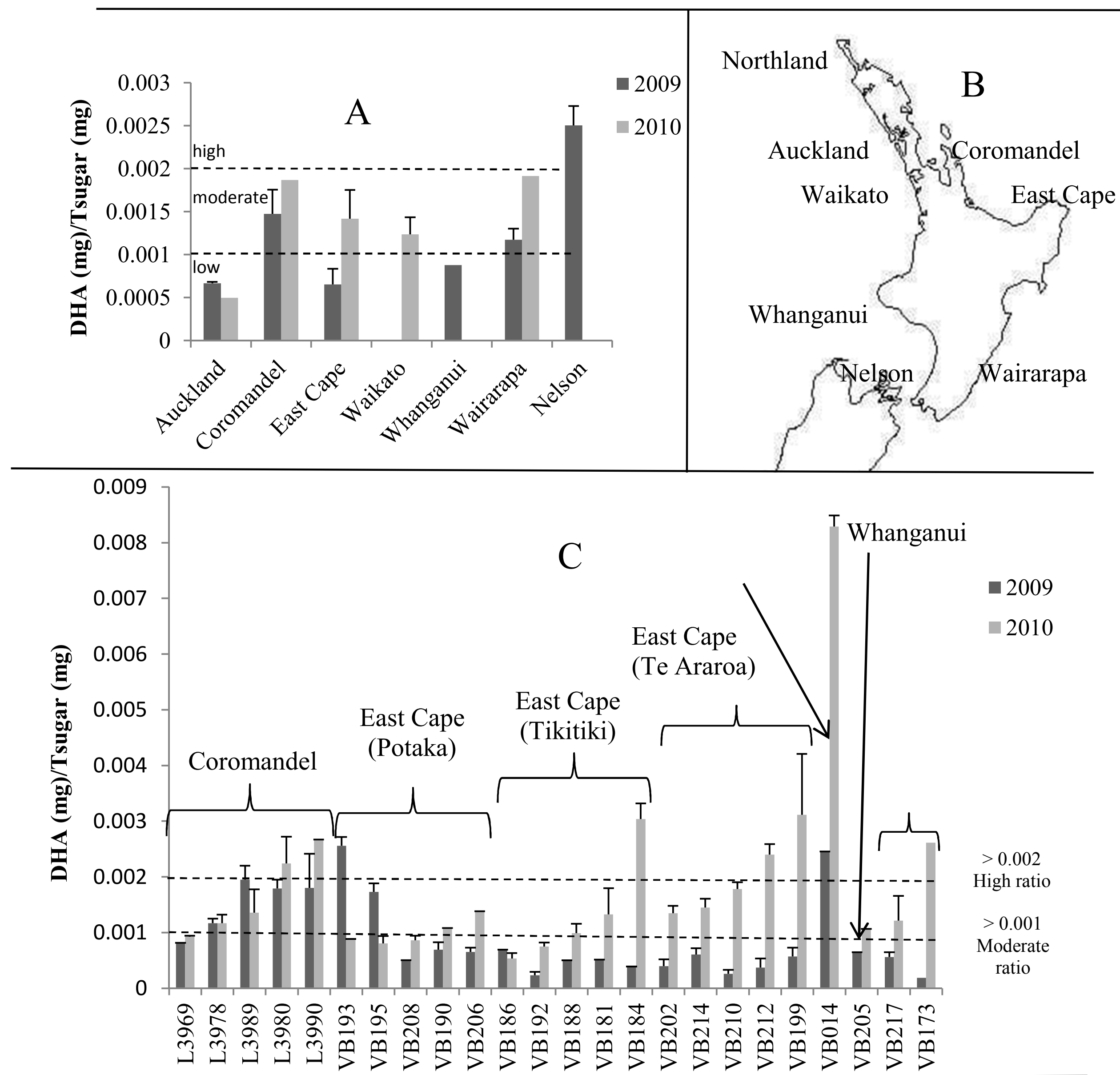

Figure 3.

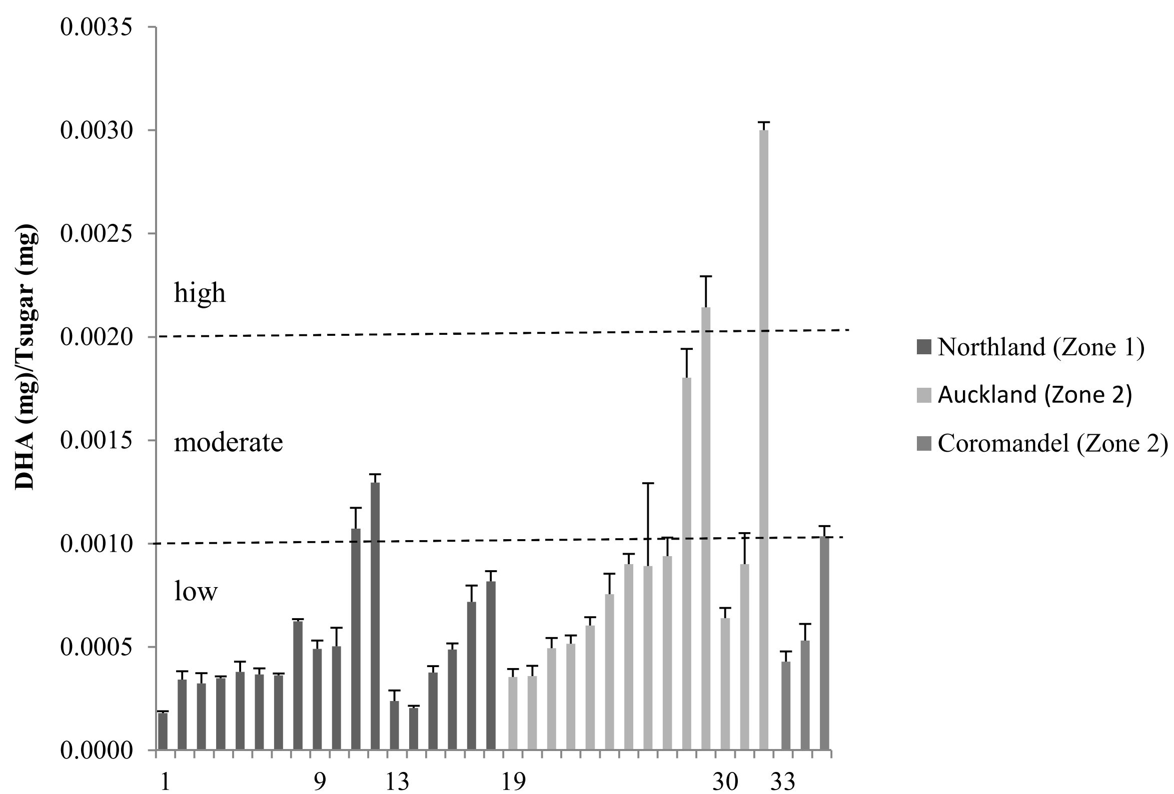

Figure 4.

Table of Contents Graphic: