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Identification and quantitative levels of antibacterial components of some New Zealand honeys☆

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Abstract

High performance liquid chromatograms of the phenolic fraction of 19 samples of New Zealand manuka honey, some with high levels of non-peroxide antibacterial activity and some with no such activity, were identical, which indicated that phenolic components of this honey are not responsible for the presence or absence of this activity in manuka honey. Similarly, the result showed that geography does not influence the phenolic composition of manuka honey. Antibacterial bee peptides and the antibacterial β-triketone leptospermone were not detected in manuka honey. Methyl syringate constituted approximately 70% w/w of the phenolic fraction of manuka honey and can be regarded as a floral marker for this honey. High performance liquid chromatographic profiles of the phenolic components of manuka, heather, clover and beech honeydew honeys were significantly different and could be used to differentiate honeys if they can be shown to be as consistent as those of manuka honey. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

In a survey of unifloral New Zealand honeys, Allen et al. (1991) found several which exhibited non-peroxide antibacterial activity. The greatest activity was observed in manuka honey, a very popular and economically important product derived from the native New Zealand "manuka" tree *Leptospermum scoparium* (Myrtaceae). However, not all manuka honey exhibits non-peroxide antibacterial activity. Instead, the bioactivity is recorded in manuka honey only from specific localities (Molan, 1995), particularly the East Cape region of the North Island of New Zealand.

Manuka honey contains a number of aromatic acids (Russell, Molan, Wilkins & Holland, 1990) of which syringic acid and phenyllactic acid are the most abundant (Wilkins, Lu & Molan, 1993). Recently we described the identification of some phenolic acids and flavonoids in bioactive manuka honey (Weston, Mitchell & Allen, 1999). Phenolic acids and flavonoids, particularly those derived from propolis, exhibit weak antibacterial activity (Marcucci, 1995) and we believed that different absolute levels of these constituents might explain why some manuka honey, especially that from the East Cape region of New Zealand, exhibits non-peroxide antibacterial activity and why manuka honey from most other regions of the country, along with nearly all other honeys, does not possess this property (Allen, Molan & Reid, 1991). Another unique feature of Eastland manuka is discussed below in Section 3.2.

Much work has been published by a Spanish group on the use of chromatographic profiles of both flavonoids and phenolic acids to relate honeys to geographical origin and floral source (Andrade, Ferreres & Amaral, 1997; Ferreres, Ortiz, Silva, Garcia-Viguera, Tomás-Barberán & Tomás-Lorente, 1992; Tomás-Barberán, Ferreres, Garcia-Viguera & Tomás-Lorente, 1993). This approach appealed to us as a potential means of distinguishing manuka honey with non-peroxide antibacterial activity, from inactive manuka and other honeys in New Zealand and whether a unique floral marker was responsible for that activity. Preliminary results of this work (see title footnote) indicated that there were no differences between any manuka honeys using the profiles of phenolic

☆ Preliminary results of this work were presented in a poster (paper 65) at the Apimondia99 congress of the International Federation of Beekeepers Associations, Vancouver, September 1999.

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components and, in fact, manuka honey was homogeneous with respect to these constituents. More comprehensive results of that work are reported in this paper. A search for other products, which conceivably might contribute to the non-peroxide antibacterial activity of manuka honey is also described. HPLC profiles of the phenolic components of one sample each of heather, clover and beech honeydew honey indicated that different unifloral honeys can be successfully distinguished by this method.

2. Materials and methods

2.1. Materials

Samples of manuka honey were obtained from Professor P.C. Molan, Honey Research Unit, Waikato University, Hamilton; Mr. T. Harvey, Comvita (NZ) Ltd., Te Puke; and W.L. and M. Bennett, SummerGlow Apiaries Ltd., Hamilton. Samples are described as having high or low non-peroxide antibacterial activity, based on the diameter of the area of inhibition of growth in the standard agar well diffusion assay for non-peroxide antibacterial activity, which was carried out by the Honey Research Unit, Waikato University, Hamilton (Allen et al., 1991; Molan & Russell, 1988). Values < 8, no detectable activity; 8–11, low; 12–15, medium; 16–19, high and 20+, exceptional activity.

2.2. Methods

2.2.1. Liquid–liquid extraction of honey

Manuka honey with a high level (18) of non-peroxide antibacterial activity (50 g) was dissolved in water, filtered through glass wool and made up to 230 ml in a liquid–liquid extractor. A solution (5 ml) of 2,4,6-tri-*tert*-butylphenol (8 mg) in ethanol (25 ml) was added as an internal standard and the mixture was extracted continuously with diethyl ether for 20 h. The ethereal solution was filtered and concentrated to dryness under vacuum at 40°C.

2.2.2. Extraction of honey on XAD-2 resin

Amberlite XAD-2 resin (60 g) was soaked overnight in a mixture of water (200 ml) and methanol (200 ml). It was then washed with water and packed into a column (25×2 cm). Manuka honey with a high level (19) of non-peroxide antibacterial activity (236 g) was dissolved in a mixture of water (1l) and concentrated hydrochloric acid (37%, 1 ml) and the solution was filtered slowly through the column, followed by acidified water (pH 2, 100 ml), distilled water (300 ml) and methanol (300 ml). The methanol extract was concentrated under vacuum at 40°C and freeze-dried to afford an extract of 749 mg (0.32%). The recovery of flavonoids with this method is 95% (Ferreres, Tomás-Barberán, Soler, Garcia-Viguera, Ortiz & Tomás-Lorente, 1994).

2.2.3. Extraction of honey with 2-butanol

Manuka honey with a high level (19) of non-peroxide antibacterial activity (200 g) was dissolved in a mixture of water (1l) and concentrated hydrochloric acid (37%, 1 ml) and the solution was extracted three times with 2-butanol (200 ml). The alcoholic extract was concentrated under vacuum at 40°C. It was then dissolved in a small volume of water and freeze-dried to leave an extract of 848 mg (0.42%) (Weston et al., 1999).

2.2.4. Methylation of honey extracts

The honey extracts were dissolved in methanol (2 ml). BF_3:MeOH (14% BF_3; 2 ml) was added and the mixture was heated under reflux for 2.5 h. The solutions were poured into saturated sodium bicarbonate solution and extracted three times with dichloromethane. The organic layer was dried, filtered, concentrated and examined by GC.

2.2.5. Isolation of leptospermone from manuka oil

A mixture of the cyclic β-triketones, leptospermone, isoleptospermone and flavesone was separated from the essential oil of manuka leaves by extraction of an ethereal solution of the oil with 5% aqueous sodium hydroxide (Briggs, Penfold & Short, 1938).

2.2.6. Gas chromatography and mass spectrometry

GC was carried out on a Hewlett-Packard 5890 Series II gas chromatograph with a Hewlett-Packard Ultra-2 glass capillary column containing 5% methylphenylsilicone (25 m×0.2 mm; film thickness, 0.33 μm) and a flame ionisation detector. Helium was used as a carrier gas at 50 ml/min. The column was programmed from 60°C (1 min) to 145°C at 7°C/min, then to 190°C at 2°C/min and finally to 300°C at 10°C/min. Combined GC–MS was performed as above, interfaced to a Hewlett-Packard mass selective detector (5970 Series) which was operated in the scan mode, at 70 eV.

2.2.7. High performance liquid chromatography

The phenolic extract (~40 mg from 20 g honey) was dissolved in MeOH (2 ml) for HPLC, which was carried out on an Alltech Econosil column (250×4.6 mm; C-18; 5 μm particle size) at 35°C with detection at 280 nm. The solvent programme was a combination of those of Ferreres et al. (1994) and Andrade et al. (1997) (Table 1). Phenolic compounds were identified by comparison of chromatographic retention times with those of authentic standards. Quantitative levels were determined from the UV absorption during HPLC and extinction coefficients, which were determined at 280 nm from Beer-Lambert graphs of the standards. The limit of detection was 0.1 μg/100 g honey.

2.2.8. Dialysis of honey

a. Manuka honey with a medium level (11.6) of non-peroxide antibacterial activity (100 g) was added