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Full Length Research Paper

A convenient new analysis of dihydroxyacetone and methylglyoxal applied to Australian *Leptospermum* honeys

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New Zealand mānuka (*Leptospermum scoparium*) honey is known to exhibit non-peroxide antibacterial activity caused by the active ingredient methylglyoxal which arises by chemical conversion of dihydroxyacetone during honey maturation. This study determines whether methylglyoxal and dihydroxyacetone are present in Australian *Leptospermum* honeys. This research developed a rapid and sensitive high-performance liquid chromatographic method for the concurrent analysis of methylglyoxal and dihydroxyacetone in honeys. Both compounds were quantified as their *O*-(2, 3, 4, 5, 6-pentafluorobenzyl) hydroxylamine. HCl derivatives on single run reversed phase high-performance liquid chromatography with diode array detection. Four species of monofloral *Leptospermum* honeys sourced from Northern Rivers Region, New South Wales, Australia contained methylglyoxal and dihydroxyacetone. The highest methylglyoxal concentrations were found in *Leptospermum polygalifolium* honeys.

Key words: High-performance liquid chromatography, dihydroxyacetone; methylglyoxal, *Leptospermum*, honey, mānuka.

INTRODUCTION

Recent increases in bacterial antibiotic resistance have led to a resurgence of interest in the use of honey as an infected wound treatment (Molan, 2006). Clinical trials of topical applications of honey have proven at least as effective in healing leg ulcers (Jull et al., 2008) and infected wounds (Robson et al., 2009) as standard medical treatments. All honeys exert antibacterial effects due to their hygroscopy, mildly acidic pH and generation of hydrogen peroxide when applied to a wound (Cooper and Jenkins, 2009). These three factors contribute to the efficacy of honey as a broad spectrum topical treatment; however honeys derived from New Zealand *Leptospermum* (L.) *scoparium* (mānuka) exhibit non-peroxide antibacterial activity due to the presence of plant-derived factors (Cooper and Jenkins, 2009; George and Cutting, 2007).

The phenolic compounds found in *L. scoparium* honey (Stephens et al., 2010) are believed to exert an anti-inflammatory action while the active non-peroxide antibacterial component is methylglyoxal (MGO) (Mavric et al., 2008; Adams et al., 2008). MGO arises by chemical conversion of dihydroxyacetone (DHA) during honey maturation; the DHA arises in the nectar of the mānuka flower and considerable variation in DHA levels was noted between different plants (Adams et al., 2009). The retail price of mānuka honey is directly dependent on

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Abbreviations: L, Leptospermum; **MGO,** methylglyoxal; **DHA,** dihydroxyacetone; **HPLC,** high-performance liquid chromate graphy; **PFBHA,** O-(2, 3, 4, 5, 6-pentafluorobenzyl) hydroxylamine; **HCl**, hydrochloric acid; **ACN,** acetonitrile; **HA,** hydroxyacetone.

the non-peroxide antibacterial activity that it exhibits and therefore upon the MGO content; thus there is considerable interest in quantifying MGO and its precursor, DHA, in honey. To date, there has been no research into the presence of DHA and MGO in other species of the *Leptospermum* genus.

The determination of underivatised MGO and DHA content of honeys has been conducted by high-performance liquid chromatography (HPLC) using refractive index which was prone to co-eluting interferences (Adams et al., 2009). HPLC ultraviolet analysis (Adams et al., 2008; Mavric et al., 2008) has been performed on *o*-phenylenediamine derivatives of MGO in honey; this afforded cleaner results than HPLC using refractive index analysis (Adams et al., 2009). This type of analysis does not detect monocarbonyl compounds such as DHA.

DHA in self-tanning creams has been derivatised with *O*-(2, 3, 4, 5, 6-pentafluorobenzyl) hydroxylamine. HCl (PFBHA) and analyzed by reversed phase HPLC ultraviolet analysis (Biondi et al., 2007). MGO and other carbonyl compounds also utilized PFBHA derivatisation and analysis of volatile oxime derivatives by gas chromatography (Bao et al., 1998).

This work reports the development of a reversed phase HPLC with diode array detection method for the simultaneous quantitation of PFBHA derivatives of MGO and DHA in *Leptospermum* honeys. This convenient new analysis technique has been applied to 34 Australian honeys. These include four different types of monofloral honey samples from *Leptospermum polygalifolium*, *Leptospermum semibaccatum*, *Leptospermum liversidgei* and *Leptospermum leavigatum* in Northern Rivers Region, New South Wales, Australia. Nine different types of difloral honeys and one type of trifloral honey from this region were also analyzed using the same new technique.

MATERIALS AND METHODS

Origin of honey samples

Honeys were obtained from Tyagarah Apiaries, Tyagarah, New South Wales, Australia, 2841. The honeys were stored in drums below 30°C until arrival at the University of the Sunshine Coast, then kept at 4°C until analysed. Analysis was carried out on honeys that had been allowed to warm to room temperature. Details of floral source and collection date of each honey sample are given in Monofloral honeys are those for which the apiarist observed bees foraging exclusively on the one floral source. Details of floral source and collection date of each honey sample are given in Table 1.

Chemicals

HPLC MilliQ grade water was used in all analyses. HPLC Chromasolv gradient grade acetonitrile (ACN) was obtained from Merck, Kilsyth, Victoria, Australia, 3137. Hydroxyacetone (HA) (90%), *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine. HCl (PFBHA) (99%), dihydroxyacetone (DHA) (dimer, 97%) and methylglyoxal (MGO) (40%) were purchased from Sigma-Aldrich, Castle Hill, New South Wales 1765, Australia.

HPLC conditions

Analyses were performed on a Perkin Elmer Series 200 Pump and Autosampler with a Flexar photo diode array detector (λ = 263 nm). HPLC separations were performed on a Synergi Fusion column (75 × 4.6 mm, 4 μm particle size).

Mobile phase A was water: ACN, 70/30, v/v and mobile phase B was 100% ACN. The following 23 min gradient elution was employed: A:B = 90:10 (isocratic 2.5 min), graded to 50:50 (8.0 min), graded to 0:100 (1.0 min), 0:100 (isocratic 7.0 min), graded to 90:10 (1.0 min), 90:10 (isocratic 4.0 min), detection at 263 nm.

Preparation of standards

HA (3.01 mg/ml) formed the HA internal standard solution. The PFBHA derivatising reagent was 19.8 mg/ml in citrate buffer (0.1 M) adjusted to pH 4 with NaOH (4M). DHA (3.88 mg/ml) formed the DHA standard solution. MGO (1.21mg/ml) formed the MGO standard solution.

Pure MGO-*bis*-PFBHA standard was prepared by mixing MGO standard with excess PFBHA standard solution and recrystalising from ACN. The derivative was analysed in triplicate and compared with the MGO calibration to assess the actual composition of the Sigma-Aldrich MGO reagent. This was shown to be 40.4%.

Sample preparation

Six standards of clover honey (0.2-0.25 g) and 34 Australian *Leptospermum* honey samples (0.1-0.15 g) were weighed into 16 × 75 mm test tubes. HA standard solution (250 µl) was added to each test tube. For the preparation of standards, DHA standard solution (550, 300, 200, 100, 50 and 0 µl) was added to tubes 1 to 6, respectively. MGO standard solution (0, 50, 100, 200, 300, 400 µl) was also added to tubes 1 to 6, respectively. Each of the 40 test tubes was thoroughly mixed and let stand for 1h to allow complete dissolution. PFBHA derivatising solution (1200 µl) was added to each test tube, which was mixed and let stand for 1hr to allow for complete derivatisation. ACN (6 ml) was added to each test tube and mixed until all crystals dissolved. Water (2 ml) was added to each test tube and mixed. A 1.5 ml aliquot of each sample was placed in an HPLC vial for analysis.

DHA and MGO calibration curves were generated from tubes 1 to 6 by linear regression using the HPLC peak area ratios of DHA: HA plotted against the mass of DHA and MGO: HA plotted against the mass of MGO in the six standards. The DHA and MGO mass content of the 34 test honeys were determined against these calibration curves.

RESULTS

The simultaneous quantitation of PFBHA derivatives of MGO and DHA in Australian honeys has been performed via reversed phase HPLC with diode array detection in this study. Figure 1 shows the HPLC separation for Sample number 12. On the basis of six concentration standards the MGO content calibration curve was constructed with an R^2 value of 0.999. The linear

Table 1. MGO and DHA content of honey samples.

Sample	Species	Extraction date	MGO (mgper kg honey)	DHA (mgper kg honey)
1	*L. polygalifolium / L. liversidgei / Guioa semiglauca*[a]	No date	331	522
2	*L. polygalifolium*	11/2005	1723	1185
3	*L. polygalifolium*	11/2005	1567	1310
4	*L. polygalifolium / Guioa semiglauca*[a]	11/2005	614	564
5	*L. polygalifolium*	12/2005	347	530
6	*L. polygalifolium / L. semibaccatum*	01/2006	553	412
7	*L. liversidgei / Corymbia intermedia*[b]	04/2006	303	441
8	*L. polygalifolium*	11/2006	474	523
9	*L. polygalifolium*	12/2006	820	816
10	*L. liversidgei / Corymbia intermedia*[b]	05/2007	344	637
11	*L. polygalifolium / Guioa semiglauca*[a]	12/2007	755	1130
12	*L. polygalifolium*	11/2008	1210	2403
13	*L. semibaccatum*	11/2008	452	1204
14	*L. polygalifolium / Lomandra longifolia*[c]	12/2008	663	1265
15	*L. polygalifolium / Guioa semiglauca*[a]	12/2008	332	725
16	*L. polygalifolium / Eucalyptus spp.*[b]	01/2009	264	910
17	*L. polygalifolium / L. liversidgei*	02/2009	416	929
18	*L. polygalifolium / L. liversidgei*	03/2009	894	1690
19	*L. polygalifolium / Corymbia intermedia*[b]	03/2009	673	1247
20	*L. polygalifolium / L. liversidgei*	04/2009	789	1527
21	*L. liversidgei / Corymbia intermedia*[b]	04/2009	455	973
22	*L. liversidgei*	05/2009	459	1310
23	*L. liversidgei*	05/2009	463	1333
24	*L. liversidgei*	08/2009	417	740
25	*L. leavigatum*	10/2009	140	702
26	*L. trinervia / Guioa semiglauca*[a]	10/2009	56	423
27	*L. trinervia / Guioa semiglauca*[a]	10/2009	43	791
28	*L. speciosum / L. semibaccatum*	11/2009	248	783
29	*L. speciosum / L. semibaccatum*	11/2009	174	1456
30	*L. polygalifolium / Guioa semiglauca*[a]	11/2009	125	1521
31	*L. polygalifolium / Guioa semiglauca*[a]	11/2009	84	1304
32	*L. polygalifolium / Guioa semiglauca*[a]	11/2009	56	1036
33	*L. liversidgei*	07/2010	289	1149
34	*L. liversidgei*	07/2010	179	908

[a]FamilySapindaceae ;[b]FamilyMyrtaceae; [c]FamilyLaximaniaceae

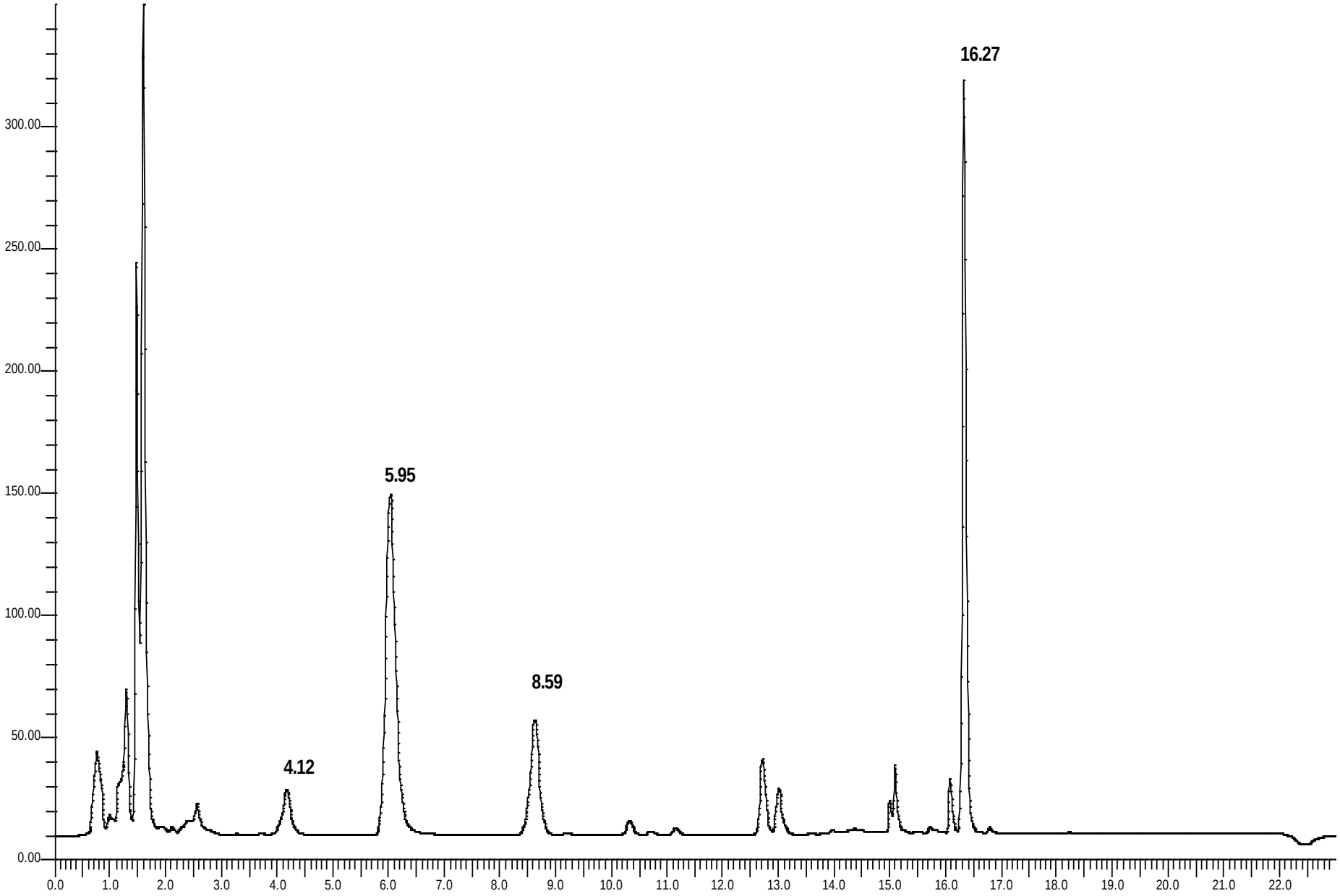

Figure 1. Reversed phase HPLC separation of honey sample #12. The elution times of DHA, PFBHA, HA and MGO are 4.12, 5.95, 8.59 and 16.27 min, respectively.

relationship between the MGO: HA peak area ratio and mass of MGO (mg) is given by Equation 1:

$$\text{MGO : HA (peak area)} = 19.598 \times \text{mass MGO (mg)} + 0.247 \quad (1)$$

On the basis of six concentration standards the DHA content calibration curve was constructed with an R^2 value of 0.999. The linear relationship between the DHA: HA peak area ratio and mass of DHA (mg) is given by Equation 2:

$$\text{DHA : HA (peak area)} = 1.329 \times \text{mass DHA (mg)} + 0.050 \quad (2)$$

The MGO and DHA content and extraction date of 34 Australian honey samples are displayed in Table 1.

DISCUSSION

During preparation, standards required 15min for complete MGO derivatisation, while *Leptospermum* honey samples required 1h for both complete dissolution and derivatisation. This suggests a crosslinking of native MGO in *Leptospermum* honeys that required hydrolysis for complete dissolution and derivatisation. Also during sample preparation excess PFBHA (1200 µl) was added to allow for complete derivatisation. Small or no excess of PFBHA reagent compromises complete derivatisation.

The useable linear range of MGO content from analysis of *o*-phenylenediamine derivatives (50-900 mg MGO per kg honey) (Adams et al., 2008) has been extended significantly in this study from analysis of PFBHA derivatives (20-1800 mg per kg honey). The preparation time has also been reduced from 12 to 16h (Mavric et al., 2008; Adams et al., 2008) to 2 h.

The results in Table 1 indicate clearly that elevated levels of MGO and its precursor DHA are present in honey from representatives of six *Leptospermum* species (*L. polygalifolium*, *L. liversidgei*, *L. semibaccatum*, *L. leavigatum*, *L. trinervia*, *L. speciosum*) and blends with other genera; the lowest level observed is 43 mg MGO per kg honey from one of these blends. In contrast Mavric et al. (2008) found that MGO levels in conventional honeys did not exceed 5 mg MGO per kg honey. It can be concluded that the phenomenon of over production of DHA with resultant development of MGO is present at the genus level and is not restricted to mānuka (*L. scoparium*). Further research into this phenomenon across the other 77 *Leptospermum* species is warranted. It seems likely that the DHA and MGO which were observed in the difloral honeys with other genera is derived from the *Leptospermum* species and the DHA and MGO amounts are probably indicative of the proportion of the *Leptospermum* species that is present. For the purposes of this discussion the MGO content can be separated into three broad levels: low (0-500 mg MGO per kg honey), medium (500-1000 mg MGO per kg honey) and high (>1000 mg MGO per kg honey).

Likewise, DHA content can be divided into three ranges: low (0-1000 mg DHA per kg honey), medium (1000-1500 mg DHA per kg honey) and high (>1500 mg DHA per kg honey). Samples number 2, 3 and 12 containing the monofloral honey sourced from *L. polygalifolium* are the only samples observed to have a high MGO content. Sample number 12 also displays a high DHA content. These results might indicate that *L. polygalifolium* has the greatest over production of DHA, with resultant development of MGO in the honey, but caution should be exercised as the factors which contribute to this could include environmental as well as genetic effects. A comparison of the six *L. polygalifolium* monofloral honeys, Samples number 2, 3, 5, 8, 9 and 12, reveals a possible environmental influence on MGO and DHA content. Analysis of the MGO content of these *L. polygalifolium* monofloral honey samples reveals: two samples in the low range; one sample in the medium range; and three in the high range. Analysis of the DHA content of these *L. polygalifolium* monofloral honey samples reveals: three samples in the low range; two samples is the medium range; and one sample in the high range.

Adams et al. (2009) reported a time dependent chemical conversion of DHA in maturing honeys to MGO. The data for MGO: DHA ratios in this study are consistent with that finding, displaying a steady proportional increase in MGO: DHA in older honey. The MGO: DHA ratio was ~1 for the average of the honey samples collected in 2005 to 2006. This ratio decreased to ~0.5 for the average of the honey samples extracted in 2007 to 2008. A further decrease in this ratio was observed in the honey samples collected in 2009 to 2010 with an average MGO: DHA ratio of ~0.25.

This convenient analysis of both DHA and MGO not only shows the current level of MGO, but also the potential of honeys to develop high levels of MGO in the future. Eight of the seventeen samples extracted in 2009, Samples number 16, 17, 21 and 24 to 28 currently have low DHA content and thus can only mature into low MGO content samples. However, the other nine samples collected in 2009, Samples number 18 to 20, 22, 23 and 29 to 32, with currently medium or high DHA content have the potential to mature into medium or high MGO content samples. It is these latter nine samples that are of interest to honey producers considering maturing honeys for the medicinal market.

The antibacterial activity of *L. scoparium* honey was found to correlate strongly to its MGO levels (R^2 = 0.92) (Adams et al., 2008). Analogously, from the MGO data obtained during this study, inferences can be made about the antibacterial activity of Australian *Leptospermum* honeys. Investigation into the total and non-peroxide activities of these Australian *Leptospermum* honeys is currently underway.

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