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Note

The origin of methylglyoxal in New Zealand manuka (*Leptospermum scoparium*) honey

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ABSTRACT

Methylglyoxal in New Zealand manuka honey has been shown to originate from dihydroxyacetone, which is present in the nectar of manuka flowers in varying amounts. Manuka honey, which was freshly produced by bees, contained low levels of methylglyoxal and high levels of dihydroxyacetone. Storage of these honeys at 37 °C led to a decrease in the dihydroxyacetone content and a related increase in methylglyoxal. Addition of dihydroxyacetone to clover honey followed by incubation resulted in methylglyoxal levels similar to those found in manuka honey. Nectar washed from manuka flowers contained high levels of dihydroxyacetone and no detectable methylglyoxal.

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Honey derived from the manuka tree (*Leptospermum scoparium*) often exhibits antibacterial activity (UMF) that is unrelated to the content of hydrogen peroxide, which is responsible for the antibacterial activity of other honeys. This unique activity of manuka honey is due to the presence of methyl glyoxal,[1,2] the origin of which has not so far been determined, although it is well known among beekeepers that non-peroxide activity and thus methylglyoxal increase with storage. Methylglyoxal must therefore be formed in honey by chemical or enzymatic means. Methylglyoxal and its relationship to glucose metabolism has been the subject of recent review;[3,4] a variety of pathways, both enzymatic and non-enzymatic, lead to the production and degradation of methylglyoxal in living tissues.

The non-enzymatic conversion of trioses to methylglyoxal was first observed in the mid twentieth century. The kinetics of the acid-catalysed transformation of trioses has been investigated;[5] and it was demonstrated that in aqueous acetic acid both D,L-glyceraldehyde and dihydroxyacetone dehydrated irreversibly to methylglyoxal. Polyvalent anions including phosphate are reputed to catalyse the conversion of dihydroxyacetone to methyl glyoxal[6] although this result has been queried due to the possible artefactual production of methylglyoxal by the derivatisation procedure used in detection.[7] Methylglyoxal has been postulated as an intermediate in the Maillard reaction between glycine and dihydroxyacetone in a computational study,[8] this is perhaps indicative of a methylglyoxal intermediate in the reaction of dihydroxyacetone with other amino acids and proteins. In addition to the non-enzymatic conversions mentioned above, methylglyoxal may be formed directly from dihydroxyacetone–phosphate by the action of the enzyme methylglyoxal synthase (E.C. 4.2.99.11).[3]

During the course of the activity-guided isolation of methylglyoxal from manuka honey[1] a second peak, which eluted after the methylglyoxal at ∼24 min, was observed in the HPLC trace (Fig. 1). This peak did not exhibit any non-peroxide antibacterial activity and was not observed in a number of samples of honey from floral sources other than manuka. The peak was isolated and shown to be dihydroxyacetone (1,3-dihydroxypropanone) by NMR and by comparison with a standard in HPLC. The area of this peak showed a linear correlation to UMF or non-peroxide antibacterial activity ($R^2 = 0.64$) albeit somewhat less strong than the correlation of methylglyoxal to UMF.[1] Given the chemical relationship between the two trioses, this was an indication of the possible origin of the methylglyoxal.[5]

Nine samples of freshly produced manuka honey from various sites (Table 1) were obtained from the comb soon after deposition by the bees and before prolonged ripening had occurred. Methylglyoxal was found only at low levels (139–491 mg/kg honey) in the samples of freshly produced honey, however dihydroxyacetone was present in varying quantities (1192–5099 mg/kg honey) in all the samples. Upon storage of the freshly produced honey at 37 °C, dihydroxyacetone was seen to decrease and methylglyoxal was seen to increase (Fig. 2a and b).

When dihydroxyacetone was added to clover honey and the honey stored at 37 °C a similar formation of methylglyoxal and disappearance of dihydroxyacetone occurred (Fig. 3a and b). The lack of mass balance between the two sets of curves is probably indicative of side reactions of dihydroxyacetone to produce other

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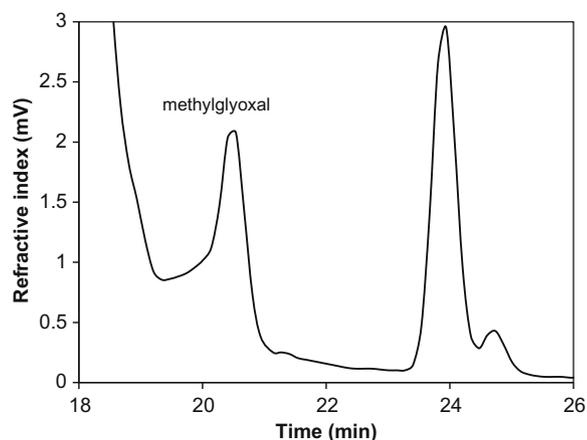
Figure 1. HPLC chromatogram of a UMF 30⁺ manuka honey: expanded view to show methylglyoxal and the peak at ∼24 min, which was isolated.

Figure 2. (a) Disappearance of dihydroxyacetone (DHA) in freshly produced honeys with time of storage at 37 °C. (b) Appearance of methylglyoxal (MGO) in freshly produced honeys with time of storage at 37 °C.

products, for example, glycolate; the latter reaction occurs in the presence of hydrogen peroxide.[9] Alternatively, methylglyoxal may be consumed by reaction with protein or hydrogen peroxide[10] in the honey. An artificial honey was created, which comprised appropriate quantities of sucrose, fructose and glucose adjusted to pH 3.8 with acetic acid; addition of dihydroxyacetone, at a level similar to that of the freshly produced honey M165, resulted, after storage, in some conversion to methylglyoxal, but at a reduced rate compared to clover honey. It is likely that, in the complex natural honey matrix, there exist possibilities for catalysis or alternative reaction routes involving amine groups.[6,8]

Addition of methylglyoxal to clover honey followed by incubation at 37 °C yielded little change in methylglyoxal concentration and no formation of dihydroxyacetone, which is expected given the lack of reversibility of the reaction by which methylglyoxal is produced from dihydroxyacetone.[5] A similar addition of D,L-glyceraldehyde to clover honey yielded methylglyoxal with results comparable with those of the dihydroxyacetone, however no dihydoxyacetone was formed. This concurs with the observation that there is no mutual isomerisation of the two trioses in acidic media.[5] D,L-Glyceraldehyde was not observed in the freshly produced honeys.

When dihydroxyacetone was added to clover honey together with the amino acids arginine or lysine, so that the added amino acid content was 0.62%, methylglyoxal formed more rapidly than when dihydroxyacetone alone was added and then began to disappear gradually; both processes were greatly accelerated when the added amino acid content was increased to 2.5% (Fig. 4). White and Rudyj[11] found protein contents ranging from 0.058% to 0.786% in 740 samples of honey, so it seems unlikely that loss of methylglyoxal due to reaction with protein is other than gradual; however this result confirms that the presence of proteins and or

Table 1
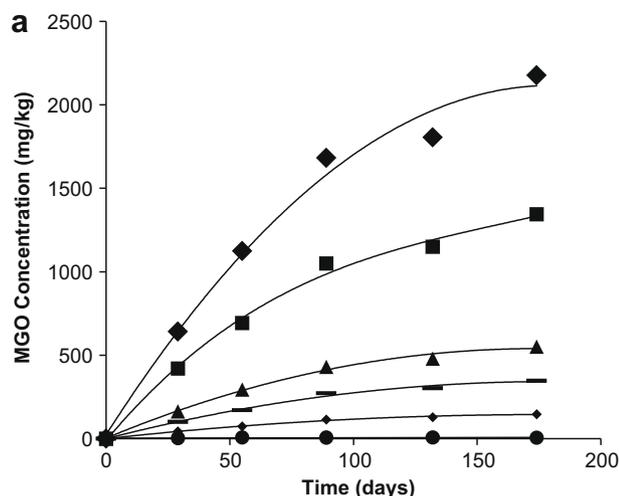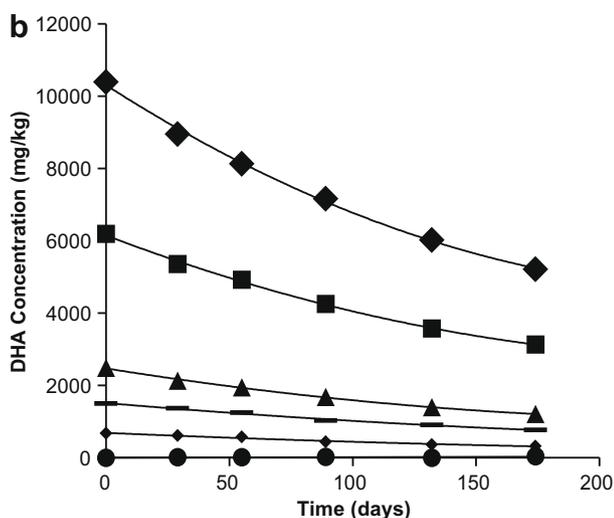Origin of freshly produced manuka honey samples

Code no.	Area
M161	Ahipara-Herekino, Northland
M162	Taemaro Mangonui, Northland
M163	Herekino, Northland
M165	Riversdale, South Wairarapa
M169	Riversdale, South Wairarapa
M177	Riversdale, South Wairarapa
M189	Tora, South Wairarapa
M197	Tiraumea, Eketahuna, Wairarapa
M212	Whangamarino Swamp, Waikato
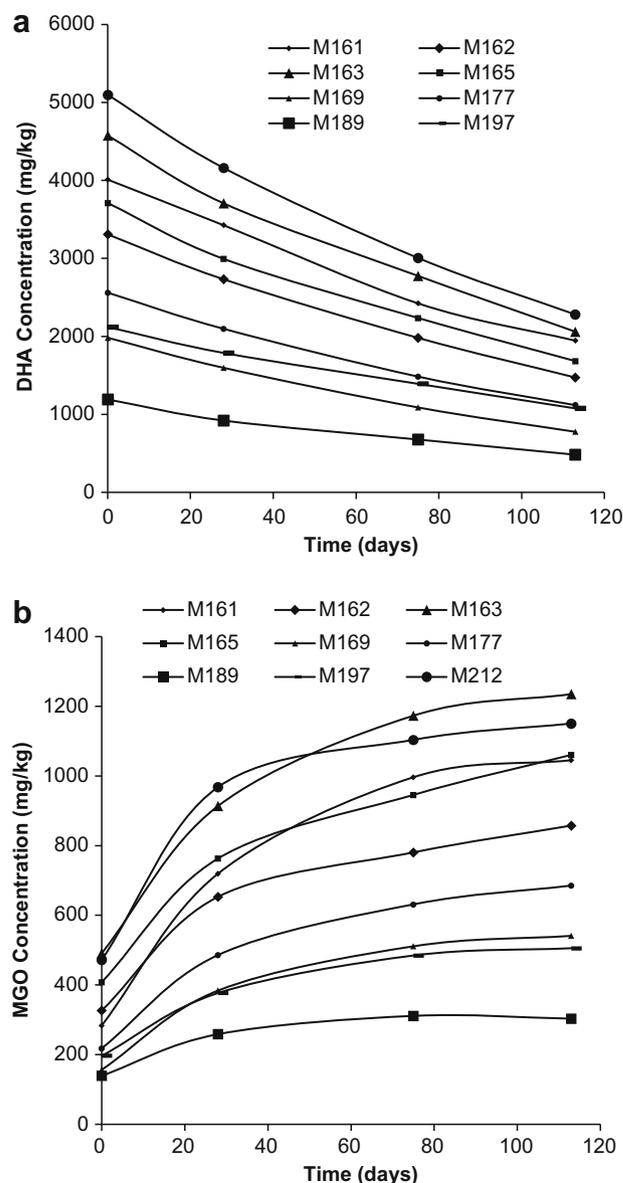
amino acids can accelerate conversion of dihydroxyacetone to methylglyoxal. Since the conversion of dihydroxyacetone to methylglyoxal is non-enzymatic it was anticipated that heating the honey would result in accelerated conversion, however at temperatures of 50 °C and above there was loss of both dihydroxyacetone and methylglyoxal compared to incubation at 37 °C, accompanied by a sharp, tenfold or greater increase in the hydroxymethylfurfural content.

Nectar samples were obtained by washing manuka flowers with water. A small selection of manuka trees from Northland and from the central Waikato area around Hamilton was sampled; this selection included some pink-flowered garden cultivars. Additionally kanuka (*Kunzea ericoides*) flowers were tested; kanuka and manuka trees commonly grow together and, although the latter flower earlier, kanuka nectar may be collected by bees that have previously collected manuka nectar if the hives are not moved immediately after the manuka ceases to flower. All the manuka nectars contained dihydroxyacetone but in varying amounts as
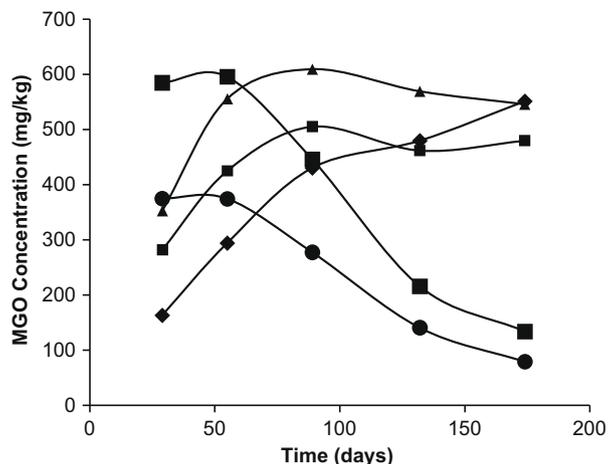
a

♦ 10400 mg/kg DHA ■ 6190 mg/kg DHA ▲ 2490 mg/kg DHA

— 1500 mg/kg DHA ♦ 680 mg/kg DHA ● 0 mg/kg DHA

b

♦ 10400 mg/kg DHA ■ 6190 mg/kg DHA ▲ 2490 mg/kg DHA

— 1500 mg/kg DHA ♦ 680 mg/kg DHA ● 0 mg/kg DHA

Figure 3. (a) Methylglyoxal concentrations (mg/kg) over time for a series of clover honey samples incubated at 37 °C in the presence of various initial concentrations of dihydroxyacetone (DHA). (b) Dihydroxyacetone concentrations (mg/kg) over time for a series of clover honey samples incubated at 37 °C in the presence of various initial concentrations of dihydroxyacetone.

→♦ 2490 mg/kg DHA   →■ 2.6 g/kg DHA + 6.2 g/kg Arg

→▲ 2.6 g/kg DHA + 6.2 g/kg Lys   →■ 2.6 g/kg DHA + 25 g/kg Lys

→● 2.6 g/kg DHA + 25 g/kg Arg

Figure 4. Formation of methylglyoxal in clover honey to which dihydroxyacetone and the amino acids lysine (Lys) and arginine (Arg) have been added.

We conclude that the methylglyoxal in New Zealand manuka honey is derived by the non-enzymatic conversion of dihydroxyacetone which occurs at high levels in the nectar for reasons which are as yet unknown. It would appear, from the limited survey conducted in this study, that there is variation in the amount of dihydroxyacetone in the nectar and that certain manuka trees have the potential to produce honeys with high non-peroxide antibacterial activity, whereas others do not. There is therefore a possibility for selective breeding to produce trees that have high levels of dihydroxyacetone in the nectar, should that be desired.

1. Experimental

1.1. Materials

Commercially available Comvita UMF®30⁺ manuka honey was used for the isolation of dihydroxyacetone. Clover (*Trifolium* spp.) honey was supplied by the New Zealand Honey Food and Ingredient Advisory Service of the National Beekeepers' Association of N.Z. (Inc.). Dihydroxyacetone, methylglyoxal (40% w/v in water), D,L-glyceraldehyde, L-arginine and L-lysine were purchased from Sigma–Aldrich.

Samples of manuka honey freshly produced by the bees were obtained from various sites in the North Island of New Zealand. These samples were kindly provided by Watson & Son Ltd. and Waitemata Honey Co. Ltd. The points of origin of these samples are given in Table 1.

1.2. Methods

1.2.1. High performance liquid chromatography (HPLC)

HPLC was carried out as described previously.[1] Dihydroxyacetone concentrations were determined from the RI signal compared to an external calibration curve. In this system methylglyoxal, dihydroxyacetone, D,L-glyceraldehyde and hydroxymethylfurfural elute at 20.4, 23.6, 18.8 and 54.5 min, respectively.

1.2.2. Nuclear magnetic resonance spectroscopy (NMR)

NMR in D_2O was carried out as described previously.[1]

best could be judged by normalisation of the chromatograms, which was achieved by setting the glucose peaks to the same height. The largest quantities were observed in the pink cultivars. A very approximate idea of the concentration of dihydroxyacetone in one of the pink cultivars was obtained by comparing the ratios of the areas of the dihydroxyacetone to that of the sugars in the nectar with a similar ratio in clover honey to which a known amount of dihydroxyacetone had been added; this crude calculation afforded a value for dihydroxyacetone in the nectar as equivalent to ~13,600 mg/kg in honey, which is more than double the largest value observed in the freshly produced honeys. None of the nectars contained measurable amounts of methylglyoxal. The kanuka nectar did not display dihydroxyacetone; although a peak eluted close to the relevant region of the chromatogram, the UV spectrum of this peak did not match that of dihydroxyacetone.

1.2.3. Measurement of antibacterial activity

Non-peroxide antibacterial activity (UMF) was tested as previously described.[1]

1.2.4. Isolation of dihydroxyacetone

Honey (0.50 g) was dissolved in HPLC grade water (1.0 mL) and fractionated by HPLC. The peak eluting at 23.6 min was collected. ^{13}C NMR (100.6 MHz, D_2O): δ_C dihydroxyacetone 214.5 (C=O), 67.3 ($-CH_2OH$), dihydroxyacetone monohydrate 97.3 (HO–C–OH), 66.3 ($-CH_2OH$).

1.2.5. Collection and extraction of nectar

Flowers of manuka and kanuka (K. ericoides) were collected after at least two days without rain. Herbarium samples were identified and collected by Ms Toni Cornes, keeper of the University of Waikato Herbarium (WAIK); voucher specimens have been deposited in the herbarium. Nectar was obtained by soaking the flowers (loosely packed volume ~500 mL) in Milli-Q water (500 mL, room temp, 4 h) followed by evaporation of the water under reduced pressure (35 °C) to ~5 mL for HPLC. Chromatograms were approximately normalised by setting the glucose peaks to the same height.

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