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...
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 α-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 monocabonyl 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 iberidgiei 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%), dihydroxycetone (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 (1.21 mg/ml) formed the MGO standard solution.

Pure MGO-bis-PFBHA standard was prepared by mixing MGO standard with excess PFBHA standard solution and recrystallising 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 1 hr to allow for complete derivatization. PFBHA derivatising solution (1200 µl) was added to each test tube, which was mixed and let stand for 1 hr to allow for complete derivatization. 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 mass and MGO mass plotted against the mass of DHA and MGO: HA plotted against the mass of DHA and MGO: HA plotted against the mass of DHA and MGO.

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 R² value of 0.999. The linear
Table 1. MGO and DHA content of honey samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Species</th>
<th>Extraction date</th>
<th>MGO (mg per kg honey)</th>
<th>DHA (mg per kg honey)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L. polygalifolium / L. liversidgei / Guioa semiglauca&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No date</td>
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<td>522</td>
</tr>
<tr>
<td>2</td>
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<td>4</td>
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<td>11/2005</td>
<td>614</td>
<td>564</td>
</tr>
<tr>
<td>5</td>
<td>L. polygalifolium</td>
<td>12/2005</td>
<td>347</td>
<td>530</td>
</tr>
<tr>
<td>6</td>
<td>L. polygalifolium / L. semibaccatum</td>
<td>01/2006</td>
<td>553</td>
<td>412</td>
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<tr>
<td>7</td>
<td>L. liversidgei / Corymbia intermedia&lt;sup&gt;b&lt;/sup&gt;</td>
<td>04/2006</td>
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<td>8</td>
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<td>474</td>
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<td>9</td>
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<td>15</td>
<td>L. polygalifolium / Guioa semiglauca&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12/2008</td>
<td>332</td>
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<td>10/2009</td>
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<td>791</td>
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<tr>
<td>28</td>
<td>L. spectus / L. semibaccatum</td>
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<td>L. spectus / L. semibaccatum</td>
<td>11/2009</td>
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<td>11/2009</td>
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<td>07/2010</td>
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<td>07/2010</td>
<td>179</td>
<td>908</td>
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</tbody>
</table>

<sup>a</sup>Family Sapindaceae; <sup>b</sup>Family Myrtaceae; <sup>c</sup>Family Laximanniaceae
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:

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

(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:

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

(2)

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

DISCUSSION

During preparation, standards required 15 min for complete MGO derivatisation, while Leptospermum honey samples required 1 h 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 16 h (Mavric et al., 2008; Adams et al., 2008) to 2 h.

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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