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Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (*Leptospermum scoparium*) honey

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Abstract—Using HPLC a fraction of New Zealand manuka honey has been isolated, which gives rise to the non-peroxide antibacterial activity. This fraction proved to be methylglyoxal, a highly reactive precursor in the formation of advanced glycation endproducts (AGEs). Methylglyoxal concentrations in 49 manuka and 34 non-manuka honey samples were determined using a direct detection method and compared with values obtained using standard *o*-phenylenediamine derivatisation. Concentrations obtained using both the methods were similar and varied from 38 to 828 mg/kg.

Keywords: New Zealand manuka honey; Bioactive fraction; Methylglyoxal

1. Introduction

Manuka honey is derived from the manuka tree (*Leptospermum scoparium*), a native of New Zealand. All honeys contain peroxide, which imbues them with antibacterial properties but certain manuka honeys exhibit antibacterial activity, which cannot be attributed to the peroxide present[1,2] and which may constitute the major part of the total antibacterial activity. The bioactive component has so far eluded isolation.[3,4] Methylglyoxal has been detected as its quinoxaline adduct by derivatisation with *o*-phenylenediamine (OPD) at relatively high levels in manuka honeys that exhibit non-peroxide antibacterial activity.[5] It has been demonstrated that the bioactivity of methylglyoxal, at the levels at which it is present in the honey, is equivalent to the non-peroxide activity.[5]

Carbohydrates in foods and beverages are susceptible to degradation by processing, cooking and prolonged storage forming reactive 1,2-dicarbonyl compounds, including methylglyoxal, glyoxal and 3-deoxyglucosone (3-DG). The non-enzymatic reactions involving these compounds are collectively referred to as either caramelisation or, if amino-containing compounds are present, Maillard reactions. Traditionally, such processes are considered to be desirable as they confer much of the desirable taste and colour upon cooked foods. Methylglyoxal reacts rapidly with the side chains of arginine, lysine and cysteine residues in proteins to form so-called advanced glycation endproducts (AGEs) and also reacts with amino-containing lipids to form lipoxidation end products (ALEs) as well as with nucleic acids.

Levels of methylglyoxal and other 1,2-dicarbonyls have been determined in a range of foodstuffs and beverages. These include wine,[6] beer,[6] roasted coffee,[7–9] bread,[9] dairy products,[10] soy sauce[8] and lipids.[11] Methylglyoxal has also been measured in cigarette smoke[12] and atmospheric pollutants.[13]

The initial aim of this work was to isolate and characterise the compound(s) responsible for the non-peroxide activity of New Zealand manuka honey. During the course of this work, Mavric et al.[5] reported the presence of large amounts of methylglyoxal in some manuka honey samples which was correlated to its non-peroxide

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antibacterial activity. The reported method of detection involved treating the honey sample with OPD, which reacts with 1,2-dicarbonyl compounds to form the corresponding quinoxaline derivatives that were analysed by HPLC using UV detection. The active HPLC fraction that was obtained in this study was shown to contain the majority of the non-peroxide antibacterial activity and its subsequent identification confirmed that it was methylglyoxal. Methylglyoxal concentrations were determined for 49 manuka and 34 New Zealand non-manuka honey samples using indirect (OPD derivatisation) and direct methods. The direct (HPLC) method involved a mixed mode size-exclusion/ligand exchange separation with refractive index detection. The results obtained from these two methods are compared.

2. Experimental

2.1. Materials

Forty-nine manuka honey samples of various non-peroxide antibacterial activities were kindly supplied by Comvita New Zealand Ltd (TePuke, New Zealand). Thirty-four New Zealand honey samples from certified sources were supplied by the New Zealand Honey Food and Ingredient Advisory Service of the National Beekeepers Association of N.Z. (Inc.); these included samples of Clover (*Trifolium* spp.), Northern Rata (*Metrosideros robusta*), Kamahi (*Weinmannia racemosa*), Vipers Bugloss (*Echium vulgare*), Tawari (*Ixerba brexioides*), Rewarewa (*Knightia excelsa*), Pohutakawa (*Metrosideros excelsa*), Clover/Gum (*Eucalyptus* spp.), Clover/Blackberry (*Rubus fruticosus*), Clover/Catsear (*Hypochaeris radicata*), Beech (*Nothofagus* spp.), Honeydew, Koromiko (*Hebe* spp.), Mingimingi (*Cyathodes juniperina*) and Penny Royal (*Mentha pulegium*). Commercially available Comvita UMF®30^{+} manuka honey (sample 6) was used for isolation of the active fraction.

Methylglyoxal (40% (w/v) in water) and *o*-phenylenediamine were obtained from Sigma–Aldrich, glacial acetic acid (analytical grade) was obtained from Ajax Finechem, phenol (AR grade) from BDH and HPLC grade MeOH from Scharlau. Catalase (C-10, 4000 units mg^{-1}) was obtained from Sigma. HPLC grade water was prepared from a Barnstead E-Pure Water System (18.2 MΩ cm).

2.2. Methods

2.2.1. Measurement of non-peroxide antibacterial activity. Honey solutions were prepared by dissolving honey (2 g) in HPLC grade water (2 mL). This solution (1 mL) was added to catalase solution (1 mL) and assayed immediately. The catalase solution was prepared by addition of catalase (20 mg) to HPLC grade water (10 mL). For low activity honeys (ca. 2% (w/v) equivalent phenol concentration), the honey solution was prepared by dissolving honey (1 g) directly into catalase solution (1 mL). The results obtained from these more concentrated samples were adjusted using an empirically determined factor,[1] in order to be able to compare them with the more dilute samples.

Non-peroxide antibacterial activity was assayed using standard well-diffusion assays on *Staphylococcus aureus* (ATCC 25923) inoculated agar plates.[1] Each sample was applied to four wells per plate. The plates were repeated in triplicate.

The clearance zones obtained were compared with a set of phenol standards (2%, 3%, 4%, 5%, 6%, 7% (w/v) phenol in HPLC grade water). Activity was then expressed as the equivalent phenol concentration (1% w/v). Commercially this value is expressed as UMF® (Unique Manuka Factor).

Fractions obtained from HPLC were concentrated and added to clover honey for assay. The final clover honey concentration was kept the same as above (i.e. equivalent to 1 g honey dissolved in 1 mL water). Catalase solution was also used, as above. Unmodified clover honey exhibited zero non-peroxide activity using the same bioassay.

2.2.2. Nuclear magnetic resonance spectroscopy (NMR). NMR was carried out using a Bruker DRX 400 MHz NMR spectrometer in D_2O referenced to external TMS. ^{1}H, ^{13}C, COSY, HSQC and HMBC experiments were carried out.

^{13}C NMR (100.6 MHz, D_2O): δ_C methylglyoxal monohydrate 211.2 (C=O), 91.8 (CH), 26.5 (CH_3), methylglyoxal dihydrate 97.2 (CCH_3), 94.0 (CH), 23.5 (CH_3).

^{1}H NMR (400 MHz, D_2O): δ_H methylglyoxal monohydrate 5.22 (s, CH), 2.26 (s, CH_3), methylglyoxal dihydrate 4.73 (s, CH), 1.33 (s, CH_3).

2.2.3. High performance liquid chromatography (HPLC). *Sample preparation for the direct method*: The honey sample was dissolved in HPLC grade water (50% (w/v)). The solution was centrifuged in an Eppendorf Centrifuge 5810R at 12,000 rpm (18,500 g) for 1 h. The supernatant was filtered with Dismic Disposable Syringe Filter Units (Cellulose Acetate 0.45 μm (hydrophilic)) and stored at −20 °C prior to HPLC analysis.

Sample preparation for derivatisation method (o-phenylenediamine (OPD) derivatisation of honey samples): Honey (0.6 g) was dissolved in HPLC grade water (30% (w/v)). The honey solution (1.5 mL) was treated with 2% (w/v) OPD in 0.5 M phosphate buffer (0.75 ml, pH 6.5) for 16 h. Reactions were performed in the dark at room temperature. Samples were analysed using HPLC system 3 (below).

2.2.4. HPLC systems. *HPLC was performed using variously three HPLC systems*: HPLC system 1 (preparative HPLC): This consisted of a Waters 515 HPLC Pump, Shodex RI-102 refractive index detector, a Rheodyne 7725i manual sample injector equipped with a 4.5 mL loop and a Waters PrepLC™ Assembly (25 × 100 mm) equipped with three 25 × 100 mm PrepPak® Cartridges (Delta-Pak® C18 15 μm 100 Å). Samples were eluted with HPLC grade water at 10 mL min^{-1}. The system was controlled using Waters Empower™ 2 Chromatography Software.

HPLC system 2 (analytical and preparative HPLC): This consisted of a Waters 515 HPLC Pump, Waters Column Heater Module, Waters 2996 Photodiode Array Detector (PDA), Waters 2414 Refractive Index (RI) Detector, a Rheodyne 7725i manual sample injector and two columns, a Shodex KS-801 and a Shodex KS-802, connected in series and maintained at 50 °C. The system was eluted with HPLC grade water at 1 mL min^{-1}. The system was controlled using Waters Empower™ 2 Chromatography Software. For analytical purposes, a 20 μL sample loop was used and the honey samples (1 g in 2 mL water) were prepared as above. For preparative HPLC a 500 μL sample loop was used, the RI detector was disconnected and fractions were collected directly from the PDA detector's outlet tube.

HPLC system 3 (for OPD derivatised samples): This consisted of a Waters 515 HPLC Pump, Waters 2996 Photodiode Array Detector (PDA), a Rheodyne 7725i manual sample injector (5 μL sample loop) and a Waters SymmetryShield RP18 5 μm 3.0 × 250 mm column. A gradient elution was performed at 0.3 mL min^{-1}. Eluent A was 0.075% acetic acid in water and eluent B was 80% MeOH in water (final solution made up 0.075% acetic acid). Gradient steps were (min, (% B)): 0 (10), 4 (10), 5 (42), 30 (55), 31 (100), 34 (100), 35 (10) and 40 (10). The system was controlled using Waters Empower™ 2 Chromatography Software.

2.3. Location of the non-peroxide antibacterial fraction

A sample of Comvita UMF®30^+ manuka honey (sample 6) (1.0 g) was dissolved in HPLC grade water (2 mL) and prepared as described earlier. This solution (ca. 2.7 mL) was injected (100 μL aliquots) over twenty-seven 50 min runs using HPLC system 2. This injection volume satisfied an acceptable compromise between resolution and the number of runs. Three fractions (0–20, 20–21.5 and 21.5–50 min) were collected, pooled and concentrated. Fraction 1 (0–20 min) contained the majority of the honey and was used directly in the bioassay. Fractions 2 (20–21.5 min) and 3 (21.5–50 min) were collected directly into clover honey solution to make sensible comparisons between the fractions and the original manuka honey sample. The three fractions were reduced in volume (1.7 mL) by a combination of rotary evaporation and drying under a stream of dry nitrogen. A sample of each of these solutions (1 mL) was added to catalase solution (1 mL) and assayed for its non-peroxide antibacterial activity as described above. The original UMF®30^+ manuka and clover honeys were also assayed for comparison. Each sample was tested in eight wells on each of the two agar plates. Aliquots of fractions 1–3 were also analysed using HPLC system 2.

2.4. Isolation of the active fraction

Sample preparation: Comvita UMF®30^+ manuka honey (sample 6) (50 g) was dissolved in HPLC grade water (100 mL). The solution was centrifuged in an Eppendorf Centrifuge 5810R at 12,000 rpm (18,500 g) for 1 h. The supernatant was removed from the pellet and centrifuged a second time under the same conditions. The resultant supernatant was filtered with a Dismic Disposable Syringe Filter Unit (Cellulose Acetate 0.45 μm (hydrophilic)) to give a final volume of 125 mL which was stored at −20 °C prior to HPLC analysis.

The active fraction was isolated using a three-step procedure:[14]

Step 1: Aliquots of the filtered honey solution (50 runs × 2.5 mL) were injected onto HPLC system 1. A fraction from 11.0–30.0 min was collected and pooled. After completion of the runs the pooled fractions were reduced in volume by a combination of rotary evaporation (30 °C) and blowing under a stream of dry nitrogen (35 °C) to a final volume of ca. 5 mL. Because elution was performed with water the column was backwashed with MeOH after every five runs to reverse any packing down effects of the C18 chains, an effect that would potentially reduce resolution.

Step 2: The sample from Step 1 was split into five 1 mL aliquots and re-injected as in Step 1. The same 11.0–30.0 min fraction was collected. The pooled fractions were reduced in volume as in Step 1 to a final volume of ca. 1.8 mL.

Step 3: An aliquot (50 μL) from Step 2 which was sufficient to give a refractive index reading of ca. 45 mV was injected onto HPLC system 2 and a fraction from 19.8–20.8 min was collected. The pooled fractions from the 36 runs were reduced in volume (ca. 1 mL) in the same manner as in Steps 1 and 2 and the sample was stored at −20 °C. To obtain a yield the sample was blown under a stream of dry nitrogen (35 °C) until a constant weight was obtained (13.2 mg). When this sample was dissolved in water and reanalysed using HPLC system 2, only one peak at 20.45 min was observed.

3. Results and discussion

3.1. Location of the non-peroxide antibacterial fraction

Figure 1a shows a typical chromatogram (HPLC system 2) of a manuka honey sample, in this case Comvita UMF®30$^+$ manuka honey (sample 6). The separation was achieved by a pair of mixed mode size exclusion/ligand exchange columns connected in series. Preliminary fractionation of this honey sample yielded three fractions which were tested for their activity. Fraction 1 (0–20 min) contained the bulk of the honey plus some of the peak at 20.45 min. Fraction 2 (20–21.5 min) contained only the peak at 20.45 min and fraction 3 (21.5–50 min) contained the remainder of the sample, including a peak eluting at 23.82 min. Fractions 2 and 3 were collected into clover honey to make sensible comparisons between the fractions and the original manuka honey sample. The results for non-peroxide antibacterial activity of the three fractions are listed in Table 1. Although the major part of the activity is located in fraction 2, fraction 1, which comprised the bulk of the sugar, also contained some of the activity. In the HPLC analyses (HPLC system 2) of fractions 1–3, fraction 1 still contained a relatively large amount of the peak at 20.45 min (Fig. 1b). Fraction 2 contained a larger peak at 20.45 min and fraction 3 contained only the peak at 23.74 min. The peak in fraction 3 displayed zero non-peroxide antibacterial activity.

A series of 49 manuka honey samples of known antibacterial activity were analysed by HPLC system 2. The peak area of the small peak centred at 20.45 min, eluting after the large fructose peak, showed a strong correlation with the non-peroxide antibacterial activity ($R^2 = 0.92$). Thirty-five New Zealand non-manuka honey samples were also analysed using the same HPLC setup. With the exception of the Kamahi samples, most had either no or very small peaks centred at 20.45 min and most demonstrated low or no detectable non-peroxide antibacterial activity. Kamahi samples displayed no detectable activity, so it is likely that there is some interference with the peak at 20.45 min in this honey.

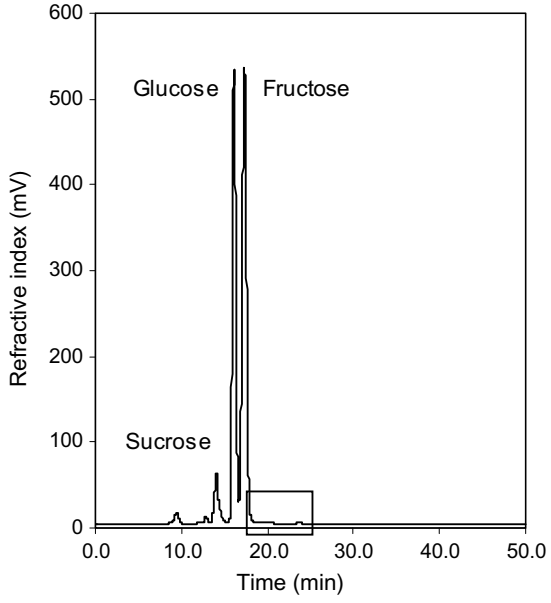

Figure 1a. HPLC (system 2) chromatogram of Comvita UMF®30$^+$ manuka honey (sample 6); box shows the region expanded in Figure 1b.

Table 1. Antibacterial activity of fractions isolated from Comvita UMF®30$^+$ manuka honey (sample 6)

Honey sample/fraction	Activity (equiv % phenol, UMF®)[a]
Fraction 1 (0–20 min)	9.4 ± 0.7
Fraction 2 (20–21.5 min)	19.4 ± 0.6
Fraction 3 (21.5–50 min)	No activity
Comvita UMF®30$^+$ manuka honey(sample 6)	27.0 ± 1.5
Clover honey (entire)	No activity

[a] Mean and standard deviation of 12 determinations.

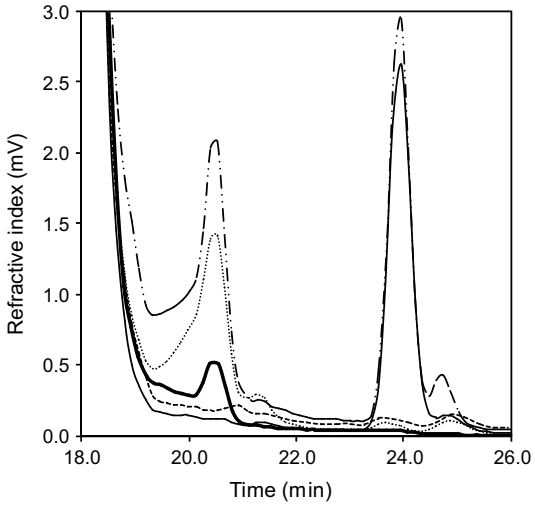

Figure 1b. HPLC (system 2) chromatogram of Comvita UMF®30$^+$ manuka honey (sample 6) (·—··), fraction 1 (bold line), fraction 2 (···), fraction 3 (line) and clover honey (---).

Treatment of the active manuka honey with alkali and titration back to the original pH resulted in irreversible loss of non-peroxide antibacterial activity and disappearance of the peak at 20.45 min.

Prolonged evaporation (high vacuum (ca. 0.02 mbar), 3 weeks) of an aqueous solution of active honey produced no change in the area of the peak at 20.45 min. This result was consistent with the active compound(s) having relatively low volatility.

3.2. Isolation and identification of the active fraction

Isolation of the peak at 20.45 min was achieved by a combination of HPLC systems 1 and 2. Elution of the honey with water over a C18 reversed phase column

(HPLC system 1) was used to remove most of the sugars while retaining a fraction with the majority of the antibacterial activity. The sugars elute early (6–11 min) as one large peak. The slower eluting compounds, including the target compound, were collected as one large fraction from 11–30 min. Using this system no obvious peak was observed which could be ascribed to the target compound. Using water as the eluent results in the target compound eluting from 7–30 min, with a maximum concentration appearing at ca. 14 min. This was determined by taking a series of 1 min fractions (5–30 min) and testing their antibacterial activity as well as injecting a small aliquot of these fractions onto HPLC system 2 and observing the changing peak area at 20.45 min. To remove the major part of the remaining sugars, the pooled 11–30 min fractions were again separated using HPLC system 1 and a fraction from 11–30 min was again collected. The fraction obtained from these two steps was eluted with water using two mixed mode size-exclusion/ligand exchange columns connected in series (HPLC system 2). The peak centred at 20.45 min was collected. Using this setup ca. 360 μg of the target compound per injection was isolated. Figure 2 shows a HPLC trace of a small aliquot of the pooled, isolated fraction. NMR of the isolated fraction was consistent with a mixture of the mono- and dihydrates of methylglyoxal. This identification was confirmed by co-injection of an authentic sample of methylglyoxal into active manuka honey, the peak at 20.45 min increasing in direct proportion to the amount of methylglyoxal added. Also, reaction of the isolated fraction with OPD resulted in one major HPLC peak (HPLC system 3) which co-eluted with the quinoxaline adduct formed by the reaction of commercial methylglyoxal with OPD. The observation of two compounds in the NMR spectrum but only one peak in the HPLC is consistent with an equilibrium between the mono- and dihydrates of methylglyoxal. A total of 13.2 mg of methylglyoxal was isolated from 50 g of Comvita UMF®30^{+} manuka honey (sample 6). This corresponds to ca. 35% yield based on an average of the methylglyoxal concentrations determined by the two analytical methods described. The majority of the losses are expected during the two reversed phase purification steps. A significant amount of methylglyoxal elutes with the sugars in the large early peak (6–11 min). Further methylglyoxal will be lost during the final HPLC step. Significant losses also occur during the various steps of sample preparation, for example, filtration and decanting after centrifugation.

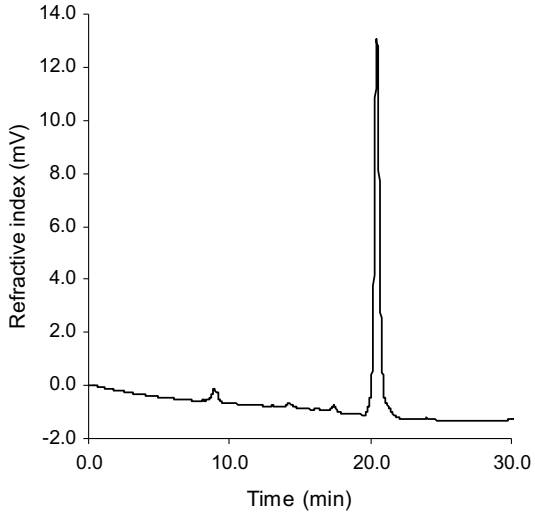

Figure 2. HPLC chromatogram of the isolated active fraction (methylglyoxal) using HPLC system 2.

Methylglyoxal has been previously detected, as its *o*-phenylenediamine derivative, at high levels in active manuka honey;[5] and it was demonstrated that the non-peroxide antibacterial activity of the honey was similar to the antibacterial activity of methylglyoxal at the levels present in the honey. The work described in this study confirms the identity of the active component as methylglyoxal. The precise form that methylglyoxal takes in the honey is unknown as it has been shown that under conditions of dehydration oligomer formation can occur[15] and honey itself is strongly dehydrating. The principal component of concentrated (40% w/v) methylglyoxal by NMR is the monohydrate. It is therefore likely that methylglyoxal is present in honey as the monohydrate and the dihydrate forms upon isolation or dilution. Nemet et al.[15] have shown that solvent, temperature and the amount of available water strongly influence the equilibrium of the different forms of methylglyoxal, therefore changing its reactivity. In D_2O, both the monohydrate (56–62%) and the dihydrate (38–44%) forms are observed.[15,16] Trace amounts of the 1,2-dicarbonyl form have been observed in water,[17] whereas significant amounts are observed in dimethylsulfoxide.[15] No dihydrate form was observed in dimethylsulfoxide.

The alkali-lability of dicarbonyls is well documented[18] and thus the reason for the loss of activity in alkaline conditions[19] is now explained.

3.3. Methylglyoxal concentrations in honey

Weigel et al.[20] have measured the 1,2-dicarbonyl compounds methylglyoxal, glyoxal and 3-deoxyglucosone in 21 multifloral honey samples. Methylglyoxal levels were low, ranging from 0.4 to 5.4 mg/kg. The same study found that storage of samples at 35 and 45 °C resulted in a linear increase of 3-DG, whereas methylglyoxal and glyoxal concentrations were unaltered. In a recent study,[5] high levels of methylglyoxal (48–743 mg/kg) were reported in manuka honey. Both of these studies used a standard OPD derivatisation method to quantify methylglyoxal levels. In this study,

Table 2. Non-peroxide antibacterial activities and methylglyoxal concentrations of New Zealand honeys

Sample no.	Source[a]	Honey type	Activity (equiv % phenol, UMF®)[b]	Concentration of methylglyoxal using direct method (mg/kg)	Concentration of methylglyoxal using derivatisation method (mg/kg)
1	Comvita	Manuka	7.7 (0.4)	142	125
2	Comvita	Manuka	11.1 (0.9)	289	190
3	Comvita	Manuka	15.8 (1.0)	354	340
4	Comvita	Manuka	17.1 (1.0)	376	350
5	Comvita	Manuka	22.9 (0.8)	649	572
6	Comvita	Manuka	27.0 (1.5)	829	679
7	Comvita	Manuka	13.8 (0.8)	390	221
8	Comvita	Manuka	17.7 (0.9)	294	361
9	Comvita	Manuka	20.0 (0.7)	398	441
10	Comvita	Manuka	27.3 (1.0)	820	709
11	HappyBee	Manuka	8.1 (0.5)	203	
12	Comvita	Manuka	14.5 (0.7)	360	
13	Comvita	Manuka	25.3 (1.2)	553	634
14	Comvita	Manuka	27.5 (0.9)	828	725
15	Comvita	Manuka	23.6 (1.3)	558	571
16	Comvita	Manuka	19.9 (0.5)	543	396
17	Comvita	Manuka	17.5 (0.6)	471	313
18	Comvita	Manuka	27.3 (0.8)	630	704
19	Comvita	Manuka	13.9 (0.4)	208	205
20	Comvita	Manuka	14.7 (0.5)	249	219
21	Comvita	Manuka	17.7 (0.5)	375	329
22	Comvita	Manuka	13.9 (0.6)	235	219
23	Comvita	Manuka	14.8 (0.7)	247	249
24	Comvita	Manuka	14.9 (0.7)	286	273
25	Comvita	Manuka	11.0 (0.4)	191	167
26	Comvita	Manuka	10.9 (0.4)	219	173
27	Comvita	Manuka	15.3 (0.7)	303	267
28	Comvita	Manuka	14.4 (0.4)	298	272
29	Comvita	Manuka	14.2 (0.4)	306	244
30	Comvita	Manuka	14.2 (0.5)	ND[c]	257
31	Comvita	Manuka	11.9 (0.6)	ND[c]	186
32	Comvita	Manuka	9.2 (0.4)	142	121
33	Comvita	Manuka	8.3 (0.6)	ND[c]	106
34	Comvita	Manuka	9.3 (0.6)	155	141
35	Comvita	Manuka	7.9 (0.8)	ND[c]	96.6
36	Comvita	Manuka	13.9 (1.1)	273	223
37	Comvita	Manuka	7.3 (0.4)	110	96.8
38	Comvita	Manuka	7.3 (0.4)	ND[c]	107
39	Comvita	Manuka	8.6 (0.2)	123	113
40	Comvita	Manuka	8.0 (0.9)	63	114
41	Comvita	Manuka	10.0 (0.8)	94	131
42	Comvita	Manuka	7.5 (0.2)	91	108
43	Comvita	Manuka	5.9 (0.4)	ND[c]	71
44	Comvita	Manuka	6.1 (0.5)	62	63
45	Comvita	Manuka	4.5 (0.2)	ND[c]	59
46	Comvita	Manuka	5.5 (0.2)	46	38
47	Comvita	Manuka	5.4 (0.1)	69	25
48	Comvita	Manuka	9.4 (0.3)	147	129
49	Comvita	Manuka	7.5 (0.2)	106	90
50	Honey NZ	Rewarewa Active 10+	ND[d]	19	24
51	NZHF&IAS	Northern Rata	ND[d]	47	10
52	NZHF&IAS	Honeydew	5.8 (0.2)	29	3.7
53	NZHF&IAS	Clover	ND[d]	33	1.6
54	NZHF&IAS	Native Wild Green Honey	ND[d]	61	2.9
55	NZHF&IAS	Kamahi	ND[d]	135	5.3
56	NZHF&IAS	Waikato Clover	ND[d]	0	3.7
57	NZHF&IAS	Vipers Bugloss	ND[d]	21	2.4
58	NZHF&IAS	Clover	ND[d]	0	2.9
59	NZHF&IAS	Tawari	ND[d]	32	5.6
60	NZHF&IAS	Tawari	ND[d]	27	3.5
61	NZHF&IAS	Vipers Bugloss	ND[d]	21	2.1

Table 2 (*continued*)

Sample no.	Source[a]	Honey type	Activity (equiv % phenol, UMF®)[b]	Concentration of methylglyoxal using direct method (mg/kg)	Concentration of methylglyoxal using derivatisation method (mg/kg)
62	NZHF&IAS	Vipers Bugloss	ND[d]	28	1.6
63	NZHF&IAS	Rewarewa	ND[d]	62	7.0
64	NZHF&IAS	Pohutakawa	ND[d]	0	2.7
65	NZHF&IAS	Kamahi	ND[c]	55	9.6
66	NZHF&IAS	Kamahi	ND[c]	80	5.3
67	NZHF&IAS	Clover	ND[d]	19	3.2
68	NZHF&IAS	Kamahi	ND[d]	91	5.9
69	NZHF&IAS	Pohutakawa	ND[d]	0	2.7
70	NZHF&IAS	Clover	5.1 (0.1)	56	13
71	NZHF&IAS	Clover/Gum (25%)	ND[d]	41	12
72	NZHF&IAS	Clover/Blackberry	6.4 (0.3)	64	17
73	NZHF&IAS	Clover/Catsear (Otaki)	ND[d]	19	2.9
74	NZHF&IAS	Multiflora (Waikanae)	ND[d]	46	13
75	NZHF&IAS	Clover	ND[d]	79	16
76	NZHF&IAS	Honeydew	4.6 (0.3)	29	3.7
77	NZHF&IAS	Kawatiri 1 Koromiko (comb)	ND[d]	90	24
78	NZHF&IAS	Kawatiri 2 Cyathodes (comb)	ND[d]	71	18
79	NZHF&IAS	Kawatiri 3 Northern Rata (comb)	ND[d]	81	9.4
80	NZHF&IAS	Penny Royal no. 1	5.4 (0.3)	92	4.5
81	NZHF&IAS	Clover/Thistle	ND[d]	0	2.1
82	NZHF&IAS	Rewarewa	ND[d]	91	6.2
83	NZHF&IAS	Pohutkawa	ND[d]	0	4.0

[a] NZHF&IAS: New Zealand Honey Food & Ingredient Advisory Service of the Beekeepers Assn. of N.Z. (Inc.).
[b] Mean and standard deviation of 12 determinations.
[c] A relatively large peak of unknown identity centred at 20.70 min prevented determination of methylglyoxal concentration.
[d] Activity too low to be detected using the method described in this study.

methylglyoxal levels were determined by a direct method and compared with those observed using the indirect OPD derivatisation method, Table 2. The direct method used is possible only due to the relatively large amounts of methylglyoxal found in manuka honey. When applied to non-manuka honey samples, somewhat ambiguous results were obtained. This was due to the relative insensitivity of the direct method where the limit of detection is ca. 50 mg/kg, corresponding to ca. UMF®5. For these honeys the indirect OPD method was needed to obtain reliable data.

For the direct method, a standard curve was generated by adding known amounts of methylglyoxal to a solution of clover honey (50–900 mg/kg). A linear response was observed in this range ($R^2 = 0.99$). All the manuka honey samples fell within this range.

For the indirect OPD derivatisation method a standard method[20] was used with minor changes to the chromatography. A linear response was observed for the standard curve ($R^2 = 0.999$). Figure 3 shows the chromatogram obtained from OPD derivatisation of Comvita UMF®30$^+$ manuka honey (sample 6). The large peak centred at 26.8 min is the OPD derivative of methylglyoxal, this was confirmed by OPD derivatisation of commercial methylglyoxal.

Figure 4 shows an overlay plot of methylglyoxal concentrations for the manuka honey samples using both

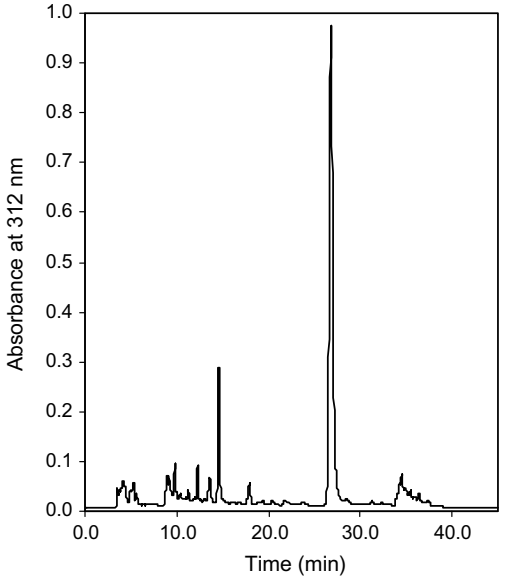

Figure 3. HPLC (system 3) chromatogram of Comvita UMF®30$^+$ manuka honey (sample 6) derivatised with *o*-phenylenediamine.

the analytical methods. The indirect method gave a tighter fit ($R^2 = 0.98$) than the direct method ($R^2 = 0.92$). Better HPLC resolution of the peak of interest can be obtained for the indirect method, the direct method being more prone to interference from co-eluting peaks. Of the

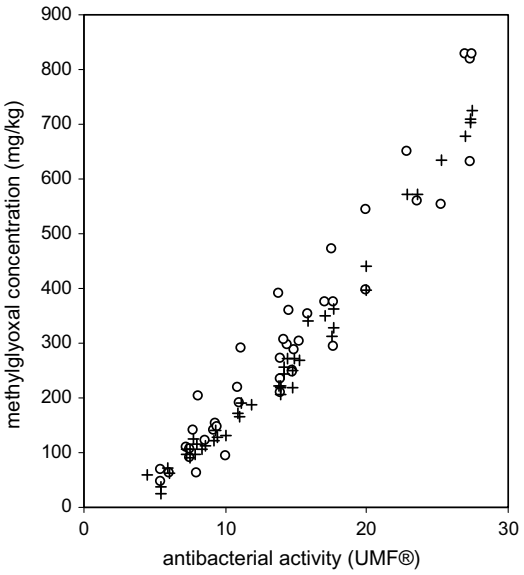

Figure 4. Non-peroxide antibacterial activity of manuka honeys versus methylglyoxal concentration determined by direct (○) and derivatisation (+) methods.

49 manuka honey samples, seven could not be determined due to the presence of a relatively large closely eluting peak (20.70 min) (sample nos. 30, 31, 33, 35, 38, 43 and 45). This peak was also observed in both tawari samples (nos. 59 and 60). Manuka and tawari flower at a similar time, so the presence of the closely eluting peak in the afore-mentioned manuka samples is consistent with those samples originating from a mixed source including Tawari. The identity of this compound has not yet been determined. Despite the lower resolution for the direct method, a relatively good agreement is observed between both sets of data. For the manuka honey samples, methylgyloxal concentrations of 38–725 mg/kg and 46–828 mg/kg were recorded using the derivatisation and direct methods, respectively. Some Vipers Bugloss (*E. vulgare*) honeys have been shown to exhibit significant levels of non-peroxide antibacterial activity.[1] The three samples (samples 57, 61 and 62) in this report contained low levels of methylglyoxal (2.4, 2.1 and 1.6 mg/kg) and also exhibited no activity using the bioassay described.

For the non-manuka honey samples methylglyoxal concentrations of 1.6–24 mg/kg and 0–135 mg/kg were recorded using the derivatisation and direct methods, respectively. Generally for these honeys the direct method gives higher methylglyoxal levels than the derivatisation method. This is likely due to the co-elution of other compound(s) interfering with integration values and is particularly observed for samples 55, 66, 68, 75, 77, 79, 80 and 82. This effect is swamped for higher activity honeys.

Of the 34 non-manuka honey samples, only five showed any antibacterial activity using the assay described. Interestingly, none of these samples showed sufficient methylglyoxal concentrations to account for their observed activity. At these low activity levels, it seems likely that other compounds are making a significant contribution to the overall effect. Some of the phenolic acids and flavonoids present in honeys are known to exhibit weak antibacterial activity.[3,21–23] Whilst most of the methylglyoxal concentrations of the non-manuka honey samples in this report are similar to those previously reported for honeys in Europe,[20] three samples are noticeably higher. Weigel et al.[20] reported methylglyoxal levels from 0.4 to 5.4 mg/kg in 21 multifloral honeys from Europe. Levels of 24, 17 and 24 mg/kg were recorded for Rewarewa (sample 50), Clover/Blackberry (sample 72) and Kawatiri (sample 76) samples, respectively. These levels are still significantly lower than those observed in the majority of the manuka samples; they correspond to UMF values less than 5, which is the lower limit of detection in the bioassay described herein.

Yadav et al.[24] have shown that methylglyoxal levels in some plants increase significantly (2–6-fold) in response to salinity, drought and cold stress. Much of the manuka honey comes from areas deemed too marginal for farming. It is possible that the soil and climatic conditions in these areas are contributing high stress levels to the plant. The excess methylglyoxal produced is then transferred to the honey.

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