JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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J. Agric. Food Chem., **Just Accepted Manuscript** • Publication Date (Web): 18 Jun 2014

Just Accepted

Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

TECHNISCHE UNIVERSITÄT DRESDEN

Faculty of Mathematics and Science

Food Chemistry
Professorship of Special Food Chemistry and Food Production

Differentiation of Manuka Honey from Kanuka Honey and from Jelly Bush Honey using HS-SPME-GC/MS and UHPLC-PDA-MS/MS

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ABSTRACT

In the present study, pollen-identical pure manuka and kanuka honeys and an Australian jelly bush honey were analyzed for the non-volatiles by UHPLC-PDA-MS/MS and for the volatiles by HS-SPME-GC/MS. A chromatographic profile matchup by means of characteristic marker compounds achieved a clear discrimination between manuka, kanuka, and jelly bush honey. UHPLC-PDA profiles of manuka honey show leptosin, acetyl-2-hydroxy-4-(2-methoxyphenyl)-4-oxobutanat, 3-hydroxy-1-(2-methoxyphenyl)-penta-1,4-dion, kojic acid, 5-methyl-3-furancarboxylic acid, and two unknown compounds as prominent, kanuka honey was characterized by 4-methoxyphenyllactic acid, methyl syringate, p-anisic acid, and lumichrome. 2-methylbenzofuran, 2'-hydroxyacetophenone, and 2'-methoxyacetophenone were markant volatiles for manuka honey, kanuka honey was characterized by 2,6,6-trimethyl-2-cyclohexene-1,4-dione, phenethyl alcohol, p-anisaldehyde, and an unknown compound in HS-SPME-GC/MS. The jelly bush honey differed from the manuka honey by higher contents of 2-methoxybenzoic acid and an individual unknown substance in the PDA profile and by lower intensities of 2'-methoxyacetophenone and higher concentrations of cis-linalool oxide and 3,4,5-trimethylphenol in the HS-SPME-GC/MS profile.

KEYWORDS: monofloral honey, UHPLC-PDA-MS/MS, HS-SPME-GC/MS, non-volatile compounds, volatile compounds, *Leptospermum scoparium* J.R. et G. Forst, *Kunzea ericoides* (A. Rich.) J. Thompson, *Leptospermum polygalifolium* Salisb.

INTRODUCTION

The New Zealand manuka honey of the species *Leptospermum scoparium* J.R. et G. Forst is currently traded as one of the most medically effective honeys due to its outstanding antibacterial activity. Mostly, this is attributed to hydrogen peroxide formed by glucose oxidase or to factors such as high concentrations of sugar (osmolarity), low pH, or the bee defensin-1, which is a proteinogenic inhibin.[1-5] Nevertheless, the high antibacterial activity of manuka honey is attributed, for the main part, to substantial quantities of the 1,2-dicarbonyl compound methylglyoxal (MGO).[3,4] This so-called non-peroxide antibacterial activity is advertised to the consumer by indicating the UMF® (Unique Manuka Factor) whereby the designated level and the price correlate positively. The value of the UMF is determined using a test system developed by Molan and his co-workers[6,7] and specifies the antibacterial effectiveness of the honey compared to a defined phenol solution. According to Kwakman et al.[4], further constituents of the manuka honey beside MGO contribute to the non-peroxide antibacterial effect. The authors detected rather considerable residual antibacterial activity in manuka honey after the deactivation of the MGO.

The great commercial relevance attributed to manuka honey as well as its limited availability has led to more manuka honey being sold on the market than actually produced, so blending and adulteration has to be assumed. Therefore, the New Zealand Government (Ministry for Primary Industries)[8] and the Official Unique Manuka Factor Honey Association (UMFHA)[9] have postulated methods to authenticate the true monofloral manuka honey in order to protect New Zealand honey from adulteration.

In the case of manuka honey, the microscopic pollen analysis (melissopalynology) foremost used for honey authentication comes to its limits as manuka pollen and kanuka (*Kunzea ericoides* (A. Rich.) J. Thompson) pollen are identical. Furthermore, in contrast to manuka honey, kanuka honey shows only weak non-peroxide antibacterial activity.[7] And as it is relatively easy to upgrade this honey by adding MGO or its precursor dihydroxyacetone, which will be converted to MGO after a short storage time, the determination of the MGO content is not really helpful.[8] These issues initiated the New Zealand project "to avoid the fake manuka honey".[10] A differentiation of the pollen-identical manuka- and kanuka honeys requires distinct, as yet missing differentiation parameters.

Therefore, in order to succeed in the authentication, chemical analytical markers must be worked out for the individual monofloral honeys. In the past, promising approaches were carried out by means of analyzing the non-volatile ingredients as well as volatile aromatic compounds. So, Oelschlaegel et al.[11] investigated 40 commercial manuka honeys. After the UHPLC analyses of the phenolic acids, flavonoids, norisoprenoides, and nitrogen-containing compounds, these honeys could be divided into three groups due to their different PDA profiles generated by prominent peaks. The first group was marked by 4-hydroxybenzoic acid, dehydrovomifoliol, and benzoic acid; in contrast, group 2 was dominated by kojic acid and 2-methoxybenzoic acid and a third group was characterized by syringic acid, 4-methoxyphenyllactic acid as well as methyl syringate (Figure 1). Impressively, the analyses of the volatiles by HS-SPME-GC/MS resulted in the same three groups analogous to the UHPLC-PDA profiles.[12,13] Benzaldeyhde, alpha-isophorone, 3,4,5-trimethylphenol, benzoic acid, and phenylacetic acid were specific for group 1 (Figure 2). The honeys of group 2 contained the highest intensities of 2-methylbenzofuran,

2'-hydroxyacetophenone, and 2'-methoxyacetophenone, and group 3 honeys contained cis-linalool oxide. Due to the high MGO content determined for group 2 which strongly differed from the two other groups, Oelschlaegel et al.[11] assumed that group 2 represents pure manuka honeys. For kanuka honey, 4-methoxyphenyllactic acid is said to be characteristic according to Stephens et al.[14]

The Australian native jelly bush (*Leptospermum polygalifolium* Salisb.) honey or berringa honey whose nectar plant is classified to the same genus as the manuka shrub needs to be considered as well. Its non-peroxide antibacterial activity caused by approximately the same quantities of MGO has already been noted as a New Zealand equivalent.[15,16] The fact of the same botanical classification but a different geographical origin constitutes a very important distinction. In the literature, only Yao et al.[17] investigated the two different manuka species concerning flavonoids and phenolic acids. They described myricetin, luteolin, and tricetin as main flavonoids for jelly bush honey and quercetin, isorhamnetin, luteolin, and an unknown flavanon for manuka honey. In regard to the phenolic acids, gallic acid was measured to be dominant in manuka honey, jelly bush honey should additionally contain coumaric acid.

The aim of this study was to establish chemical marker compounds for the three different monofloral honeys in order to differentiate between them. This was achieved by analyzing both the non-volatile compounds such as phenolic substances or norisoprenoides obtained by UHPLC-PDA-MS/MS and the volatile compounds by HS-SPME-GC/MS. By a subsequent comparison of the resulting PDA- and GC-MS-profiles, marker compounds could be worked out for the first time allowing for a clear

differentiation of the three honeys derived from monocultures among themselves on the basis of chemometrics.

MATERIAL AND METHODS

Honey Samples. Eight monofloral manuka honeys (New Zealand), seven monofloral kanuka honeys (New Zealand), and one jelly bush honey (Australia) were received from UMFHA, which also performed pollen-analytical studies. According to the information obtained from UMFHA, the monofloral honeys were harvested in regions with only one of the three honey cultivars in 2012. All the samples were stored in a refrigerator at 6 °C.

Chemicals. Methanol (HPLC grade and LC-MS grade) and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Schwerte, Germany). Daidzein standard, 2'-hydroxyacetophenone, 2'-methoxyacetophenone, and 2,6,6-trimethyl-2-cyclohexene-1,4-dione were acquired from Alfa Aesar (Karlsruhe, Germany). The 4-methoxy-^{13}C, d_3-benzoic-2,3,5,6-d_4 acid as internal standard for LC-MS/MS was supplied by Isotec via Sigma Aldrich (Steinheim, Germany). Acetic acid 100% glacial was from Fluka via Sigma-Aldrich (Steinheim, Germany). Sodium chloride was purchased from VWR International (Darmstadt, Germany) and benzaldehyde-d_6, phenethyl alcohol, cis-linalool oxide, p-anisaldehyde, and 2-methyl-6-propyl phenol from Sigma-Aldrich (Steinheim, Germany). 3,4,5-trimethylphenol and 2-methylbenzofuran were purchased from Dr. Ehrenstorfer GmbH via VWR International (Darmstadt, Germany). All the chemicals used in this study were of analytical grade. Bidistilled water was generated by the Bi-Distilling Apparatures Bi 18E QSC GmbH (Maintal, Germany).

Head Space Solid Phase Microextraction (HS-SPME). The HS-SPME-GC/MS was carried out as described by Oelschlaegel et al.[12] Briefly, honey was homogenized with sodium chloride and water in a SPME vial. Deuterated benzaldehyde was used as internal standard. Before measuring, the DVB/CAR/PDMS fiber which is described

as the most suitable fiber for honey volatiles was conditioned.[18] Subsequently, the analytes were desorbed in the PTV injector. The HS-SPME holder was part of a Combi PAL (CTC Analytics, Zwingen, Swiss).

GC/MS Detection. A Thermo Scientific Trace GC Ultra interfaced with a Thermo Scientific DSQ II (Thermo Fisher, Dreieich, Germany) was used. The MS was operated in the positive EI mode at 70 eV, the mass range was m/z 50-600. EI source temperature was set at 200 °C. Spectral data recording started only after 4 min due to the huge solvent peak. Identification of the compounds based on a comparison of the MS spectra with those of the NIST-08-mass spectral library, with linear retention indices (LRI) and with reference substances (retention time, MS spectrum).

UHPLC-PDA-MS/MS Detection.

Prior to the UHPLC analyses, a solid phase extraction was carried out and has already been described.[11] Briefly, 2 g of honey were dissolved in 2% sodium chloride (pH 2-3) with subsequent SPE clean-up using Chromabond® cartridges. Chromatographic analyses were performed with an Acquity Ultra Performance UPLC-PDA system (Waters, Eschborn, Germany) on a Nucleodur C18 column with a water/methanol/formic acid gradient within 20 min. Mass spectrometric investigations were carried out using a coupled TSQ Quantum Access MAX (Thermo Fisher, Dreieich, Germany). The identification of the compounds was realized via retention time, UV maxima, and also timed SRM (single reaction monitoring).

Statistical Analyses. The data matrix consists of rows representing the different honeys (objects) and columns, which includes SPE-UHPLC-PDA-MS/MS data of the substances (variables) previously described as characteristic. For PCA and k-means,

144 the matrix covered 16 objects x 13 variables. For this, SPSS 15.0 was used. All data

145 were standardized (z-values) before chemometrics.

RESULTS AND DISCUSSION

Comparison of the HS-SPME-GC/MS Profiles.

The volatiles were examined in seven manuka, two kanuka, and one jelly bush honey using the HS-SPME-GC/MS method by Oelschlaegel et al.[12] Due to the rather small sample size this investigation comprised less honeys than those analyzed by the UHPLC-PDA-MS/MS method. The chromatographic profiles of the three honey sorts were compared; the resulting characteristic compounds are listed in Table 1.

In the samples of the monofloral manuka honey mostly the compounds of Oelschlaegel's group 2 were detected as well (Figure 3).[13] The following volatile substances could be confirmed as characteristic for manuka honey: 2-methylbenzofuran, 2'-hydroxyacetophenone, and 2'-methoxyacetophenone. In contrast, for kanuka honey subsequent compounds proved to be prominent: 2,6,6-trimethyl-2-cyclohexene-1,4-dione, phenethyl alcohol, p-anisaldehyde, and an unknown compound, which refers to a similarity to Oelschlaegel's group 3 (Figure 4).[13] 2,6,6-trimethyl-2-cyclohexene-1,4-dione and phenethyl alcohol, although already known in honey in general, were now described for kanuka honey for the first time.

2'-methoxyacetophenone as characteristic for manuka honey has already been reported by Daher et al.[19] whereas Tan et al.[20] described this compound for manuka/ kanuka honeys without differentiation. In one Portuguese multifloral honey 2'-methoxyacetophenone was analyzed with an equivalent high peak intensity as well, however, this honey showed a completely different MS-profile.[21] Due to our findings that this compound could only be detected in negligible intensities for kanuka honey under the chosen conditions, 2'-methoxyacetophenone can be used as a criterion distinguishing manuka honey from kanuka honey. Nevertheless, 2-methylbenzofuran

and 2'-hydroxyacetophenone are present exclusively in manuka honey in reference to the three monofloral honeys. For kanuka honeys, there are two characteristic peaks which are present in other honeys, especially in European ones, in definitely small quantities: one has been identified as anisaldehyde, the second is unknown.[22-29] The latter was first assumed to be p-propyl anisole which was proposed by Visser et al.[29] and later by Daher et al.[19] as a marker for manuka honey. However, both groups of authors identified this component solely via the NIST database; Visser et al.[29] analyzed one manuka honey, Daher et al.[19] two commercial manuka honeys.

Due to our investigations using a reference standard, p-propyl anisole could not be confirmed neither as a component in manuka nor in kanuka honey. Subsequent studies with a further suggested compound by the NIST database - with 2-methyl-6-propyl phenol as a reference substance, also failed, although the MS spectrum was the same. The investigations concerning the identification are still ongoing.

An Australian jelly bush honey also provided by UMFHA was analyzed to do a profile matchup with manuka honey as well (Table 1). Due to the notably lower intensities of 2'-methoxyacetophenone and higher concentrations of cis-linalool oxide and 3,4,5-trimethylphenol in the jelly bush honey profile, a differentiation towards manuka honey was possible (Figure 5). However, a confirmation of the distinctive features would require further analyses of more jelly bush honeys.

Comparison of the UHPLC-PDA Profiles.

In all, eight manuka-, seven kanuka- and one jelly bush honey were investigated according to the UHPLC-PDA-MS/MS method described by Oelschlaegel et al.[11] (Table 2). Comparing the PDA profiles (λ = 254 nm), an unambiguous distinction among the pure manuka and pure kanuka honeys can immediately be recognized.

194 Additionally, all the investigated samples of manuka-, kanuka-, and jelly bush honey
195 were analyzed by UHPLC-HRMS and evaluated by Braggins et al. (Analytica
196 Laboratories Ltd., Waikato, New Zealand).[30] Results were noted in Table 2.

197 Leptosin was detected as the principal analyte of manuka honey (Figure 6). In
198 contrast, kanuka honey profiles were dominated by 4-methoxyphenyllactic acid and
199 also by methyl syringate (Figure 7). Leptosin, a glycoside of methyl syringate, was
200 already described exclusively for the *Leptospermum*-species by Kato and co-writers[31]
201 and, therefore, could be confirmed as a marker for manuka honey. 4-
202 methoxyphenyllactic acid was found in manuka/ kanuka honeys by Tan et al.[20] and in
203 one of six commercial manuka honeys analyzed by Le Gresley et al.[32] According to
204 Stephens et al.[14] the substance is uniquely characteristic for kanuka honey, which, in
205 turn, corresponds to our results. A large number of other characteristic substances in
206 the profiles of pure monofloral honeys were worked out as follows: acetyl-2-hydroxy-
207 4-(2-methoxyphenyl)-4-oxobutanat, 3-hydroxy-1-(2-methoxyphenyl)-penta-1,4-dion,
208 and still unknown compounds M4 and M7 for manuka honey and, for kanuka honey
209 p-anisic acid and lumichrome (Figure 8). Kojic acid and 5-methyl-3-furancarboxylic
210 acid which are well-marked for manuka may be a further distinction criteria. Acetyl-2-
211 hydroxy-4-(2-methoxyphenyl)-4-oxobutanat and 3-hydroxy-1-(2-methoxyphenyl)-
212 penta-1,4-dion were proved exclusively for manuka honey and had been isolated and
213 subsequently identified via NMR-spectroscopy by Oelschlaegel et al.[11] Although
214 lumichrome is currently discussed controversially as a marker substance, both, for
215 the country-specific Italian thistle honey (*Galactites tomentosa* Moench), and for the
216 cornflower honey (*Centaurea cyanus*) (recent studies reported two- to threefold
217 higher contents in cornflower honey), the compound constitutes an important
218 distinguishing criterion in the manuka/kanuka comparison due to its uniqueness to
219 kanuka honey.[33-36] With a retention time similar to lumichrome, the unknown peak M7

existing in manuka honey with UV maxima at λ = 245 nm and 306 nm compared to lumichrome (λ = 259, 349 nm) has a markedly deviating UV spectrum and, after its identification, could serve as a further marker substance for manuka honey.

P-anisic acid as well as methyl syringate could be used as differentiation criteria in the profile matchup for kanuka honey versus manuka honey due to the uniqueness or to the tendentially higher peak intensities. Their comparatively low occurrence in honeys from Europe and other honey from New Zealand can be neglected.[14,26,37-41] Senanayake[42] divided his manuka honeys into active and inactive samples on the basis of their UMF contents. The inactive samples contained higher 4-methoxyphenyllactic acid and methyl syringate than the active samples. Based on our results, kanuka honey, which verifiably does not have any non-peroxide antibacterial activity, might be contained in these honeys in larger quantities. According to Oelschlaegel et al.[11] group 2 represents the true manuka honeys (Figure 6). In contrast, group 3 includes honeys with a higher proportion of kanuka honey, and honeys of group 1 very probably contains only low proportions of both manuka honey and kanuka honey (Figure 7).

Distinguishing the so-called Australian manuka honey (jelly bush honey) from the New Zealand manuka honey is also of interest. Although both honey sorts show a giant leptosin peak, the PDA-profiles are characterized by distinct differences (Figure 8). Whereas acetyl-2-hydroxy-4-(2-methoxyphenyl)-4-oxobutanat, 3-hydroxy-1-(2-methoxyphenyl)-penta-1,4-dion, and two unknown peas M4 and M7 are present in New Zealand manuka honey, the Australian manuka honey is characterized by 2-methoxybenzoic acid and an unknown substance J2 (Figure 8). Based on these substances, it is possible to achieve a differentiation of these two kinds of honey, however, the prominent compounds of jelly bush honey would have to be ascertained

by additional samples. The results reported by Yao et al.[17] who had analyzed honeys of both kinds of manuka honey could not be confirmed as already demonstrated by Oelschlaegel et al.[11]. Neither could any high contents of abscisic acid nor gallic acid, which was declared as prominent for manuka honey, nor any different flavonoids be detected.

Final assessment of honey characterization by chemometrics

A PCA was calculated with the results of UHPLC-PDA-MS/MS. So, the marker potential of the abovementioned characteristic substances for each of the three honeys was investigated. About 82% of the variation could be explained by the first two principal components (PC). Thereby, the first PC in negative is loading with lumichrome, 4-methoxyphenyllactic acid, and p-anisic acid; in positive through kojic acid, acetyl-2-hydroxy-4-(2-methoxyphenyl)-4-oxobutanat, 3-hydroxy-1-(2-methoxyphenyl)-penta-1,4-dion, unknown M4, and unknown M7 (Figure 9). If moving to the higher values of PC 2, 2-methoxybenzoic acid and unknown J2 are located. Looking at the scatter plot, the individual honeys are separated from each other. Negative values of PC 1 present the characteristic substances of kanuka honey while positive values mark the typical manuka honey compounds. The analytes which are explained by PC 2 are referring to jelly bush honey (Figure 10). A few substances, which are listed below, do not permit a clear allocation: methyl syringate, leptosin, and 5-methyl-3-furancarboxylic acid. This is caused by the fact that the compounds are not clearly assigned only to one honey and, therefore, this kind of behavior in the loading plot was to be expected (Figure 9). Nonetheless, due to their huge peak height and individuality, a further consideration is indispensable in profile matchup. These results confirm that potential marker suitability is assured.

By means of the 13 declared substances, a cluster analysis was carried out to cluster the investigated honeys on the basis of their measured data. For this, a k-means with the requirement of 3 clusters was used. Before clustering, a successful test of dissimilarity was carried out. On the basis of Euclidean distance and subsequent iteration, a corresponding classification after cluster membership was generated. The aim to classify similar honeys in one cluster was achieved. Therefore, all manuka honeys were assigned to cluster 1, all kanuka honeys were assigned to cluster 2, and the last one, cluster 3, contains only the jelly bush honey. Chemometrics confirmed the characteristic substances for each honey that had already been worked out by profile matchup, allowing for a classification in three clusters to which only one kind of honey was assigned. A definite classification of the kind of honey requires numerous further investigations with a larger number of samples as well as with samples from different years and the various areas of New Zealand.

This is now a first approach to differentiating the manuka honeys from New Zealand and Australia as well as distinguishing the kanuka honeys amongst themselves by UHPLC-PDA-MS/MS and HS-SPME-GC/MS profile matchup.

ABBREVIATIONS USED

jell, jelly bush honey; kan, kanuka honey; man, manuka honey; MGO, methylglyoxal; PC, principal component

ACKNOWLEDGEMENTS

We thank UMFHA for providing the samples. Our sincere thanks go to Gesa Heuser (TU Dresden, Germany) for sample preparation.

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Figure Captions

Figure 1: UHPLC-PDA-MS/MS example profiles, λ = 272 nm: group classification extracted from the publication by Oelschlaegel et al.[11] of commercially available manuka honeys; numbered peaks are characteristic compounds of each group; (A) group 1 (25%), (B) group 2 (25%), (C) group 3 (50%); (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

Figure 2: HS-SPME-GC/MS example profiles: group classification of commercially available manuka honeys according to Oelschlaegel et al.[13]; (A) group 1 (25%), (B) group 2 (50%), (C) group 3 (25%); (1) cis-linalool oxide, (2) benzaldehyde, (3) alpha-isophorone, (4) 2-methylbenzofuran, (5) 2'-hydroxyacetophenone, (6) 2'-methoxyacetophenone, (7) 3,4,5-trimethylphenol, (8) benzoic acid, (9) phenylacetic acid; * peak cut

Figure 3: HS-SPME-GC/MS profiles: comparison of Oelschlaegels's group 2[13] (B) with pure manuka honeys (Mi), (Mii); (4) 2-methylbenzofuran (5) 2'-hydroxyacetophenone (6) 2'-methoxyacetophenone; * peak cut

Figure 4: HS-SPME-GC/MS profiles: comparison of Oelschlaegels's group 3[13] (C) with pure kanuka honeys (Ki), (Kii); (1) cis-linalool oxide, (10) 2,6,6-trimethyl-2-cyclohexene-1,4-dione, (11) phenethyl alcohol, (12) p-anisaldehyde, (13) unknown; * peak cut

Figure 5: HS-SPME-GC/MS profiles: comparison of pure kanuka honey (K), pure manuka honey (M) and pure jelly bush honey (J); (1) cis-linalool oxide, (4) 2-methylbenzofuran, (5) 2'-hydroxyacetophenone, (6) 2'-methoxyacetophenone, (7)

427 3,4,5-trimethylphenol, (10) 2,6,6-trimethyl-2-cyclohexene-1,4-dione, (11) phenethyl
428 alcohol, (12) p-anisaldehyde, (13) unknown; * peak cut

429 Figure 6: UHPLC-PDA-MS/MS profiles (254 nm): comparison of Oelschlaegels's
430 group 2[11] (B) with pure manuka honeys (Mi); (Mii); (B1=M1) kojic acid, (B2) benzoic
431 acid, (M2) 5-methyl-3-furancarboxylic acid, (M3) leptosin, (M4) unknown, (M5) acetyl-
432 2-hydroxy-4-(2-methoxyphenyl)-4-oxobutanat, (M6) 3-hydroxy-1-(2-Methoxyphenyl)-
433 penta-1,4-dion, (M7) unknown; * peak cut

434 Figure 7: UHPLC-PDA-MS/MS profiles (254 nm): comparison of Oelschlaegels's
435 group 3[11] (C) with pure kanuka honeys (Ki), (Kii); (C1) syringic acid, (C2=K1) 4-
436 methoxyphenyllactic acid, (C3=K3) methyl syringate, (K2) p-anisic acid, (K4)
437 lumichrome; * peak cut

438 Figure 8: UHPLC-PDA-MS/MS profiles (254 nm): comparison of pure kanuka honey
439 (K) with pure manuka honey (M) and pure jelly bush honey (J); (K1) 4-
440 methoxyphenyllactic acid, (K2) p-anisic acid, (K3) methyl syringate, (K4) lumichrome,
441 (M1) kojic acid, (M2) 5-methyl-3-furancarboxylic acid, (M3) leptosin, (M4) unknown,
442 (M5) acetyl-2-hydroxy-4-(2-methoxyphenyl)-4-oxobutanat, (M6) 3-hydroxy-1-(2-
443 methoxyphenyl)-penta-1,4-dion, (M7) unknown, (J1) 2-methoxybenzoic acid, (J2)
444 unknown; * peak cut

445 Figure 9: Loading plot (varimax rotation) for the first two principal components (PC1
446 with 61.9% of the total variance and PC2 19.6% of the total variance) of the
447 determined UHPLC-PDA-MS/MS data; (K1) 4-methoxyphenyllactic acid, (K2) p-anisic
448 acid, (K3) methyl syringate, (K4) lumichrome, (M1) kojic acid, (M2) 5-methyl-3-
449 furancarboxylic acid, (M3) leptosin, (M4) unknown 1, (M5) acetyl-2-hydroxy-4-(2-

methoxyphenyl)-4-oxobutanat, (M6) 3-hydroxy-1-(2-methoxyphenyl)-penta-1,4-dion, (M7) unknown 3, (J1) 2-methoxybenzoic acid, (J2) unknown 2

Figure 10: PCA score plot of manuka honeys (man), kanuka honeys (kan) and the jelly bush honey (jell) of the determined UHPLC-PDA-MS/MS data; number = sample number

Table 1: HS-SPME-GC/MS: Characteristic Compounds of Monofloral Honey Samples, Including LRI and Identification Method (via Reference Standard: STD, via NIST Library: MS); Percentage of Peak Area in Total Peak Area, Average of n Samples (- not detected; + 0-1%; ++ >1-5%, +++ >5-45%)

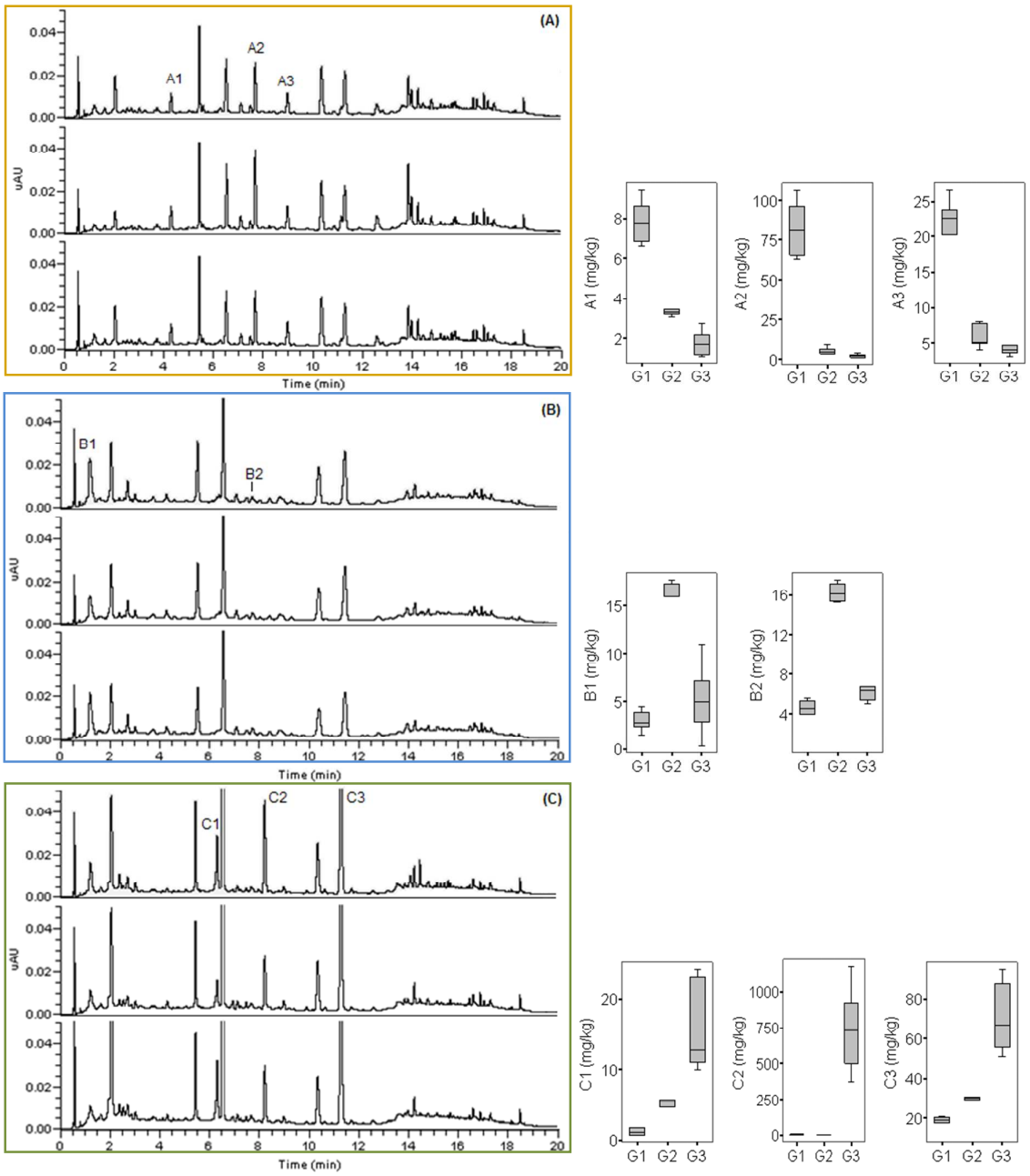
compound	LRI FFAP	identification	manuka (n = 7)	kanuka (n = 2)	jelly bush (n = 1)
cis-linalool oxide	1443	STD, LRI	+	++	+++
2-methylbenzofuran	1579	STD	++	-	-
2,6,6-trimethyl-2-cyclohexene-1,4-dione	1676	STD, LRI	-/+	++	+
2'-hydroxyacetophenone	1785	STD	+	-	-
phenethyl alcohol	1892	STD, LRI	-/+	++	-
2'-methoxyacetophenone	1979	STD	+++	+	+
p-anisaldehyde	2013	STD, LRI	-/+	+++	-
unknown	2056	-	-	+++	-
3,4,5-trimethylphenol	> 2100	STD	-/+	+	+++

Table 2: UHPLC-PDA-MS/MS: Characteristic Compounds of Monofloral Honey Samples, Including Retention Time and UV Maxima; Percentage of Peak Area in Total Peak Area at λ = 254 nm, Average of n Samples (- not detected, + 0-1%; ++ >1-2%, +++ >2-6%, ++++ >6-35%)

compound[a]	t_R (min)	UV_{max} (nm)	manuka n = 8	kanuka n = 7	jelly bush n = 1
kojic acid	1.13	266	+++	+	++
5-methyl-3-furancarboxylic acid[b]	2.68	266	+	-/+	++
leptosin[c,e]	6.52	263	++++	++	++++
unknown M4	6.90	252, 309	+	-	-
2-methoxybenzoic acid[e]	7.53	295	++	+	++++
acetyl-2-hydroxy-4-(2-methoxyphenyl)-4-oxobutanat[d]	8.02	249, 309	+	-	-
4-methoxyphenyllactic acid	8.18	274	-/+	++++	-
3-hydroxy-1-(2-Methoxyphenyl)-penta-1,4-dion[b,e]	9.32	249, 309	++	-	-
unkown J2	10.55	245	-	-	+++
p-anisic acid	10.73	256	-	+++	-
methyl syringate	11.50	274	+++	++++	+++
lumichrome[f]	12.79	259, 349	-	+++	-
unkown M7	13.96	245, 306	++	-	-

[a] Identification via reference standard; [b] Identification via isolated standard was proven by NMR and MS; [c] MS/MS identification via m/z 559 = $[M+Na+H]^+$; [d] MS/MS identification via 225 = $[M-Ac+H]^+$; [e] Described as prevalent for manuka honey and jelly bush honey by Analytical Laboratories Ltd., Waikato, New Zealand; [f] Described as prevalent for kanuka honey by Analytical Laboratories Ltd., Waikato, New Zealand

Figure 1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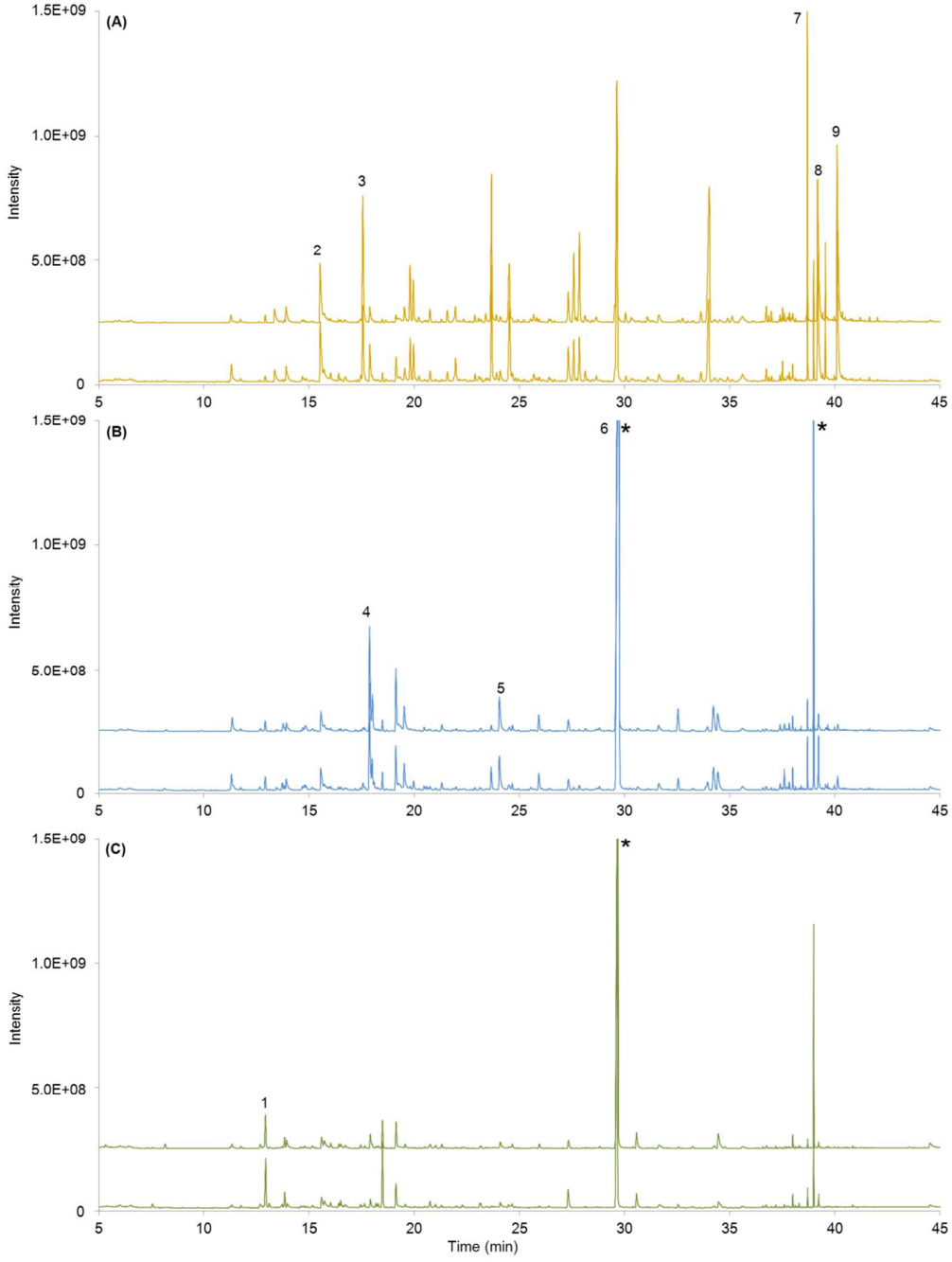
A1
A2
A3
uAU
Time (min)
A1 (mg/kg)
A2 (mg/kg)
A3 (mg/kg)
G1 G2 G3
(B)
B1
B2
B1 (mg/kg)
B2 (mg/kg)
(C)
C1
C2
C3
C1 (mg/kg)
C2 (mg/kg)
C3 (mg/kg)

Figure 2


(A)
(B)
(C)
Intensity
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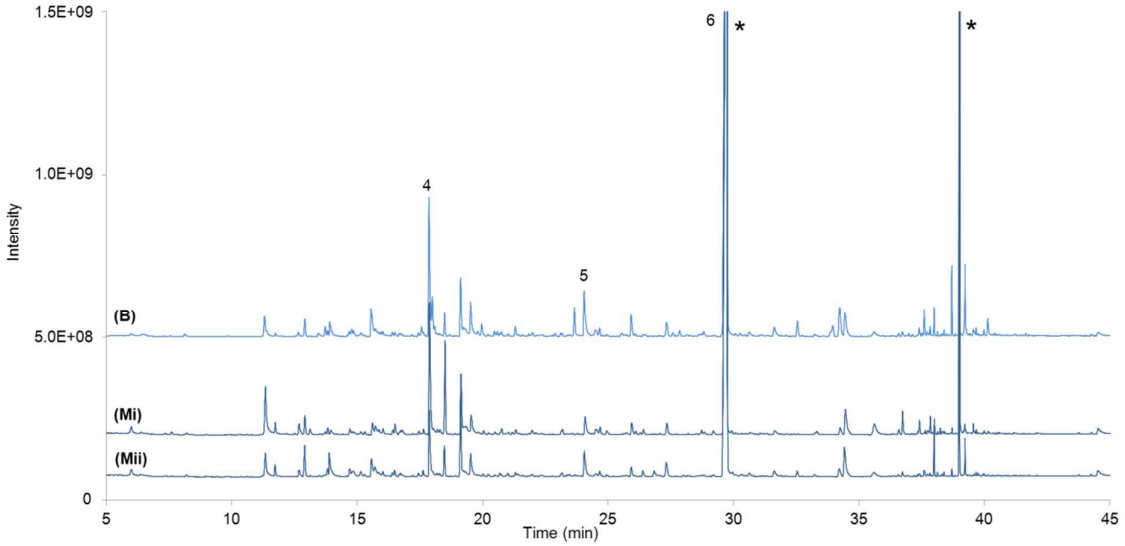
1.0E+09
5.0E+08
0
1
2
3
4
5
6
7
8
9
*
5
10
15
20
25
30
35
40
45
Time (min)

Figure 3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1.0E+09
5.0E+08
0
Intensity
(B)
(Mi)
(Mii)
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5
6
*
*
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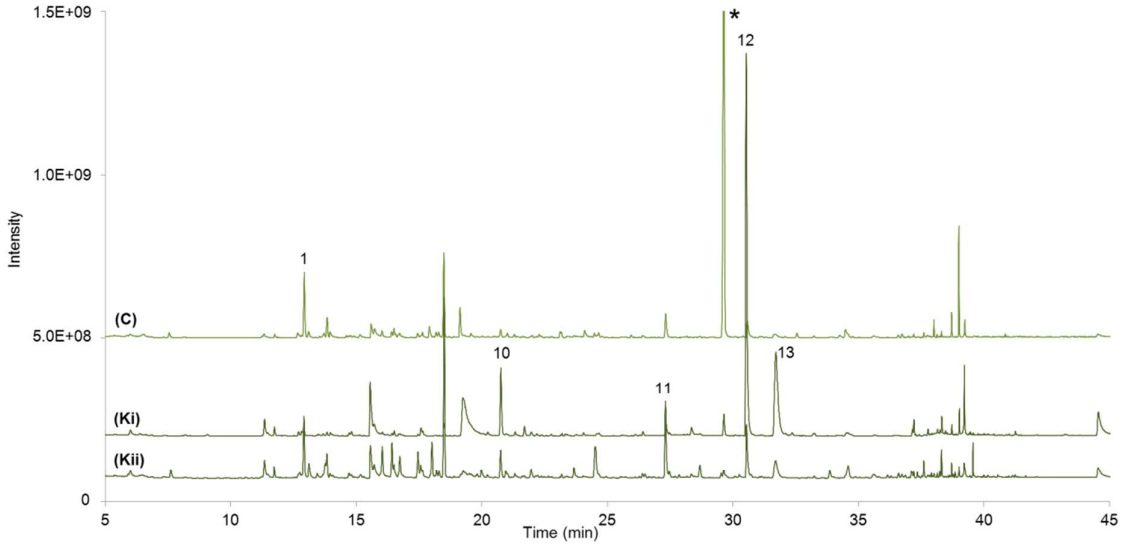
10
15
20
25
30
35
40
45
Time (min)

Figure 4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(C)
(Ki)
(Kii)
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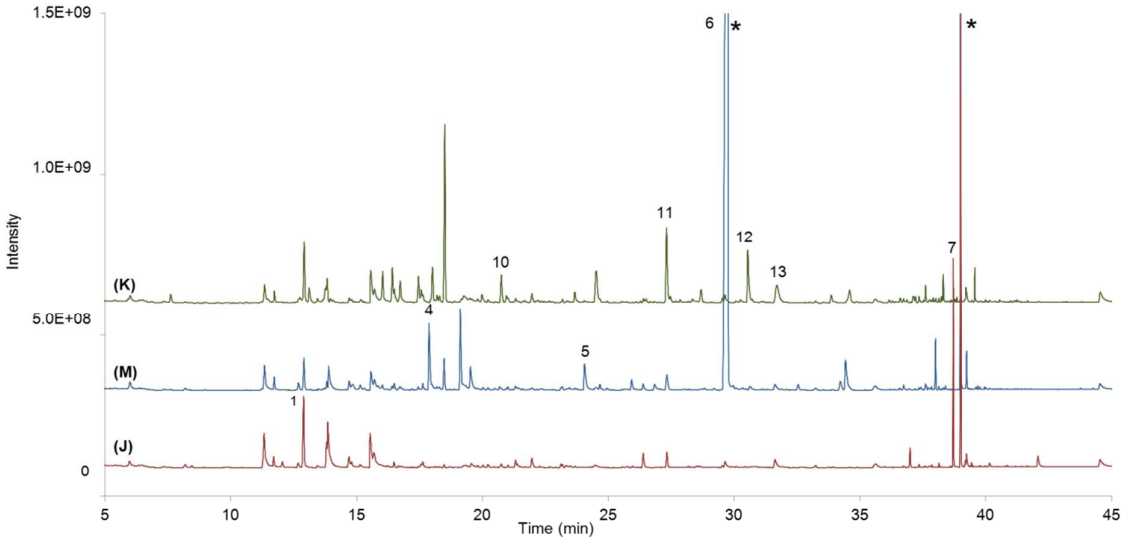
5
10
15
20
25
30
35
40
45
Time (min)

Figure 5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5.0E+08
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Intensity
(K)
(M)
(J)
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4
5
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*
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12
13
7
*
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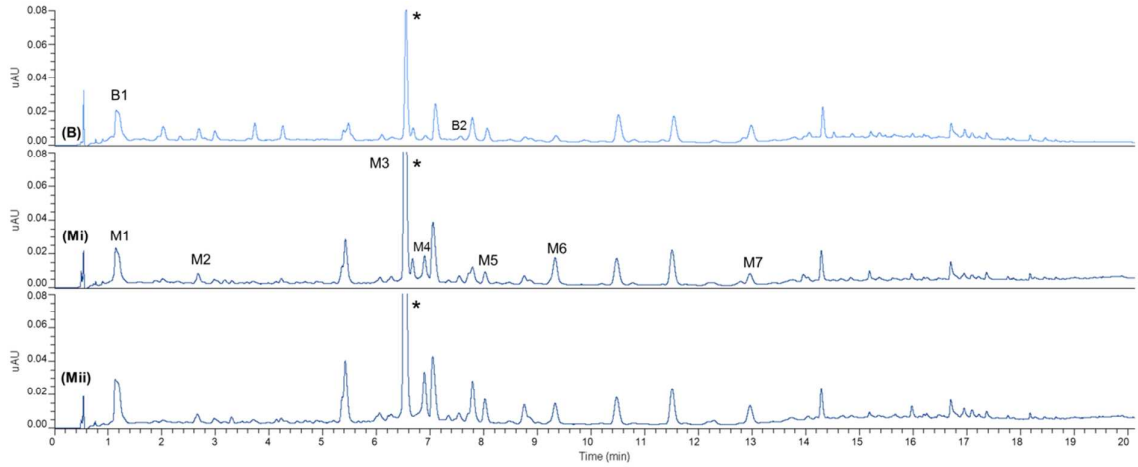
30
35
40
45
Time (min)

Figure 6



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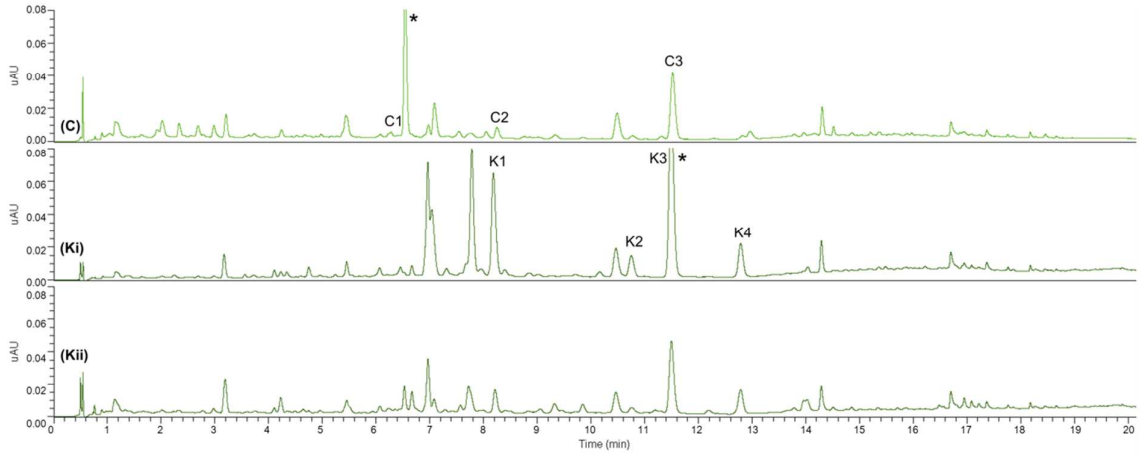

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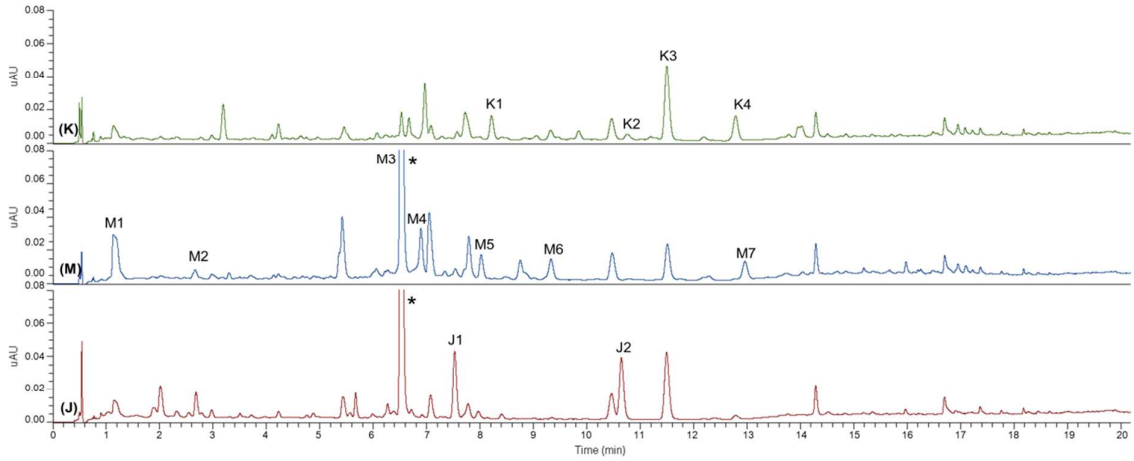


Figure 9

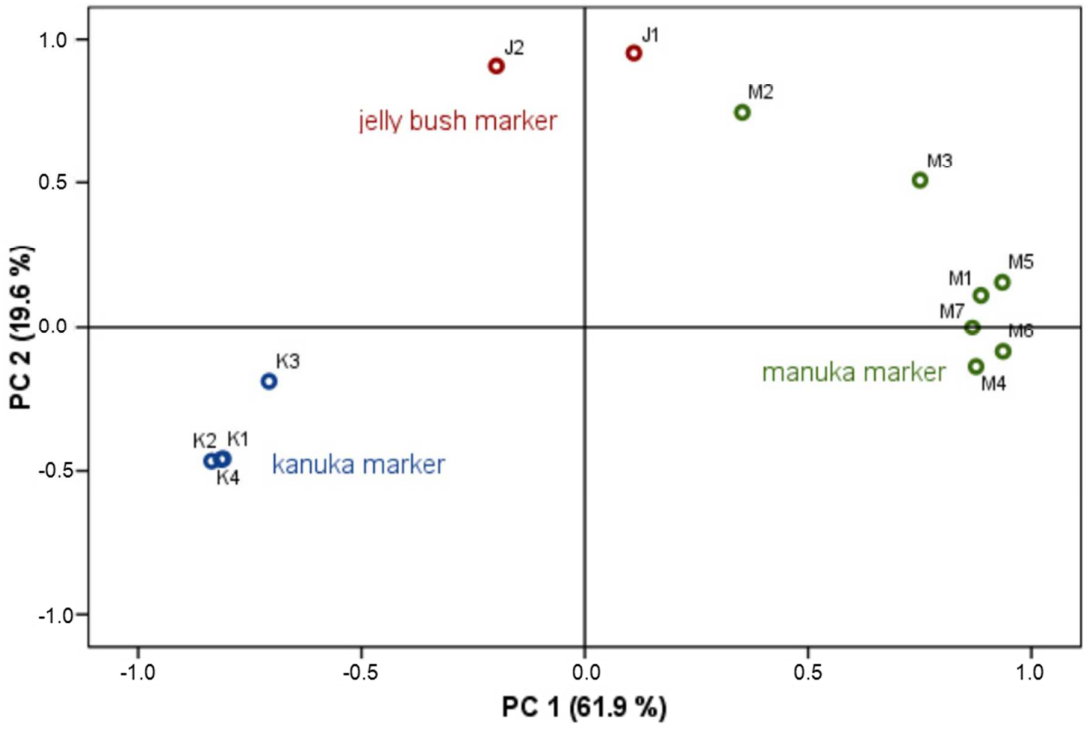
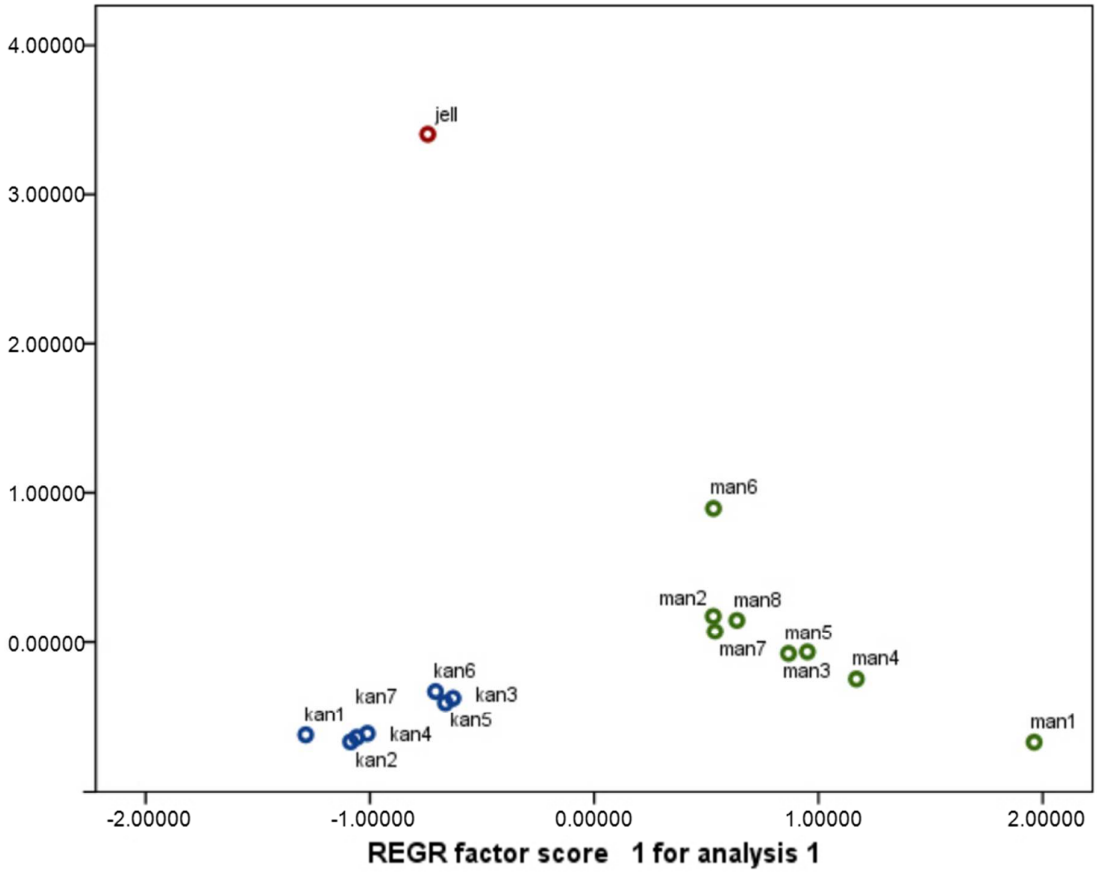

J2
J1
M2
jelly bush marker
M3
M5
M1
M7
M6
K3
manuka marker
M4
K2
K1
K4
kanuka marker
1.0
0.5
0.0
-0.5
-1.0
PC 2 (19.6 %)
-1.0
-0.5
0.0
0.5
1.0
PC 1 (61.9 %)

Figure 10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3.00000
2.00000
1.00000
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man2
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kan6
kan7
kan3
kan1
kan5
kan4
kan2
-2.00000
-1.00000
0.00000
1.00000
2.00000
REGR factor score 1 for analysis 1

Table of Contents