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Article *in* Journal of Agricultural and Food Chemistry · June 2012

Impact Factor: 2.91 · DOI: 10.1021/jf300888q · Source: PubMed

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Classification and Characterization of Manuka Honeys Based on Phenolic Compounds and Methylglyoxal

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ABSTRACT: Manuka honey from New Zealand is often considered to be a medicinal product of special value due to its high level of antimicrobial activity. Therefore, the distinct authentication of its botanical origin is of great importance. Aside from the common pollen analysis, it is in this respect particularly the analysis of the phenolic acids, flavonoids, and norisoprenoids that is described as useful. In the present study, numerous manuka honeys were analyzed by UPLC-PDA-MS/MS after solid-phase extraction and compared to other kinds of honey to define marker substances characteristic for manuka honeys. The PDA profiles obtained differed markedly from each other so that the individual honey samples could be assigned to three groups. For the honeys of group 1 the comparably high concentrations of 4-hydroxybenzoic acid, dehydrovomifoliol, and benzoic acid proved to be typical, whereas the profiles of group 2 showed high kojic acid and 2-methoxybenzoic acid intensities. The manuka honeys of group 3, on the other hand, yielded high amounts of syringic acid, 4-methoxyphenyllactic acid, and methyl syringate. Furthermore, the comprehensive comparison of manuka honeys to other unifloral honeys revealed that especially kojic acid, 5-methyl-3-furancarboxylic acid, leptosin, unedone, 2-methoxybenzoic acid, 4-methoxyphenyllactic acid, 3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione, and methyl syringate were useful for distinguishing manuka honeys from the other kinds of investigated honeys. Moreover, kojic acid, unedone, 5-methyl-3-furancarboxylic acid, 3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione, and lumichrome were identified in manuka honey for the first time.

KEYWORDS: *flavonoids, manuka honey, methylglyoxal, norisoprenoids, phenolic acids, UPLC-PDA-MS/MS, solid-phase extraction*

INTRODUCTION

Manuka honey is derived from the manuka tree, *Leptospermum scoparium*. Manuka belongs to the Myrtaceae family and grows as a shrub or small tree throughout New Zealand[1] and eastern Australia.[2,3] In traditional medicine, different extracts of manuka are used as sedatives, as a wound-healing remedy, and against diarrhea due to their astringent properties.[4] Manuka honey itself has long been employed for clearing up infections including abscesses, surgical wounds, traumatic wounds, burns, and ulcers of varied etiology.[5] The effectiveness of manuka honey as a wound dressing seems to be due to its nonperoxide antimicrobial properties.[6,7] On the basis of microbiological assays compared to phenol, a common antiseptic, the so-called Unique Manuka Factor (UMF) was established.[8] In 2008, Mavric et al.[9] identified methylglyoxal (MGO) as the bioactive compound responsible for the antibacterial activity of some manuka honeys. In this, the level of MGO was directly proportional to the level of nonperoxide antibacterial activity. The demonstrated antibacterial activity of manuka honey is an important commercial property, expressed as UMF or MGO. Therefore, it is necessary to confirm the quality of this type of honey, especially because not all manuka honeys exhibit the nonperoxide antibacterial activity.[10,11] The quality of honey is judged by its botanical origin and its chemical composition; the price of honey, on the other hand, is based on its quality. Traditionally, the floral source of a unifloral honey is identified by analyzing the bee pollen present in the honey. However, under- or over-represented or similar pollen grains such as pollen of the same subfamily (e.g., manuka and kanuka[12]) complicate the microscopic analysis. Consequently, chemical approaches need to be found for an objective characterization of the botanical source. There are approximately 200,000 known secondary plant products that lead to the uniqueness of plants. Phenolic acids, flavonoids, and norisoprenoids turned out to be very suitable for the differentiation of the unifloral honeys.[13−25] They are exclusively built in plants and incorporated into honey via the nectar, the honey dew, and the pollen.[26]

With regard to manuka honey, several compounds such as 4-methoxyphenyllactic acid and phenyllactic acid as well as 2-methoxybenzoic acid and methyl syringate[16,27−30] were assigned as marker substances. Yao et al.[23] proposed gallic acid and the abscisic acid isomers as characteristic substances. Just recently, Fearnley et al.[31] and Kato et al.[32] identified leptosin as specific to manuka honey. Because differing extraction and detection methods were applied, the obtained literature data are rarely comparable. Hence, the objectives of this study were to compare 40 manuka honeys with each other and, subsequently, with other unifloral honeys to prove the capability of the reported markers. Therefore, the analytes were extracted and enriched by solid-phase extraction (SPE) and subsequently analyzed by UPLC-PDA-MS/MS to gain sort-specific fingerprints and to quantify selected compounds via a selected reaction monitoring method (SRM).

Received: March 1, 2012
Revised: June 6, 2012
Accepted: June 7, 2012
Published: June 7, 2012

dx.doi.org/10.1021/jf300888q | *J. Agric. Food Chem.* 2012, 60, 7229−7237

Table 1. Honey Samples, Floral and Geographical Origins, Year of Production, and Ratio of the Eponymous Pollen

honey samples	NZL/year of production	pollen (%)	honey samples	origin	year of production	pollen (%)
manuka (M) 1, group 1	2007	66	**heather (H) 1**	Spain	2008	47[a]
M2	2007	59	H2	Spain	2008	26[b]
M3	2007	56	H3	Spain	2009	50[a]
M4	2007	67	H4	France	2010	48[a]
M5	2007	74	H5	Norway	2010	33[b]
M6	2008	73	H6	Norway	2010	50[a]
M7	2008	81	H7	Spain	2010	50[a]
M8	2008	95	H8	Spain	2010	49[a]
M9	2009	65	H9	Spain	2010	50[a]
M10	2010	70	H10	Spain	2010	22[b]
manuka group 2			**chestnut (C) 1**	Italy	2008	93
M11	2007	42	C2	Italy	2009	97
M12	2007	76	C3	Italy	2009	95
M13	2007	55	C4	Italy	2009	96
M14	2007	82	C5	unknown	2009	93
M15	2007	93	C6	Italy	2010	96
M16	2008	76	C7	Italy	2010	97
M17	2008	87	C8	Italy	2010	97
M18	2008	77	C9	unknown	2010	94
M19	2008	76	C10	unknown	2010	95
M20	2008	93	**eucalyptus (E) 1**	Australia	2009	82
manuka group 3			E2	Australia	2009	92
M21	2008	58	E3	Uruguay	2009	94
M22	2008	59	E4	Uruguay	2009	85
M23	2008	64	E5	Uruguay	2009	74
M24	2008	47	E6	Uruguay	2009	80
M25	2008	70	E7	Argentina	2010	83
M26	2008	55	E8	Brazil	2010	90
M27	2008	71	E9	Brazil	2010	86
M28	2008	46	E10	Uruguay	2010	84
M29	2008	46	**honeydew (D) 1**	Italy	2009	1.86[c]
M30	2009	59	D2	Italy	2009	0.85[c]
M31	2009	58	D3	Spain	2009	0.81[c]
M32	2009	72	D4	Spain	2009	0.78[c]
M33	2009	74	D5	Spain	2009	1.29[c]
M34	2009	76	D6	Spain	2009	0.83[c]
M35	2009	93	D7	Spain	2009	0.76[c]
M36	2009	48	D8	Spain	2009	1.13[c]
M37	2009	95	D9	unknown	2009	0.95[c]
M38	2010	87	D10	unknown	2009	0.84[c]
M39	2010	96				
M40	2011	74				

[a] *Erica* heather honey. [b] *Calluna* heather honey. [c] σ, conductivity (mS/cm).

MATERIALS AND METHODS

Honey Samples. Several unifloral honeys were used for a comparative study in which the assigned markers were quantified in manuka, honeydew, heather, chestnut, and eucalyptus honeys. These honeys were of various geographical origins and ages (Table 1). Before analysis, the honey samples were stored at 8 °C. The pollen ratios of the eponymous plants were provided by Intertek Food Services GmbH (Bremen, Germany) and Quality Services International GmbH (Bremen, Germany).

Chemicals. Methanol HPLC grade, acetic acid 100% glacial, and sodium chloride were purchased from VWR (Darmstadt, Germany); methanol LC-MS grade was ordered from Fisher Scientific (Schwerte, Germany) and DMSO-d_6 from Armar Chemicals (Doettingen, Switzerland). Methylglyoxal (35% w/v aqueous), *o*-phenylenediamine, kojic acid, syringic acid, benzoic acid, methyl syringate, daidzein, luteolin, and *cis*,*trans*-abscisic acid were acquired from Alfa Aesar (Karlsruhe, Germany). The isomer *trans*,*trans*-abscisic acid was obtained from *cis*,*trans*-abscisic acid after a defined period of exposure to UV light. Ethyl acetate anhydrous, formic acid LC-MS grade, gallic acid, caffeic acid, β-phenyllactic acid, 4-hydroxybenzoic acid, 5-hydroxymethylfurfural, caffeine, 2-methoxybenzoic acid, 1-hydroxy-4-keto-2-ionone (dehydrovomifoliol), phenylacetic acid, lumichrome, and 4-methoxy-C13 d3-benzoic acid-d_4 were purchased from Sigma-Aldrich (Steinheim, Germany). Unless specified otherwise, all chemicals used were of analytical grade. Bidistilled water was generated by the Bi-Distilling Apparatus Bi 18E from QCS GmbH (Maintal, Germany). The compounds 5-methyl-3-furancarboxylic acid, 3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione, and acetyl-2-hydroxy-4-(2-methoxy-phenyl)-4-oxobutanat were isolated from manuka honey.

Solid-Phase Extraction and Liquid Chromatography. In recent years, solid-phase extraction has been employed frequently. As different sorbent materials were available, it could be proved that polymeric sorbents were most suitable.[33] Hence, Chromabond HR-X cartridges (Macherey-Nagel, Düren, Germany) were chosen for the

Table 2. Quantified Compounds, Retention Times (t_R), Molecular Ions, SRM Transitions, and UV Maxima (UV_{max})

	t_R (min)	$[M - H]^-$	SRM transitions	UV_{max} (nm)
gallic acid	1.6	169.031	79.587, 81.392, 125.200	270
caffeic acid	5.6	179.060	107.156, 134.094, 135.119	322
phenyllactic acid	7.0	165.013	101.117, 119.119, 147.060	256
4-methoxyphenyllactic acid	8.1	195.068	133.181, 149.158, 177.124	274
		$[M + H]^+$		
kojic acid	1.2	143.056	69.304, 97.208	266
5-hydroxymethylfurfural	2.1	127.054	53.319, 81.167, 109.088	284
5-methyl-3-furancarboxylic acid	2.5	127.026	53.237, 81.192, 109.068	264
4-hydroxybenzoic acid	4.2	139.061	PDA detection	255
caffeine (IS[a])	5.5	195.052	109.941, 137.838	274
syringic acid	6.2	198.993	PDA detection	274
leptosin[b]	6.5	559.148	PDA detection	263
unedone	6.6	241.130	111.243, 137.183, 153.131	246
2-methoxybenzoic acid	7.4	153.100	77.160, 92.060, 134.890	298
dehydrovomifoliol	7.8	223.073	93.163, 121.102, 205.066	240
acetyl-2-hydroxy-4-(2-methoxy-phenyl)-4-oxobutanat[c]	7.9	225.012	77.154, 135.054, 207.045	250/310
phenylacetic acid	8.1	137.031	PDA detection	256
3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione	8.7	223.014	92.094, 135.050, 205.080	250/310
benzoic acid	8.9	123.080	77.182, 95.071	274
4-methoxy-C13 d3-benzoic acid-d_4 (IS)	10.1	161.050	98.149, 117.161, 143.101	256
methyl syringate	11.1	213.026	121.093, 154.031, 181.016	274
lumichrome	12.5	243.066	172.153, 198.124	260/352
2-*trans*,4-*trans*-abscisic acid	12.6	265.048	201.077, 229.034, 247.065	262
2-*cis*,4-*trans*-abscisic acid	13.8	265.048	201.077, 229.034, 247.065	262
daidzein (IS)	14.2	255.025	198.664, 254.299	302
luteolin	15.2	287.029	89.241, 135.124, 153.113	345

[a]IS, internal standard. [b]559 = $[M + Na + H]^+$. [c]225 = $[M - Ac + H]^+$.

Table 3. Linearity Range, Correlation Coefficient, Limit of Detection (LOD), Limit of Quantification (LOQ), and Recovery of the Analytes

	linearity range				recovery (R)			
	mg/kg	R^2	LOD (mg/kg)	LOQ (mg/kg)	level 1 (mg/kg)	level 2 (mg/kg)	$R_{level\ 1}$ (%)	$R_{level\ 2}$ (%)
2-methoxybenzoic acid	0.03−125	0.9990	0.04	0.13	0.1	16.0	95	97
3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione	nd[b]	nd	0.02	0.05	0.5	4.0	104	104
4-hydroxybenzoic acid	0.60−20	0.9930	0.18	0.60	3.5	20.0	103	91
5-hydroxymethylfurfural	0.03−125	0.9962	0.01	0.03	2.3	45	98	99
4-methoxy-C13 d3-benzoic acid-d_4 (IS[a])	0.50−125	0.9969	0.15	0.50	0.1	6.0	100	95
4-methoxyphenyllactic acid	0.25−250	0.9958	0.03	0.10	0.5	250.0	104	105
acetyl-2-hydroxy-4-(2-methoxyphenyl)-4-oxobutanat	nd	nd	0.02	0.05	0.5	10.0	100	101
benzoic acid	0.40−940	0.9986	0.12	0.40	3.8	75.0	102	99
β-phenyllactic acid	0.25−1250	0.9994	0.08	0.25	187.5	750.0	98	99
caffeic acid	0.05−25	0.9990	0.03	0.10	5.0	20.0	96	99
caffeine (IS)	0.10−125	0.9997	0.15	0.50	1.0	25.0	102	105
2-*cis*,4-*trans*-abscisic acid	0.03−125	0.9943	0.01	0.03	5.0	50.0	103	104
daidzein (IS)	0.50−125	0.9991	0.15	0.50	1.0	25.0	100	105
dehydrovomifoliol	0.05−650	0.9958	0.02	0.05	62.5	250.0	102	101
gallic acid	0.25−125	0.9948	0.08	0.25	2.5	50.0	103	102
kojic acid	0.13−125	0.9934	0.08	0.25	0.5	20.0	93	95
lumichrome	0.03−190	0.9957	0.01	0.03	7.5	30.0	105	108
luteolin	0.10−30	0.9987	0.04	0.12	0.1	0.8	91	88
methyl syringate	0.03−140	0.9976	0.15	0.50	5.8	115.0	105	105
phenylacetic acid	0.13−125	0.9968	0.15	0.50	187.5	750.0	108	96
syringic acid	0.40−160	0.9990	0.15	0.50	6.3	25.0	102	103
unedone	nd	nd	0.01	0.03	2.50	nd	100	nd

[a]IS internal standard. [b]nd, not determined.

dx.doi.org/10.1021/jf300888q | J. Agric. Food Chem. 2012, 60, 7229−7237

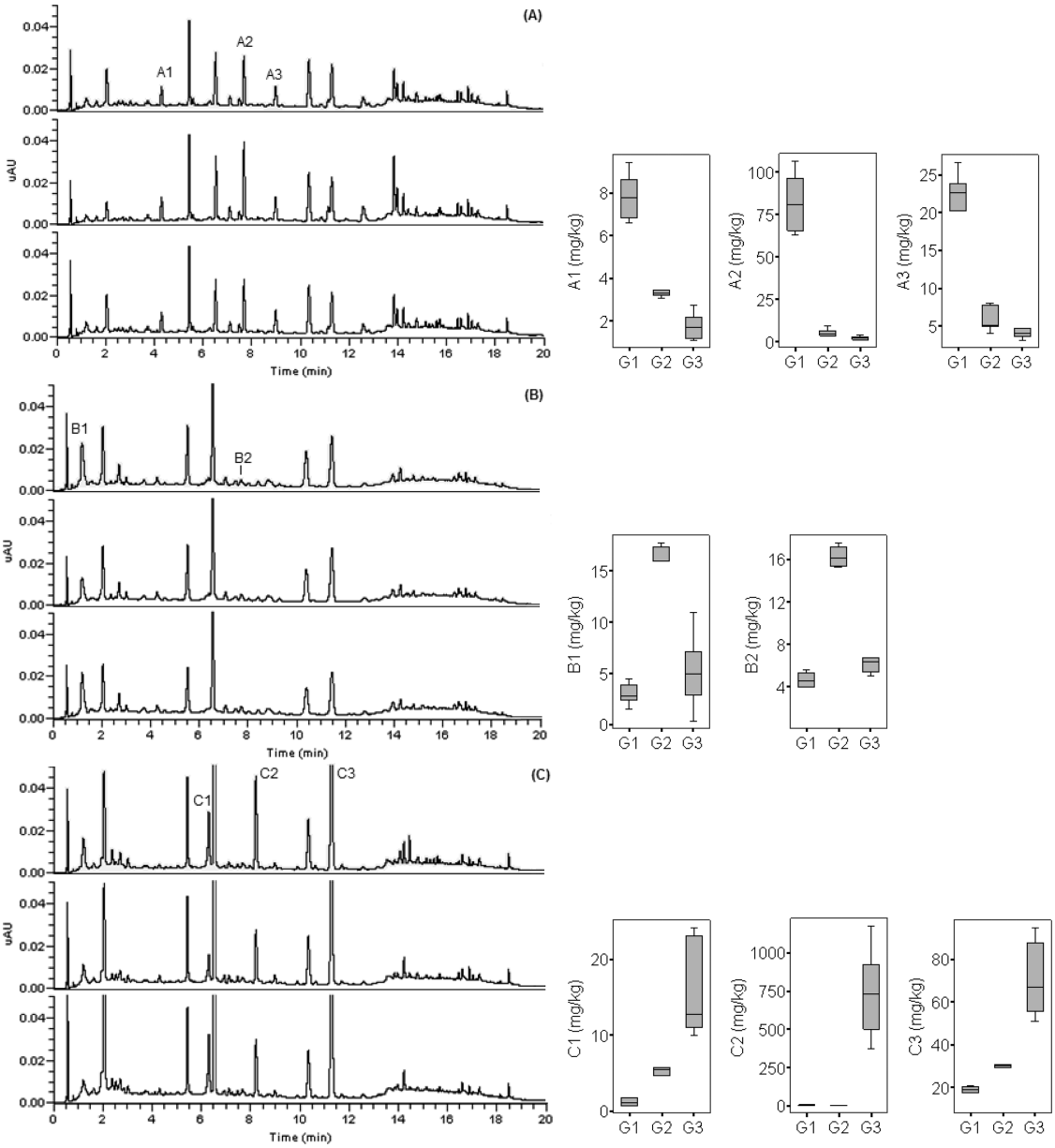

Figure 1. UPLC chromatograms, λ = 272 nm; nine representative manuka honey samples grouped according to their UPLC profile: group 1 (A), group 2 (B), group 3 (C). Numbered peaks are typical compounds of each group: (A1) 4-hydroxybenzoic acid, (A2) dehydrovomifoliol, (A3) benzoic acid, (B1) kojic acid, (B2) 2-methoxybenzoic acid, (C1) syringic acid, (C2) 4-methoxyphenyllactic acid, (C3) methyl syringate. Box plots were compiled on the basis of the analyzed samples M1−M40.

extraction of phenolic acids, flavonoids, and norisoprenoids from aqueous honey. Two grams of honey was dissolved in 3 mL of 2% NaCl and the pH adjusted to pH 1. Prior to extraction, the cartridge (85 μm, 200 mg, 6 mL) was conditioned with methanol and acidulated bidistilled water. After the honey solution (30%, w/v) spiked with 50 μL of internal standards (0.4 mg/mL caffeine, 0.4 mg/mL 4-methoxy-C13 d3-benzoic acid-d_4, 0.2 mg/mL daidzein) for tracking the extraction efficiency was loaded, the interfering sugars were removed with acidulated water, and the analytes were eluted with 75% methanol into a 5 mL flask. An aliquot was subjected to UPLC-PDA (Acquity, Waters, Eschborn, Germany) after membrane filtration (0.2 μm).

The separation was achieved on a Nucleodur C18 Pyramid column (100 × 2.1 mm, 1.8 μm) (Macherey-Nagel) within 25 min. The temperature was set at 40 °C and the flow rate at 0.4 mL/min, and 0.1% formic acid and 0.05% formic acid in methanol were used as eluents in the gradient mode. The monitoring wavelengths were 254, 272, and 300 nm.

Mass Spectrometry and Quantification. The UPLC-PDA was attached to a TSQ Quantum Access MAX (Thermo Fisher, Dreieich, Germany). Electrospray ionization was applied either in the negative or in the positive mode. The vaporizer temperature was set at 350 °C and the capillary temperature at 270 °C, the spray voltage at 3500 V for measurements in the positive mode and at 3000 V in the negative mode; the sheath gas was set at 50 mTorr, and the auxiliary gas at 10 mTorr. The analytes were detected by SRM. Compounds were identified by their retention time, UV spectrum, and characteristic SRM transitions (Table 2). To quantify the analytes, a calibration was set up in a model honey solution. For this, a model honey consisting of 40% glucose, 40% fructose, and 20% water was subjected to SPE and the standard mixtures were subsequently spiked to the extract. All standards were dissolved in methanol (HPLC grade). Prior to the

comparative quantification study, the linearity range of the analytes, the limit of detection (S/N = 3), the limit of quantification (S/N = 10), and the recoveries for two spiking levels were determined by referring to the sample weight of the SPE (Table 3). To consider possible matrix effects, the determination was accomplished in acacia honey containing a limited amount of polyphenols, ideal for investigating broad concentration ranges.

Liquid Extraction and Semipreparative HPLC for the Fractionation of Unknown Compounds. To obtain sufficient concentrations for accomplishing the identification experiments, approximately 1 kg of honey had to be processed. Hence, the extraction of the polyphenols was carried out by liquid extraction using ethyl acetate as extracting agent on the basis of the method of Trautvetter et al.[25] Thirty grams of honey was homogenized with 20 mL of 2% NaCl solution using a Turrax blender (T18 basic IKA-Werke, Germany). The suspension was shaken twice with 40 mL of ethyl acetate, once at the natural pH value of the honey and once at pH 1−2. The combined extracts were dried over Na_2SO_4 and concentrated to dryness under vacuum. The residues of 35 extractions were resolved in 5 mL of methanol/water (50:50, v/v), respectively, and combined. The unknown substances were isolated with a semipreparative HPLC-DAD system equipped with a fraction collector (VWR, Darmstadt, Germany). The separation was achieved on a Synergi Polar-RP column (Phenomenex, Aschaffenburg, Germany; 250 × 10 mm, 4 μm), at 30 °C and a flow rate of 2.8 mL/min and applying 2% (v/v) aqueous acetic acid and methanol as mobile phase.

NMR Spectroscopy. To elucidate the structure of the unknown compounds, the NMR spectra were acquired on a Bruker Avance-600 spectrometer. 1D-^{1}H and 1D-^{13}C experiments were carried out with a sweep width of 20 and 240 ppm and TMS = 0 ppm, respectively. The following 2D NMR spectra were acquired: HSQC, HMBC, COSY, NOESY, and ROESY. The isolated fractions were concentrated to dryness and subsequently resolubilized in DMSO-d_6.

Quantification of Methylglyoxal. The MGO was analyzed as its corresponding quinoxaline after derivatization with *o*-phenylenediamine (OPD) according to the method of Weigel et al.[34] with some slight modifications. Three grams of honey was dissolved in 10 mL of bidistilled water and diluted. For derivatization, 1.0 mL of the 1.5% (w/v) honey solution was mixed with 0.4 mL of aqueous OPD solution (0.5%). After a reaction time of at least 8 h in the dark and at room temperature, the samples were membrane-filtered (0.2 μm) and, subsequently, 5 μL was injected into an Elite LaChrom system (VWR). The separation was performed on a LiChroCART 125-4 Purospher RP-18 end-capped column (120 Å, 5 μm, Macherey-Nagel) at a flow rate of 0.4 mL/min and with the column oven temperature set at 30 °C. The separation was carried out with 0.1% aqueous acetic acid and methanol. The detection wavelength of the UV-DAD (VWR L-2455) was set at 316 nm. The limit of detection (S/N = 3) was 5 mg/kg, and the limit of quantification (S/N = 10) was 13 mg/kg, considering the sample weight. The calibration ranged from 35 to 1400 mg/kg with a correlation coefficient of 0.9998.

Statistical Analysis. Spearman's rank correlation was used to evaluate the relationship between the MGO content determined and selected biomarkers.

RESULTS AND DISCUSSION

UPLC-PDA Profiles and Methylglyoxal Content of the Three Types of Manuka Honeys. For the comparison of the UPLC pattern 40 manuka honey samples were applied to SPE. Their color ranged from light brown to intense dark brown, and the consistency ranged from very viscous to fluid. By comparison of the UPLC chromatograms of the extracted manuka honeys, the mentioned variation within the samples became apparent also in the profile and intensity of the phenolic acids, norisoprenoids, and flavonoids. As a result of this analysis the manuka honey samples could be divided into three groups: group 1 contained 25%, group 2 contained 25%, and group 3 contained 50% of the investigated samples.

The groups mainly differed in the intensity and only slightly in the composition of the phenolic acids and norisoprenoids. Whereas the profiles of groups 2 and 3 showed certain similarities, the profile of group 1 differed greatly. The manuka honeys of group 1 could be characterized by high 4-hydroxybenzoic acid, dehydrovomifoliol, and benzoic acid yields (Figure 1A). Furthermore, the average amount of 4-hydroxybenzoic acid was at least doubled in the honey samples of group 1 (7.5 mg/kg) compared to the other two groups (3.4 and 2.0 mg/kg); the average yield of dehydrovomifoliol was at least 8 times higher (82.1 mg/kg) and that of benzoic acid was 4 times higher (21.5 mg/kg).

The samples of group 2 showed significantly higher concentrations of kojic acid (mean = 17.0 mg/kg) and 2-methoxybenzoic acid (mean = 16.3 mg/kg) (Figure 1B), whereas the high contents of syringic acid, 4-methoxyphenyllactic acid, and methyl syringate were typical for the honeys of group 3 (Figure 1C). In this, no correlation was observed between phenyllactic acid and 4-methoxyphenyllactic acid concentrations. The average level of 4-methoxyphenyllactic acid was at least 130 times and that of methyl syringate 2.5 times higher in the honey samples of group 3.

A possible explanation for the detected trisection of the manuka honey profiles might be the occurrence of three chemotypes of *L. scoparium* in New Zealand.[28,35] Hence, the essential oil profiles of *L. scoparium* leaf material from the Northland and Waikato populations revealed a high level of eudesmols (type 1), those from the East Coast populations contained a high triketone level (type 2), and the oil from the Central and Coromandel populations was characterized by the absence of triketones (type 3).

Furthermore, there are four major *L. scoparium* varieties: *L. scoparium* var. *incanum* is mainly present in the Northland, Coromandel, and the East Coast; *L. scoparium* var. *linifolium* grows in Waikato and Coromandel; *L. scoparium* var. *myrtifolium* is spread throughout the Central North Island; and *L. scoparium* var. *triketone* is typical for the East Coast. With regard to the essential oil profiles and the morphology, *L. scoparium* var. *linifolium* is closely related to *L. scoparium* var. *incanum*.[28] Hence, the essential oil type 1 is generated by *L. scoparium* var. *incanum* and *linifolium*, type 2 by *L. scoparium* var. *triketone*, and type 3 by *L. scoparium* var. *myrtifolium*.

On the other hand, it should be taken into account that manuka and kanuka flourish shortly after one another and that the pollen grains are indistinguishable, which inevitably leads to blended honeys, depending on the floral availability in the different regions of New Zealand. The results of Stephens et al.[30] indicate that the high 4-methoxyphenyllactic acid contents are due to the presence of kanuka honey. Hence, the manuka honey samples of group 3 seem to be contaminated with kanuka honey, and because *Kunzea ericoides* is rather common on the East Coast, these samples probably originate from there. However, to prove 4-methoxyphenyllactic acid as a marker for kanuka honey, several kanuka honey samples need to be analyzed. The different kanuka honey ratio also explains the various MGO contents of the manuka honeys samples in group 3 (Table 4).

Furthermore, Senanayake[29] determined higher levels of 2-methoxybenzoic acid in manuka honeys from the Northland. Therefore, the samples of group 2 were supposedly harvested in this region. This assumption could be confirmed with the obtained MGO contents. In accordance with Senanayake, a correlation between 2-methoxybenzoic acid and MGO was

Table 4. Methylglyoxal Yields Depending on the Manuka Honey Group

	MGO content (mg/kg)			
manuka honey group[a]	mean	median	minimum	maximum
1 (10)	123	131	58	178
2 (10)	655	634	507	897
3 (20)	349	329	41	1178

[a]The number of samples is given in parentheses.

observed (r = 0.804, p = 0.002) (Figure 2), in which the highest yield of 634 mg/kg MGO was on average detected for the

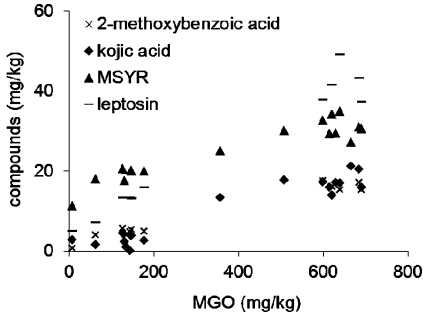

Figure 2. Positive correlation between 2-methoxybenzoic acid, kojic acid, methyl syringate (MSYR), leptosin, and methylglyoxal of the manuka honey samples of groups 1 and 2 (samples of group 3 were excluded due to high MGO variation).

manuka honeys of group 2. Consequently, it is very likely that the manuka honey samples of group 1 originate from the central and southern division. This hypothesis could again be supported by the MGO contents determined. As reported by Stephens,[28] the honeys of this region showed the lowest antibacterial activity. Referring to the MGO contents of the honeys of groups 1 and 2, positive relationships were additionally determined for kojic acid (r = 0.794, p = 0.000), methyl syringate (r = 0.797, p = 0.000), and leptosin (r = 0.848, p = 0.000).

Characteristic Manuka Honey Markers. To assign distinctive marker compounds for manuka honeys in general, the three manuka honey groups were compared to other kinds of honey. Because manuka honeys consist of nectar as well as of honey dew, mainly heather, chestnut, eucalyptus, and honeydew honeys were included in the chromatographic comparison. Compounds were considered as markers for manuka honey if they were exclusively detected in manuka honey, showed significantly higher concentrations, or were profile-defining.

The potential markers were then quantified via UPLC-MS/MS in the SRM mode or, if the compounds were poorly ionizable, via UPLC-PDA (Table 2). For the final comparison of the obtained yields from the different unifloral honeys, box plots were compiled (Figure 3).

According to the box plots, kojic acid, 5-methyl-3-furancarboxylic acid, leptosin, unedone, 2-methoxybenzoic acid, 4-methoxyphenyllactic acid, methyl syringate, and 3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione showed the highest yields in manuka honey (Figure 3). Hence, these compounds are especially useful for distinguishing manuka honeys from the other kinds of investigated honey. In these, kojic acid, 5-methyl-3-furancarboxylic acid, and 3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione were identified for the first

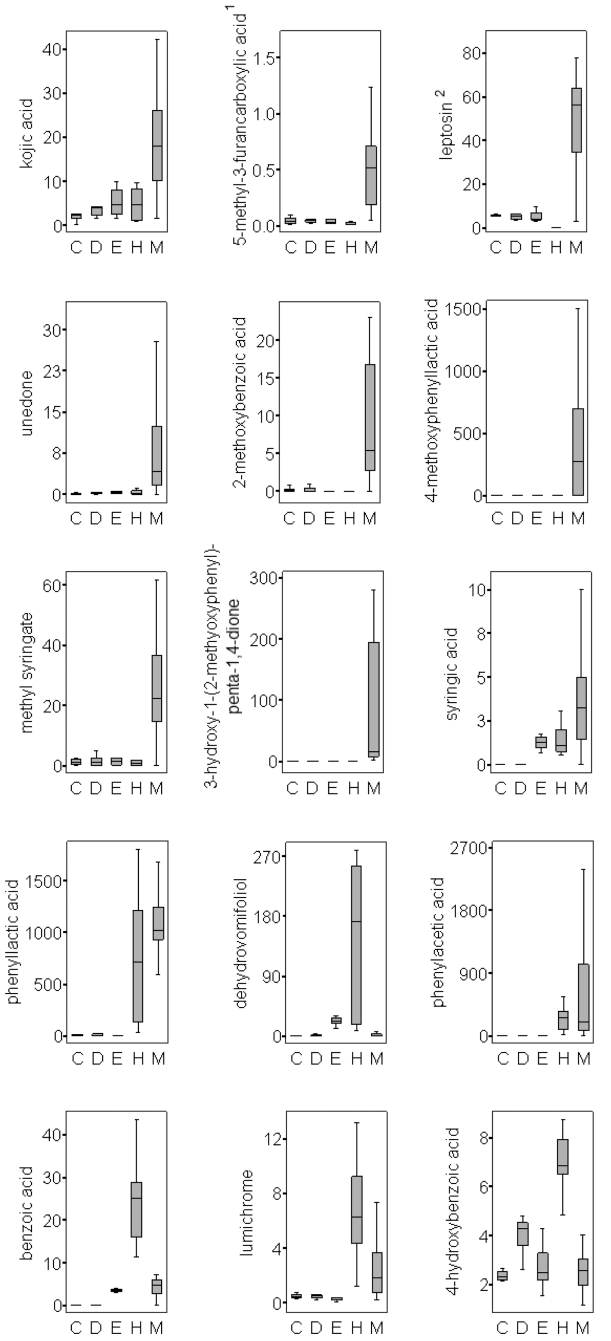

Figure 3. Box plots of quantitative data, yields in milligrams per kilogram. C, chestnut honey (n = 10); D, honeydew honey (n = 10); E, eucalyptus honey (n = 10); H, heather honey (n = 10); M, manuka honey (n = 30, M1−M30). Quantified as [1] HMF or [2] syringic acid via PDA.

time. The glycoside leptosin was recently reported as a typical molecule in manuka honeys.[31] Unedone is known as a characteristic norisoprenoid in strawberry tree honey. However, with regard to the presented comparison, it is also distinctive for manuka honeys. It could further be reconfirmed that 2-methoxybenzoic acid, 4-methoxyphenyllactic acid, and methyl syringate are typical manuka honey constituents.[16,29,30]

Furthermore, in consideration of the UPLC-PDA profile depending on the manuka honey group, the yields of syringic acid, phenyllactic acid, dehydrovomifoliol, phenylacetic acid,

benzoic acid, and lumichrome can also be used to characterize manuka honey.[36] Even though some compounds were detected at higher concentrations in other unifloral honeys, such as lumichrome in thistle honey[37] or dehydrovomifoliol and benzoic acid in heather honey, they still contribute to the peak pattern of manuka honey (Figure 4). For 4-hyroxybenzoic

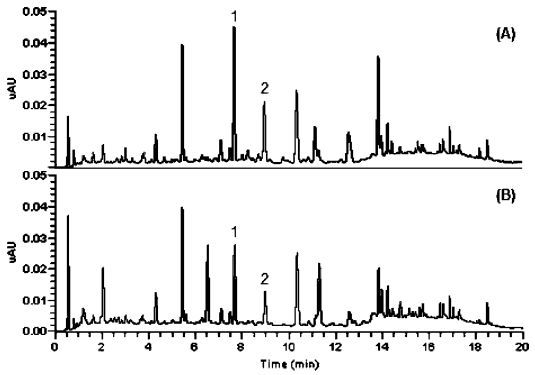

Figure 4. UPLC chromatograms, λ = 272 nm; profiles of heather honey (A) and manuka honey group 1 (B). Peaks:1, dehydrovomifoliol; 2, benzoic acid.

acid, similar amounts were detected in almost all of the investigated honey samples, whereas the yield itself could not be considered to be characteristic for manuka honey, in general. However, it is still a distinctive component for the manuka honeys of group 1.

According to the determined average amounts, phenylacetic acid (727 mg/kg), phenyllactic acid (661 mg/kg), 4-methoxyphenyllactic acid (417 mg/kg), leptosin (56 mg/kg), and methyl syringate (45 mg/kg) are the dominating compounds in manuka honey. Previous studies by Senanayake[29] and Stephens et al.[30] also demonstrated that 4-methoxyphenyllactic acid, phenyllactic acid, and methyl syringate were major components of manuka honey and determined similar yields. However, it should be noted that only the manuka honeys of group 3 contained considerable amounts of 4-methoxyphenyllactic acid, which might be due to the contamination with the kanuka honey.

The results of Yao et al.,[23] who detected gallic acid with 50% of the phenolic acid content and 2-*cis*,4-*trans*-abscisic acid with yields of up to 310 mg/kg, are rather contrasting. In the present study, gallic acid was determined to be <4.5 mg/kg and *cis*,*trans*-abscisic acid to be <3.5 mg/kg in the analyzed manuka honeys. In accordance with Senanayake,[29] the amounts of the 2-*cis*,4-*trans*-abscisic acid were slightly higher than those of *trans*,*trans*-abscisic acid (<1 mg/kg).

Newly Identified Substances in Manuka Honey. Within this research seven compounds could be identified in manuka honey for the first time (Figure 5). The presence of kojic acid (**1**) and lumichrome (**2**) was confirmed with reference substances. Kojic acid is a degradation product of carbohydrates, mainly glucose,[38] via gluconolactone and 3-ketogluconic acid lactone. Lumichrome is supposed to stimulate photosynthesis and vegetation[39] and is a degradation product of riboflavin.[40]

4-Methoxyphenyllactic acid (**3**) was assigned as a potential marker for manuka honeys by Wilkins et al.,[27] who described the methoxy group in the para position. Unedone (**4**) was proposed as a marker for strawberry tree honey.[41] Because the

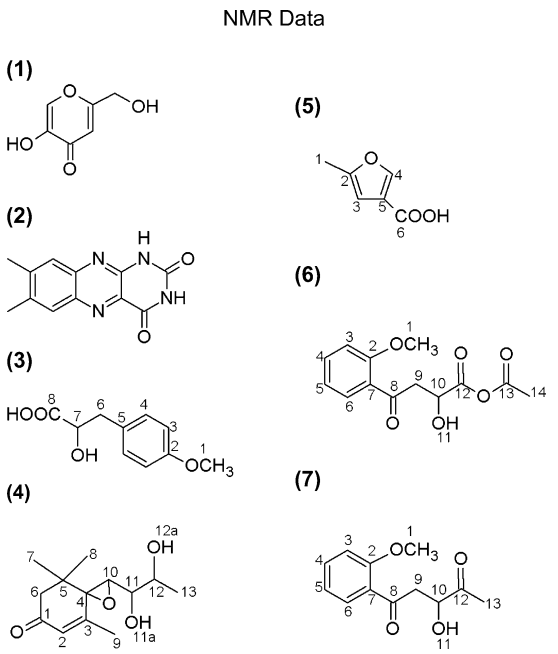

Figure 5. Newly identified compounds in manuka honey. (**3**) IUPAC, ethyl-2-carboxy-2-hydroxy-1-(4-methoxyphenyl): ^{1}H NMR (600.13 MHz, DMSO) δ 2.92 (1H, m, H6b), 3.11 (1H, m, H6a), 3.83 (3H, s, OCH_3-C2), 4.38 (1H, s, H7), 6.90 (2H, d, H3, H3′), 7.24 (2H, d, H4, H4′); ^{13}C NMR (150.92 MHz, DMSO) δ 40.79 (C6), 55.62 (OCH_3-C2), 73.23 (C7), 114.66 (C3), 130.91 (C5), 131.56 (C4), 159.91 (C2), 175.23 (C8); NMR purity of 98% determined with regard to decamethylcyclopentasiloxane. (**4**) IUPAC, 2-(1,2-dihydroxypropyl)-4,8,8-trimethyl-1-oxaspiro[2.5]oct-4-en-6-one: ^{1}H NMR (600.13 MHz, DMSO) δ 0.97 (3H, s, CH_3-C5), 1.06 (3H, CH_3-C5), 1.07 (3H, s, $^3J_{13,12}$ = 6.48 Hz, CH_3-C12), 1.74 (3H, s, CH_3-C3), 2.24 (1H, d, $^2J_{6b,6a}$ = 16.89 Hz, H6b), 2.74 (1H, d, H6a), 3.03 (1H, d, $^3J_{10,11}$ = 8.76 Hz, H10), 3.75 (1H, m, H12), 3.81 (1H, m, H11), 4.78 (1H, d, $^3J_{12a,12}$ = 5.28, OH-C12), 5.18 (1H, d, $^3J_{11a,11}$ = 6.06 Hz, OH-C11), 6.00 (1H, s, H2); ^{13}C NMR (150.92 MHz, DMSO) δ 17.45 (CH_3-C3), 18.97 (CH_3-C12), 25.45 (CH_3-C5), 26.26 (CH_3-C5), 36.82 (C5), 50.24 (C6), 65.73 (C10), 66.49 (C4), 68.12 (C12), 70.57 (C11), 128.37 (C2), 162.50 (C3), 192.85 (C1); UV (254 nm) purity 78%. (**5**) IUPAC, 5-methyl-3-furancarboxylic acid: ^{1}H NMR (600.13 MHz, DMSO) δ 2.24 (3H, s, CH_3-C2), 6.24 (1H, s, H3), 7.97 (1H, s, H4); ^{13}C NMR (150.92 MHz, DMSO) δ 21.1 (CH_3-C2), 112.1 (C3), 139.3 (C4), 145.5 (C5), 165.0 (C2), 174.0 (COOH-C5); not enough isolate to determine any purity. (**6**) IUPAC, acetyl-2-hydroxy-4-(2-methoxyphenyl)-4-oxobutanat: ^{1}H NMR (600.13 MHz, DMSO) δ 1.91 (3H, s, CH_3-C13), 3.18 (1H, dd, $^2J_{9a,9b}$ = 16.7 Hz, H9b), 3.30 (1H, m, $^3J_{9a,10}$ = 4.9 Hz, H9a), 3.88 (3H, s, OCH_3-C2), 4.41 (1H, t, $^3J_{10,9b}$ = 7.5 Hz, H10), 5.34 (1H, br s, H11), 7.03 (1H, t, H5), 7.17 (1H, t, H3), 7.55 (2H, t, H4, H6); ^{13}C NMR (150.92 MHz, DMSO) δ 21.11 (C14), 48.03 (C9), 55.85 (OCH_3-C2), 66.74 (C10), 112.5 (C3), 120.54 (C5), 127.55 (C7), 129.71 (C4), 133.94 (C6), 158.32 (C2), 172.07 (C13), 175.13 (C12), 198.63 (C8); UV (254 nm) purity 85%. (**7**) IUPAC, 3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione: ^{1}H NMR (600.13 MHz, DMSO) δ 2.19 (3H, s, CH_3-C12), 3.15 (1H, dd, H9b), 3.34 (1H, m, H9a), 3.88 (3H, s, OCH_3-C2), 4.34 (1H, s, $^3J_{10,9b}$ = 6.6 Hz, H10), 5.50 (1H, s, $^3J_{10,11}$ = 5.4 Hz, H11), 7.03 (1H, t, H5), 7.17 (1H, d, H3), 7.55 (2H, m, $^4J_{4,6}$ = 1.8 Hz, H4, H6); ^{13}C NMR (150.92 MHz, DMSO) δ 26.04 (C13), 47.38 (C9), 55.83 (OCH_3-C2), 73.02 (C10), 112.48 (C3), 120.50 (C5), 127.51 (C7), 129.71 (C4), 133.93 (C6), 158.28 (C2), 198.87 (C8), 210.84 (C12); UV (254 nm) purity 67%.

dx.doi.org/10.1021/jf300888q | J. Agric. Food Chem. 2012, 60, 7229−7237

standard substances were not commercially available, the presumed peaks were isolated from manuka honey and the structure was verified by NMR spectroscopy.

To the best of our knowledge 5-methyl-3-furancarboxylic acid (**5**), acetyl-2-hydroxy-4-(2-methoxyphenyl)-4-oxobutanat (**6**), and 3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione (**7**) were detected in honey for the first time. In this, compound **6** was not assigned as characteristic for manuka honey. It showed a remote UV activity and thus contributed marginally to the UPLC pattern. Furthermore, the detected amounts of 5.5−35 mg/kg were in the same range of those determined for heather honey (6.5−16.1 mg/kg).

The structures of compounds **5**−**7** were likewise identified via NMR analyses. As the isolated fractions contained slight impurities, it was not possible to determine each coupling constant. The numbering of the structures is independent of the IUPAC numbering.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Our sincere thanks go to Intertek Food Services GmbH, Dr. K.-P. Raezke (Applica, Bremen, Germany), and Quality Services International GmbH, Dr. C. Luellmann (QSI Bremen, Germany), who provided the honey samples.

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