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[Continued on next page]

(54) Title: MARKER COMPOUNDS OF LEPTOSPERMUM HONEYS AND METHODS OF ISOLATION AND ASSAYING THEREOF

(57) Abstract: Described herein are novel isolated compounds from Leptospermum honey and methods of assay thereof for use in the verification of the place of origin, authenticity and content of Leptospermum honeys such as mānuka honey. The inventors screened flower nectar and honeys of various floral types found in New Zealand to identify chemicals that were either unique to or in significantly higher concentrations in mānuka nectar and mono-floral mānuka honey compared to other predominantly mono-floral nectars and honeys. As a result of the screening exercise, the inventors discovered a marker compounds that could distinguish Leptospermum honey nectar and honey from other floral sources.

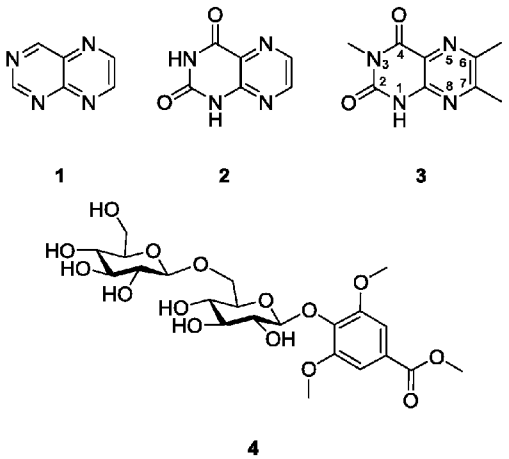

FIGURE 1

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MARKER COMPOUNDS OF LEPTOSPERMUM HONEYS AND METHODS OF ISOLATION AND ASSAYING THEREOF

RELATED APPLICATIONS

This application derives priority from New Zealand patent application number 715094 incorporated herein by reference.

TECHNICAL FIELD

Described herein are marker compounds of *Leptospermum* honeys, novel isotopically labelled marker compounds of *Leptospermum* honeys and methods of isolation, chemical synthesis and assaying thereof, for use in the verification of the quality and purity of *Leptospermum* honeys such as Mānuka honey.

BACKGROUND ART

The use of honey to augment the healing of wounds was first documented by the ancient Egyptians[1] and is currently a clinical wound treatment[2,3,4,5,6,7]. New Zealand mānuka honey, derived from the nectar of *Leptospermum scoparium*, demonstrates non-peroxide based antibacterial activity largely due to the presence of methyl glyoxal[2-6, 8]. Mānuka honey is active against methicillin-resistant *Staphylococcus aureus* (MRSA)[9, 10] and increases the susceptibility of MRSA to rifampicin[11] and oxacillin[12].

Currently, genuine mānuka honey is identified using the Unique Mānuka Factor (UMF) scale, which equates the bactericidal activity of a given honey sample with that of a given concentration of phenol[13]. Nonetheless, lack of clarity regarding what constitutes genuine mānuka honey has left the industry susceptible to counterfeiting[14, 15, 16]. Mānuka honey is central to the growth of the New Zealand honey industry, whose exports totalled $145 million (NZD) in 2013[17]. Due to its medical and economic importance, compounds that could serve as unique markers for genuine mānuka honey are of scientific and commercial importance.

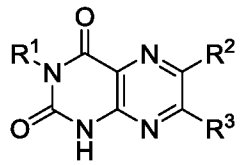
It is an object of the present invention to address the foregoing problems or at least to provide the public with a useful choice in the quality assurance of *Leptospermum* honeys to ensure that it is true to labelled specifications in terms of nutritional and/or medical potency of the honey.

Further aspects and advantages of the process and product will become apparent from the ensuing description that is given by way of example only.

SUMMARY OF THE INVENTION

Pteridines (**1**, Figure 1) are derivatives of the pyrazine[2,3-*d*]pyrimidine ring system, the first examples of which were isolated from butterfly wings[18, 19]. 2,4-Diketopteridines are known as lumazines (**2**), examples of which have been isolated from a range of organisms[20, 21, 22, 23, 24]. Herein we report the isolation, structural elucidation, and synthesis of lepteridine (**3**)[25], a known pteridine derivative from *Leptospermum* honey, with the systematic name 3,6,7-trimethyllumazine[26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36].

In a first aspect there is provided a marker compound of Type I isolated from a *Leptospermum* honey and represented by the following structural formula **I**:

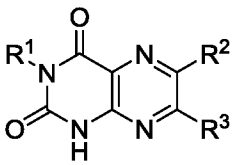


I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

The phrase '*Leptospermum* honey ' or grammatical variations thereof refers to flowers, nectar or honey of the *Leptospermum* plant species including *Leptospermum scoparium, Leptospermum scoparium var. exinium, Leptospermum polygalifolium, Leptospermum subtenue*.

In a second aspect there is provided a marker compound of Type II represented by the following structural formula **I**:



I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group where the compound of Type II contains at least one isotope not having an identical atomic mass to that of the most abundantly occurring isotope of that element in nature.

The term "isotope" refers to an isotope not having an identical atomic mass to that of the most abundantly occurring isotope of that element in nature, which is understood to have been introduced by means known to those skilled in the art of organic synthesis from commercially available isotopically enriched starting materials. Examples of isotopes not having an identical atomic mass to that of the most abundantly occurring isotope of that element in nature include but are not limited to ^2H, ^{13}C and ^{15}N.

In a third aspect there is provided a method of isolation of at least one compound of the Type I as described above, comprising the following step:

a. a chromatography step in which a fraction is collected by eluting a solution of a honey derived from nectar collected from a *Leptospermum* flower with at least one elution solvent.

The term 'comprise' and grammatical variations thereof shall have an inclusive meaning - i.e. that it will be taken to mean an inclusion of not only the listed components it directly references, but also other non-specified components or elements.

In a fourth aspect there is provided a method of assaying and quantifying a *Leptospermum* honey comprising the following steps:

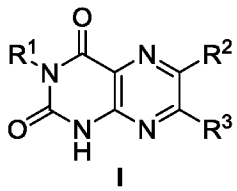
a. deriving a calibration curve for the concentration of a compound of Type II using mass spectrometry of the *Leptospermum* honey;
b. generating a calibration curve mass spectrum of a *Leptospermum* honey that contains a known concentration of at least one compound of Type II as an internal standard; and
c. deriving the concentration of at least one compound of Type I native to the *Leptospermum* honey via interpolation using the calibration curve generated in step (a) and the mass spectrum generated in step (b).

In a fifth aspect there is provided a method of assaying and quantifying a *Leptospermum* honey, comprising the following steps:

a. subjecting a Leptospernum honey to a stimulus sufficient to cause fluorescence of at least one compound of Type I as described above present in the Leptospernum honey; and
b. measuring an amount of fluorescence of the at least one compound of the Type I in the Leptospernum honey.

In a sixth aspect there is provided a test kit for testing the purity of a *Leptospermum* honey, the kit comprising at least one compound of Type I or Type II as described above.

In a seventh aspect there is provided the use of at least one compound of Type I isolated from *Leptospermum* honey and represented by the following structural formula **I**:

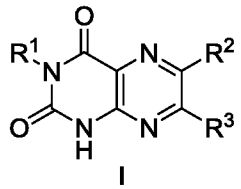


I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group in the manufacture of a medicament for the treatment of disease.

In an eighth aspect there is provided a composition comprising of at least one compound of Type I isolated from a *Leptospermum* honey and represented by the following structural formula **I**:

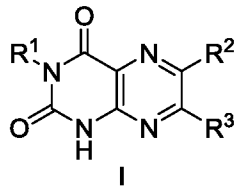
or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or



I

$-C_4H_9$ alkyl group for use in the treatment of wounds.

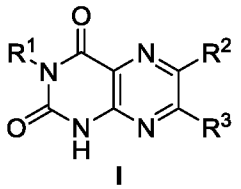
In a ninth aspect there is a provided a food supplement to improve physiological oxidative stress comprising at least one compound of Type I isolated from a *Leptospermum* honey and represented by the following structural formula I:



I

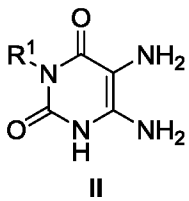
or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

In a tenth aspect there is provided the manufacture of at least one compound represented by the following structural formula I:



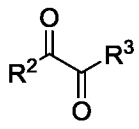
I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group produced via a condensation of an intermediate of the following structural formula **II**



II

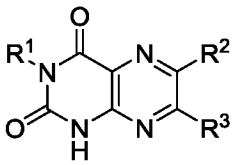
wherein R^1 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group with a compound of the following structural formula **III**:



III

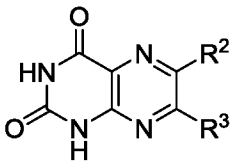
wherein R^2 and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

In an eleventh aspect there is provided the manufacture of at least one compound represented by the following structural formula I:



I

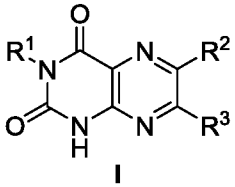
or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group produced via an alkylation of the compound represented by the following structural formula IV at position N-3:



IV

wherein R^2 and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

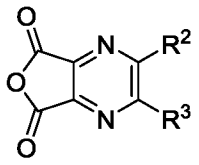
In a twelfth aspect there is provided the manufacture of at least one compound represented by the following structural formula I:



I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-$

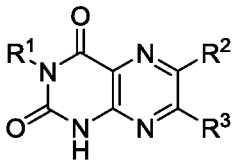
C_4H_9 alkyl group produced via the generation of a transient isocyanate species from a compound of the following structural formula V:



V

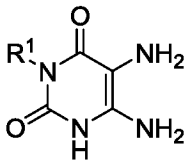
wherein R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

In a thirteenth aspect there is provided the manufacture of at least one compound of Type II represented by the following structural formula I:



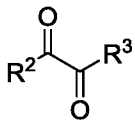
I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group, wherein the compound of Type II contains at least one isotope produced via a condensation of an intermediate of the following structural formula **II**:



II

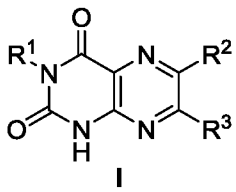
wherein R^1 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group with a compound of the following structural formula **III**:



III

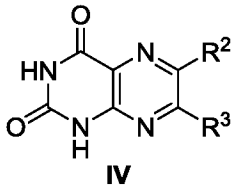
wherein R^2 and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

In a fourteenth aspect there is provided the manufacture of at least one compound represented by the following structural formula I:



I

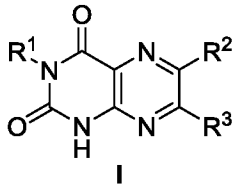
or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group, produced via an alkylation of the compound represented by the following structural formula IV at position N-3:



IV

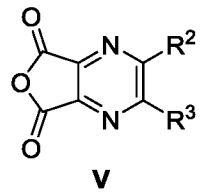
wherein R^2 and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

In a fifteenth aspect there is provided the manufacture of at least one compound represented by the following structural formula I:



I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represent either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group, produced via the generation of a transient isocyanate species from a compound of the following structural formula V:



V

wherein R^2, and R^3 independently represent either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

In summary, advantages of the marker compounds of *Leptospermum* honeys and methods of isolation and assaying thereof described herein may comprise:

- improved accuracy of determination of the floral purity and quality of *Leptospermum* Honeys in terms of its place of origin, authenticity and content; and
- improved reliability in determining the floral purity and quality of *Leptospermum* Honeys in terms of its place of origin, authenticity and content.

BRIEF DESCRIPTION OF THE DRAWINGS

Further aspects of the marker compounds of *Leptospermum* honeys and methods of isolation and assaying thereof will become apparent from the following description that is given by way of example only and with reference to the accompanying drawings in which:

Figure 1 shows the chemical structure of pteridine (1), lumazine (2), lepteridine (3), leptosperin (4);

Figure 2 shows the HMBC (heteronuclear multiple bond correlation) of lepteridine;

Figure 3 shows a scheme for the synthesis of lepteridine;

Figure 4 shows the 1H NMR spectra of natural and synthetic lepteridine;

Figure 5 shows a scheme for the synthesis of 3,6,7-(3-$^{2}H_{3}$)trimethyllumazine;

Figure 6 shows (A) Emission spectra of lepteridine standard (——), manuka honey (- - -), and manuka nectar (· · ·) at 330nm excitation wavelength. "X' indicates a non-diagnostic peak. Data shows mean spectra from experiments performed in duplicate. (B) Correlation between lepteridine concentration and fluorescence intensity for 27 manuka honey samples. Experiments were performed in duplicate, standard errors not shown. (C) Correlation between lepteridine concentration and fluorescence intensity for 6 manuka nectar samples. Data shown as mean ± standard deviation. (D) Compared the correlation curved between manuka honey samples (▼) against spiked manuka honeys (○).

Figure 7 shows a mass spectrum of a typical manuka honey sample before (A) and after (B) supplementation of lepteridine;

Figure 8 HPLC peak and MS/MS spectrum of endogenous lepteridine (A & B) and the heavier lepteridine isotope (C & D);

Figure 9 (A) Correlation between lepteridine concentration quantified by LC-MS/MS and HPLC (R2=0.9517). (B) Correlation between lepteridine concentration quantified by LC-MS/MS and fluorescence intensity at 330nm–470nm (R2=0.8995);

Figure 10 shows a table of principal enzymes from the bee hypopharyngeal glands, namely glucose oxidase, α-glucosidase, and β-glucosidase, supplemented into nectars in three treatment groups;

Figure 11 shows independently the HPLC chromatograms for leptosperin (262 nm) and lepteridine (320 nm) in the undiluted *L. scoparium* nectar; and

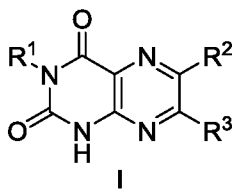
Figure 12 (A and B) shows normalisation of the nectar concentrations with reduced concentrations in both leptosperin and lepteridine in the fully ripened honey compared to the corresponding nectar at start of the dehydration process; (C) illustrates the distribution of leptosperin and lepteridine loss as a mean percentage of the initial concentration in the nectar; and (D and E) illustrate, respectively, the normalised concentration changes of leptosperin and lepteridine over time at 100% (●), 66% (■), and 33% (▲) *L. scoparium* nectar content; (F) illustrates the comparison of the % compound loss between the individual nectar groups for both leptosperin (shaded bars) and lepteridine (unshaded bars).

DETAILED DESCRIPTION OF THE INVENTION

As noted above, described herein are novel isolated compounds from *Leptospermum* honey and methods of assay thereof for use in the verification of the place of origin, authenticity and content of *Leptospermum* honeys such as mānuka honey. The inventors screened flower nectar and honeys of various floral types found in New Zealand to identify chemicals that were either unique to or in significantly higher concentrations in mānuka nectar and mono-floral mānuka honey compared to other predominantly mono-floral nectars and honeys. As a result of the screening exercise, the inventors discovered a marker compounds that could distinguish *Leptospermum* honey nectar and honey from other floral sources.

It was discovered for the first time that compounds represented by formula **I** are present in significantly enriched proportions in *Leptospermum* honey compared to other predominantly mono-floral honeys[25]. These compounds can be easily extracted and measured so can be used alone or in combination to evaluate mānuka honey as part of a certification protocol to verify its place of origin, authenticity and content.

In a first aspect there is provided a marker compound of Type I isolated from a *Leptospermum* honey and represented by the following structural formula **I**:



I

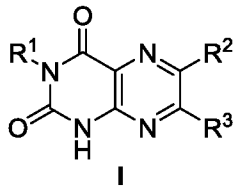
or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

Preferably, R^1, R^2, and R^3 are $-CH_3$ (this compound is referred to as 3,6,7-trimethyllumazine or lepteridine).

Preferably, the *Leptospermum* honey is selected from the flower group comprising: *Leptospermum scoparium, Leptospermum scoparium var. exinium, Leptospermum polygalifolium, Leptospermum subtenue.*

A *Leptospermum* honey certification protocol may include at least one of these Lepteridine analogue as described above either in absolute amounts or in a relative ratio of the marker compounds.

In a second aspect there is provided a marker compound of Type II represented by the following structural formula **I**:



I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group where the marker compound of Type II contains at least one isotope not having an identical atomic mass to that of the most abundantly occurring isotope of that element in nature.

In a third aspect there is provided a method of isolation of the compounds of the Type I as described above, comprising the following step:

a. a chromatography step in which a fraction is collected by eluting a honey derived from nectar collected from a *Leptospermum* flower with at least one elution solvent.

The compound represented by the formula I can be further purified with a second and subsequent chromatography or other purification steps. Preferably the organic elution solvent is a solution of acetic acid or a solution of acetonitrile. A person skilled in the art would recognise that other organic elution solvents could be used without departing from the scope of the method of manufacture described above such as formic or trifluoroacetic acid or organic alcohols such as C1-4 linear or branched chain alcohols. By varying the proportions of elution solvent and water it is possible to determine the most appropriate proportions for elution. The form of chromatography in the first chromatography step is not limited. It is not limited to column chromatography and may take various other forms. Likewise the means for detecting the marker compounds are not particularily limited. An ordinary UV absorption detector may be used, or a detection means (such as an MS detector).

In a fourth aspect there is provided a method of assaying and quantifying a *Leptospermum* honey comprising the following steps:

a. deriving a calibration curve for the concentration of a compound of Type II using mass spectrometry of the *Leptospermum* honey;
b. generating a calibration curve mass spectrum of a *Leptospermum* honey that contains a known concentration of a compound of Type II as an internal standard; and
c. deriving the concentration of a compound of Type I native to the *Leptospermum* honey via interpolation using the calibration curve generated in step (a) and the mass spectrum generated in step (b).

In a fifth aspect there is provided a method of assaying and quantifying a *Leptospermum* honey, comprising the following step:

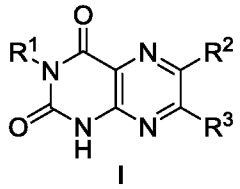
a. subjecting a Leptospernum honey to a stimulus sufficient to cause fluorescence of a compound of Type I as described above present in the Leptospernum honey; and
b. measuring an amount of fluorescence of the at least one compound of the Type I in the Leptospernum honey.

In one embodiment, a manuka honey certification protocol may include at least two or three marker compounds to increase its robustness and reliability.

In another embodiment, the method of assying and quantifying would also include the method step c) of determining the authenticity of the *Leptospermum* honey based on the measured amount of the at least one compound represented by the formula I. Either the absolute amounts could be used in the determination step or the relative ratio of the marker compounds (depending upon the variations of the marker compounds from different regions and/or climatic conditions).

In a sixth aspect there is provided a test kit for testing the purity of a *Leptospermum* honey, the kit comprising at least one compound of the Type I or Type II as described above.

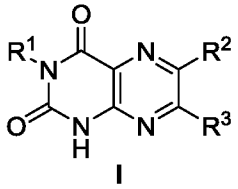
In a seventh aspect there is provided the use of at least one compound of Type I isolated from a *Leptospermum* honey and represented by the following structural formula I:



I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkylgroup, in the manufacture of a medicament for the treatment of disease.

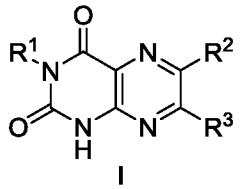
In an eighth aspect there is provided a composition comprising of at least one compound of Type I isolated from mānuka honey and represented by the following structural formula I:



I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group for use in the treatment of wounds.

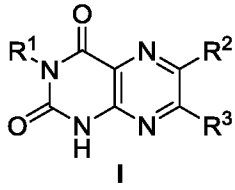
In a ninth aspect there is a provided a food supplement to improve physiological oxidative stress comprising at least one compound of Type I isolated from a *Leptospermum* honey and represented by the following structural formula I:



I

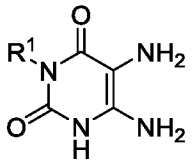
or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

In a tenth aspect there is provided the manufacture of at least one compound represented by the following structural formula **I**:



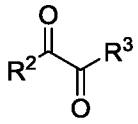
I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group produced via a condensation of an intermediate of the following structural formula **II**:



II

wherein R^1 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group with a compound of the following structural formula **III:**

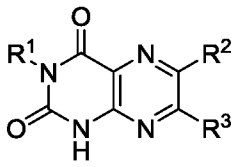


III

wherein R^2 and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

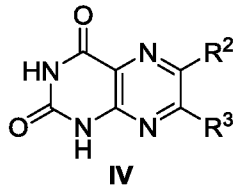
In a preferred embodiment the compound of the structural formula **III** is in the presence of an acid in a liquid carrier.

In an eleventh aspect there is provided the manufacture of at least one compound represented by the following structural formula **I**:



I

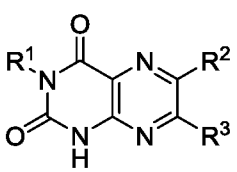
or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group produced via an alkylation of the compound represented by the following structural formula **IV** at position N-3:



IV

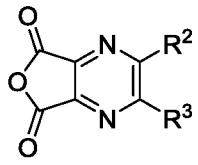
wherein R^2 and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

In a twelfth aspect there is provided the manufacture of at least one compound represented by the following structural formula **I**:



I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group produced via the generation of a transient isocyanate species from a compound of the following structural formula **V**:

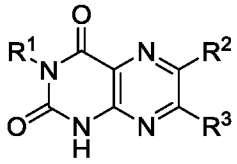


V

wherein R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

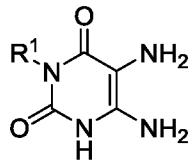
Preferably, the transient isocyanate species is that generated by a Curtius, Hofmann, Lossen or Schmidt rearrangement.

In a thirteenth aspect there is provided the manufacture of at least one compound of Type II represented by the following structural formula **I**:



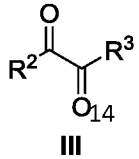
I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group, where the compound of Type II contains at least one isotope produced via a condensation of an intermediate of the following structural formula **II**:



II

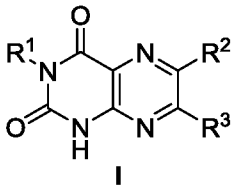
wherein R^1 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group with a compound of the following structural formula **III**:



III

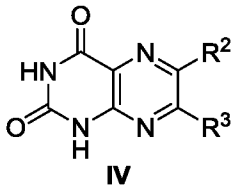
wherein R^2 and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

In a fourteenth aspect there is provided the manufacture of at least one compound of Type II represented by the following structural formula **I**:



I

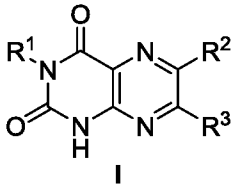
or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group, produced via an alkylation of the compound represented by the following structural formula IV at position N-3:



IV

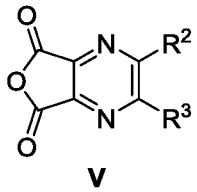
wherein R^2 and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

In a fifteenth aspect there is provided the manufacture of at least one compound represented by the following structural formula **I**:



I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represent either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group, via the generation of a transient isocyanate species from a compound of the following structural formula V:



V

wherein R^2, and R^3 independently represent either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

In this aspect it is understood that isotopes not having an identical atomic mass to that of the most abundantly occurring isotope of that element in nature, such as ^2H, ^{13}C or ^{15}N, have been introduced by means known to those skilled in the art of organic synthesis from commercially available isotopically

enriched starting materials.

In one embodiment, at least one composition represented by formula **I** as described above could be used as an internal control to be added as a positive control to determine that the verification protocol is working correctly.

The embodiments described above may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, and any or all combinations of any two or more said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to which the embodiments relates, such known equivalents are deemed to be incorporated herein as of individually set forth.

Where specific integers are mentioned herein which have known equivalents in the art to which this invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

WORKING EXAMPLES

The above-described compositions, methods of isolation and methods of measurement are now described by reference to the Figures and specific examples.

In the following examples, where specific reagents, solvents, acids, bases etc. are mentioned, it is understood that other suitable reagents, solvents, acids, bases etc. may be used and are included within the scope of this invention.

Example 1: Chemical Isolation of Lepteridine

Raw mānuka honey (51.3 g) was dissolved in H_2O + 0.1% HCOOH (150 mL) and sonicated for 20 min. The resulting suspension was filtered through celite and the filtrate used in the next step.

The filtrate was divided into two portions of 100 mL and each portion was subjected to SPE using MeOH-H_2O + 0.1% HCOOH (1:9, 80 mL) to remove undesired substances. The desired fraction was then eluted using MeOH-H_2O + 0.1% HCOOH (4:1, 80 mL). The two fractions were combined and concentrated to give the crude extract (0.23 g) which was further purified by flash chromatography (pet. ether-EtOAc 1:4) to give purified extract (3 mg) as a brown solid.

Several purified extracts were combined (6 m g total) and further purified by preparative TLC (pet. ether-EtOAc 1:3, 4 runs) to give **3** (4 m g) (as shown in Figure 1) as a colouless solid.

While using HPLC to examine New Zealand and Australian honeys derived from species of *Leptospermum*, *Eucalyptus*, *Kunzea* and *Knightia* for the presence of leptosperin (**4**)[37], [38], [39] (as shown in Figure 1) a proposed biomarker for *Leptospermum* honey, an unexpected UV absorbance was noted at 320 nm. This peak was observed only in *Leptospermum* honeys (*L. scoparium, L. scoparium var. exinium,*

L. polygalifolium, L. subtenue), including honey derived from *L. subtenue* in which no leptosperin was detected. The use of solid phase extraction (SPE) followed by reverse-phase HPLC enabled purification of the compound that exhibited the UV absorbance at 320 nm. However this method was time consuming, low yielding and not scalable, hence a more efficient isolation method was sought. Subjection of mānuka honey to SPE, followed by normal-phase flash chromatography and preparative TLC enabled isolation of the unknown compound as a colourless solid in sufficient quantity to conduct spectroscopic analysis.

Example 2: Structure Elucidation of Lepteridine

Table 1. ^{1}H, ^{13}C and ^{15}N NMR data for **3**[a]

Position	δ_C/δ_N, type	δ_H	HMBC[b]
1	NH	8.42 br	
2	149.9, C		
3	154.1, N		
4	161.1, C		
4a	123.7, C		
5	292.0, N		
6	158.9, C		
7	150.6, C		
8	329.9, N		
8a	144.8, C		
9	28.5, CH_3	3.50, s	2, 3, 4
10	22.8, CH_3	2.63, s	4a, 5, 6, 7
11	21.9, CH_3	2.67, s	6, 7, 8

[a] ^{1}H (400 MHz); ^{13}C (100 MHz); ^{15}N (60.8 MHz), chemical shift indirectly determined from ^{1}H-^{15}N HMBC NMR data. [b] HMBC correlations are from protons stated to the indicated carbon or nitrogen.

Referring to Table 1 above, the molecular formula of the unknown compound was established as $C_9H_{10}N_4O_2$ by positive ion HRESIMS. The compound was soluble in CD_3OD and $CDCl_3$; the latter was used for recording NMR spectra due to the presence of a broad resonance at δ 8.55 ppm (H-1) that was not present in spectra recorded in CD_3OD. This peak was assigned as an amide proton on the basis of its chemical shift and the absence of a distinctive hydroxyl absorption in the IR spectrum. Two singlets at δ 2.63 ppm (H-10) and δ 2.67 ppm (H-11) were assigned as heteroaryl methyl groups on the basis of their chemical shift, and the remaining singlet at δ 3.50 ppm (H-9) was assigned as an *N*-methyl group due to HMBC correlations of equal intensity to two quaternary carbonyl ^{13}C signals (C-2, C-4, Figure 2) and an HSQC correlation to a carbon signal at δ 28.5 ppm (C-9).

^{1}H-^{15}N HMBC correlations from H-10 and H-11 to N-5 and N-8 at δ 292.0 ppm and δ 329.9 ppm respectively, suggested that these two methyl groups were attached to a pyrazine ring. A 2,3-dimethyl substitution pattern was assigned based on ^{1}H-^{13}C HMBC correlations from H-10 to C-7 and from H-11 to C-6.

Given the high degree of unsaturation in the structure and the presence of a pyrazine ring, a fused heterocyclic structure was proposed for the unknown compound. Furthermore, a similarity was noted between the chemical shifts of carbons C-2, C-4 and C-4a and shifts reported for analogous carbons in natural products containing lumazine structures[20-24]. This observation, coupled with HMBC correlations from H-9 to C-2 and C-4 and an additional four bond coupling from H-10 to C-4a, led to the tentative assignment of the structure of the isolated compound as 3,6,7-trimethyllumazine (**3**).

3,6,7-Trimethyllumazine (**3**) was first synthesized in 1958[26]. Since then it has been reported in several studies on related lumazines[27-35]. Characterization data for lumazine **3** is limited to a melting point[26, 28], elemental analysis[26] and UV-vis peaks[28, 29, 32]; no NMR, MS or IR data have been reported to date.

Example 3 - Synthesis of Lepteridine

Referring to Figure 3 and following the work of Gala *et al*[40], *N*-methylation of 6-aminouracil (**5**) at position 3 was accomplished via silylation of the exocyclic amino and carbonyl groups upon treatment with hexamethyldisilazane (HDMS) in the presence of a catalytic amount of sulphuric acid (H_2SO_4). Ammonium sulphate could also be used as a catalyst. Methylation was then effected using iodomethane (MeI) in the presence of dimethylformamide (DMF) as an organic solvent in a 71% yield over two steps. Dimethylsulfate could also be used as a methylating agent. Subsequent desilylation during aqueous workup afforded 6-amino-3-methyluracil (**6**) in 78% yield.

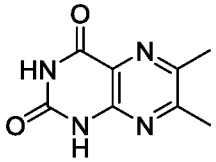
Amino uracil (**6**) was then treated with sodium nitrite ($NaNO_2$) and acetic acid (AcOH) solution, followed by reduction with sodium dithionite ($Na_2S_2O_4$) in the aqueous solvent ammonia (NH_3) at 70 °C[41] to give 5,6-diamino-3-methyluracil (**7**) in 31% yield over two steps. Alternative acids which could be used in the nitrosation first step include hydrochloric acid. An alternative to the first step reduction with sodium nitrite and acetic acid is actalytic hydrogenation using a catalyst such as palladium on carbon or platinum dioxide in an aqueous or organic solvent.

Condensation of diamino uracil (**7**) with 2,3-butanedione (**8**) in ethanol (EtOH) and acetic acid (AcOH) solution gave 3,6,7-trimethyllumazine (**3**) as a colourless solid. An alternative condensation agent which could be used here is 1,2-diketone and an alternative acid for use in the condensation step is hydrochloric acid. Spectroscopic data (UV-vis, IR, ^{1}H NMR, ^{13}C NMR) of synthetic 3,6,7-trimethyllumazine was in excellent agreement with that of the isolated natural product (see Figure 4). Furthermore, the ^{1}H NMR spectrum of combined natural and synthetic products was identical to the ^{1}H NMR spectra of separate natural and synthetic material. Thus the structure of lepteridine (**3**) was definitively established

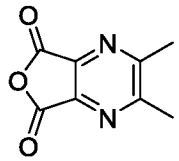
as 3,6,7-trimethyllumazine.

Given that 3,6,7-trimethyllumazine is a pteridine derivative isolated from *Leptospermum* honey the isolated compound was named lepteridine.

Alternative synthesis of compound (9) as shown in Figure 5 is via methylation at N-3 of the



intermediate compound shown above or via transformation of the intermediate compound shown below into a transient isocyanate species, including but not limited to those generated by a Curtius, Hofmann, Lossen or Schmidt rearrangement.



Referring to Figure 5 and Example 3, *N*-deuteromethylation of 6-aminouracil (**5**) at position 3 was accomplished via silylation of the exocyclic amino and carbonyl groups upon treatment with hexamethyldisilazane (HDMS) in the presence of a catalytic amount of sulphuric acid (H_2SO_4). Methylation was then effected using iodomethane-d_3 (CD_3I) in the presence of dimethylformamide (DMF) as an organic solvent in a 71% yield over two steps. Subsequent desilylation during aqueous workup afforded 6-amino-3-($^2H_3$)methyluracil (**9**) in 78% yield.

Amino uracil (**6**) was then treated with sodium nitrite ($NaNO_2$) and acetic acid (AcOH) solution, followed by reduction with sodium dithionite ($Na_2S_2O_4$) in the aqueous solvent ammonia (NH_3) at 70 °C[40] to give 5,6-diamino-3-($^2H_3$)methyluracil (**10**) in 31% yield over two steps. Alternative acids which could be used in the nitrosation first step include hydrochloric acid. An alternative to the first step reduction with sodium nitrite and acetic acid is actalytic hydrogenation using a catalyst such as palladium on carbon or platinum dioxide in an aqueous or organic solvent.

Condensation of diamino uracil (**10**) with 2,3-butanedione (**8**) in ethanol (EtOH) and acetic acid (AcOH) solution gave 3,6,7-(3-$^2H_3$)trimethyllumazine (**11**) as a colourless solid.

Materials and Methods

All reactions were carried out in flame- or oven-dried glassware under a dry nitrogen atmosphere. All reagents were purchased as reagent grade and used without further purification. Dimethyl formamide was degassed and dried using an LC Technical SP-1 solvent purification system. Ethanol was distilled over

$Mg(OEt)_2$. Ethyl acetate, methanol, and petroleum ether were distilled prior to use. All other solvents were used as received unless stated otherwise. Solid Phase Extraction (SPE) was performed using Strata C_{18} E 70 Å, 55 µm 20 g/60 mL columns. RP-HPLC was performed with an Agilent 1100 using a Jupiter C_{18} 300 Å, 5 µm, 2.0 mm x 250 mm column at a flow rate of 0.2 $mLmin^{-1}$ with a DAD Detector operating at 262, 280 and 320 nm. A suitably adjusted gradient of 5% B to 100% B was used, where solvent A was 0.1% HCOOH in H_2O and B was 20 % A in MeCN. Flash chromatography was carried out using 0.063-0.1 mm silica gel with the desired solvent. Thin layer chromatography (TLC) was performed using 0.2 mm Kieselgel F254 (Merck) silica plates and compounds were visualised using UV irradiation at 254 or 365 nm and/or staining with a solution of potassium permanganate and potassium carbonate in aqueous sodium hydroxide. Preparative TLC was performed using 500 µm, 20 x 20 cm Uniplate™ (Analtech) silica gel TLC plates and compounds were visualised using UV irradiation at 254 or 365 nm. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Infrared spectra were obtained using a Perkin-Elmer Spectrum 100 FTIR spectrometer on a film ATR sampling accessory. Absorption maxima are expressed in wavenumbers (cm^{-1}). NMR spectra were recorded as indicated on either a Bruker Avance 400 spectrometer operating at 400 MHz for ^1H nuclei and 100 MHz for ^{13}C nuclei, a Bruker DRX-400 spectrometer operating at 400 MHz for ^1H nuclei, 100 MHz for ^{13}C nuclei, a Bruker Avance AVIII-HD 500 spectrometer operating at 500 MHz for ^1H nuclei, 125 MHz for ^{13}C nuclei or a Bruker Avance 600 spectrometer operating at 600 MHz for ^1H nuclei, 150 MHz for ^{13}C nuclei. ^1H and ^{13}C chemical shifts are reported in parts per million (ppm) relative to $CDCl_3$ (^1H and ^{13}C) or $(CD_3)_2SO$ (^1H and ^{13}C). ^{15}N chemical shifts were referenced using the unified Ξ_i scale[42] as implemented by the Bruker library function "xiref." ^1H NMR data is reported as chemical shift, relative integral, multiplicity (s, singlet; assignment). Assignments were made with the aid of COSY, NOESY, HSQC and HMBC experiments where required. High resolution mass spectra were recorded on a Bruker micrOTOF-Q II mass spectrometer with ESI ionisation source. Ultraviolet-visible spectra were run as H_2O solutions on a Shimadzu UV-2101PC scanning spectrophotometer.

Example 4: Detection of Lepteridine in a Leptospermum honey

In an effort to develop a certification protocol for assaying and quantifying a component of a *Leptospermum* honey, the fluorescence of 3,6,7-trimethyllumazine or lepteridine was used to produce a calibration curve.

In a first step, a Leptospernum honey in the form of Manuka honey was diluted in sterilised distilled water to a concentration of 2 % (w/v). The diluted Manuka honey is placed in microtiter plates and measured in a spectrofluometer (for example a Gemini EM Dual Scanning Microplate Spectrofluorometer manfactured by Molecular Devices Inc coupled to an external computer equiped with SoftMax Pro software) with top down reading for better signal to noise ratio. All samples were incubated and read at room temperature.

The diluted honey sample was subjected to a stimulus in the form of an excitation wavelength of 330 nm sufficient to cause fluorescence of lepteridine present in the Leptospernum honey. The presence of lepteridine was detected by measuring an emmission wavelength of 470 nm in a spectrophotmeter.

By measuring the fluorescence of known positive control mono-floral honeys and negative control non-Manuka honeys as reference honeys, a calibration profile can be derived which can be used in the interpretation of the 470 nm fluorescence value for the purposes determining the authencity of unknown honey samples.

Example 5: Lepteridine as the compound responsible for manuka honey fluorescence

In order to confirm that lepteridine is a fluorescence marker compound of manuka honey, the emission spectrum of lepteridine were plotted at 330 excitation wavelength alongside manuka honey and manuka nectar (see Figure 6A). As can be seen from the figure, the pattern of all emission spectrum were almost identical, showing elevated fluorescence ranging from 390nm to 590nm and peak fluorescence at 470nm. It is important to reiterate that fluorescence spectrometry is a highly specific analytical technique controlled by two independent wavelengths. Thus this level of similarity provides strong support for lepteridine the responsible compound for MM2 fluorescence (as indicated by the arrow on Figure 6A). It should be noted that another emission band can be observed at 330-650 ex-em wavelength. However, this peak was non-diagnostic and it is present in all samples including blanks. This is likely to be caused by background interference arising from surface reflection or auto-fluorescence of the wells (Lakowicz, 2006). Therefore, this emission peak should not be interpreted as fluorescence signal from samples.

Further investigation were carried out on 27 manuka honey samples collected from 5 different geographical locations throughout New Zealand using both fluorescence spectrometry and HPLC. In all cases, the presence of lepteridine were confirmed using both analytical techniques, while the concentration ranges from 5mg/kg to 52mg/kg. Figure 6B plotted the fluorescence generated by manuka honey against the concentration of lepteridine detected by HPLC. As shown in the figure, a strong linear relationship was found between lepteridine concentration and fluorescence intensity (R^2=0.929). This positive association further corroborates lepteridine as the fluorescent compound at the MM2 wavelength. In addition, this high level of linear correlation demonstrated the possibility to accurately estimate the amount of lepteridine in manuka honey samples based on fluorescent spectrometry.

Analysis were carried out on 6 available manuka nectar samples using the same procedure as described above for manuka honey (Figure 6C). In comparison with manuka honey, the results from nectar samples can also be fitted into a linear correlation (R^2=0.611), again demonstrating positive association between lepteridine concentration and fluorescence intensity. While the detection of lepteridine in manuka nectar further reinforced the plant origin of lepteridine. However, lepteridine concentration in nectar samples appears to be considerably higher when compared with manuka honey, which ranges from

80mg/kg to 205mg/kg compared with 5mg/kg to 52mg/kg for honey samples. One possible explanation may be the hydrolysation of lepteridine during honey ripening, while some degree of lepteridine loss should be linked with floral dilution by other honeys.

At this point of the study, it may be reasonable to infer that lepteridine is directly relevant to fluorescence displayed at the MM2 wavelength. However, there are chances that multiple compounds could be present in manuka honey which fluoresce at similar wavelengths. If that is the case, spiking lepteridine into manuka honey should not lead to the expected increase in fluorescence (according to Figure 6B). In order to validate whether lepteridine is the main compound responsible for MM2 fluorescence, 0.05µg, 0.10µg, and 0.15µg of lepteridine standard were spiked into 3 manuka honey samples with pre-quantified lepteridine concentration at 0.302µg/kg, 0.513µg/kg, and 0.709µg/kg. Figure 6D plotted fluorescence against lepteridine concentration for spiked manuka honeys samples (—o—), and compared with earlier results from natural manuka honey samples (····x····). As shown in figure 6D, manuka honey samples generated a strong linear correlation (R^2=0.942), this correlation was almost identical compared with natural manuka honey samples ($p>0.05$). Our results demonstrate the addition of lepteridine directly led to the expected level of increase in fluorescence. Thus indicating that lepteridine is probably to be the principle compound responsible for MM2 fluorescence.

Example 6: Quantification of lepteridine in manuka honey using mass spectrometry

As described above, lepteridine may be utilised as a fluorescent marker compound for manuka honey. The fluorescence intensity ($_{ex}$330nm – $_{em}$470nm) demonstrated strong linear correlation with lepteridine concentration quantified by HPLC (area under curve). However, these results are best validated using a separate quantitative approach.

Described is a quantitative technique to measure lepteridine concentration using tandem mass spectrometry (LC-MS/MS). A heavier lepteridine isotope was synthesized and employed as an internal standard to compensate the matrix effect from manuka honey. There was no interference from endogenous compound in manuka honey and the 3 Da mass difference can be clearly distinguished on the mass spectrum. The results described further below of LC-MS/MS strongly correlates with previous data from HPLC quantification and fluorescence spectrometry. Therefore lepteridine can be accurately determined using all three methods. Results from LC-MS/MS quantification was comparatively lower than previous data from HPLC, this may be resulted from minor co-eluting compounds under the same HPLC peak. These findings demonstrate that quantitative mass spectrometry may be used as a stand-alone or complimentary approach for manuka honey authentication.

To validate the LC-MS/MS method, the mass spectrum of a typical manuka honey was obtained before and after the supplementation of the heavier lepteridine isotope (Figure 7). As shown, there was no significant interfering peaks from endogenous compounds in manuka honey from m/z 210–212 (Figure

7A). In Figure 7B, the 3 Da mass difference between the isotopes may be clearly identified on the mass spectrum. The final testing concentration of manuka honey was determined at 0.2% w/v to reduce sugar concentration while retaining relatively high mass spectrum resolution.

LC-MS/MS quantification

During the LC-stage, the endogenous lepteridine and the heavier isotope co-eluted at the exact same time (12.85 min) (Figure 8). These isomers displayed almost identical MS/MS spectrum, while only differentiated by a 3 Da mass shift from m/z 189 (Figure 8B) to m/z 192 (Figure 8D). The most abundant common ion was observed at 148.05 m/z. The heavy isotopes were not present on the part of the structure represented by this fragment ion. This common ion is employed for lepteridine quantification to reduce background interference.

Comparing LC-MS/MS and HPLC quantification

Endogenous lepteridine concentration was quantified as 3–44 mg/kg using mass spectrometry quantification. The results demonstrated strong linear correlation with previous data from HPLC analysis on the same set of manuka honey samples (R^2=0.9517) (Figure 9A). It should be noted that the mass spectrometry result was comparably lower than previous HPLC quantification (5–52 mg/kg). This suggests that other UV-absorbing compounds may have co-eluted with lepteridine under the same HPLC peak.

The results from mass spectrometry quantification also correlates well with the signature fluorescence at $_{ex}$330nm – $_{em}$470nm (R^2=0.8995) (Figure 9B).

Example 7: Stability of Leperidine as a marker of manuka honey

As above, New Zealand manuka (*Leptospermum scoparium*) honey contains unique nectar-derived compounds useful for its identification. Chemical alterations to these compounds during the honey ripening process are currently unknown. Relative concentration changes of lepteridine and leptosperin (a known marker in the art and used as a reference standard herein) were examined during *L. scoparium* nectar to honey conversion. Concentration changes of these compounds were often non-linear with respect to increasing sugar concentration. Normalisation relative to an 80 °Brix sugar solution showed a mean percentage loss of 13.66 ± 0.77% for leptosperin and 9.62 ± 1.03% for lepteridine. These two compound losses appeared to be non-enzymatic and independent of floral dilution. The lack of a floral dilution effect on leptosperin and lepteridine losses during nectar to honey conversion strongly reinforces the use of these compounds as chemical markers for authentication of manuka honey.

The conversion of nectar to honey is essentially a two-step process: the hydrolysis of sucrose to glucose and fructose followed by evaporation of excess water. A laboratory simulation of the honey ripening process was carried out to examine the chemical changes that occur during *L. scoparium* nectar conversion to honey. Whilst leptosperin and lepteridine are stable over prolonged storage and heat

treatment in honey, the chemical stability of these compounds in *L. scoparium* nectar during the honey ripening process has not been examined. The inherently higher level of leptosperin and lepteridine in nectar suggest chemical changes or physical processes during nectar to honey transformation that result in loss of these compounds in the final honey product. It is possible that these manuka-specific floral markers are modified or broken down following bee enzymatic activity or the physico-chemical changes that take place during the conversion process.

Nectar dehydration

The honey ripening process was simulated in the laboratory by dehydrating nectar in 2.5 ml wax-coated cylindrical containers (diameter, 18 mm; height, 10 mm). To represent floral dilution encountered in the natural environment, a series of three nectar dilutions (100%, 66%, and 33% v/v *L. scoparium* nectar content) was set up using an artificial nectar solution (8 °Brix) comprising 38% fructose, 30% glucose, and 12% sucrose in water as the diluent source.

The principal enzymes from the bee hypopharyngeal glands, namely glucose oxidase, α-glucosidase, and β-glucosidase, were supplemented into nectars in three treatment groups as indicated in Figure 10. The final enzyme concentration of glucose oxidase, α-glucosidase, and β-glucosidase were 0.1, 0.0005, and 0.0005 mg/ml respectively. A no-enzyme treatment consisting of nectar only was included as a control.

The dehydration process was carried out in a dehydrator at 37 °C with a starting volume of 480 µl. The experiment was carried out in duplicate, and the partially evaporated nectar was subsampled at different time points during the process. The nectar was considered fully ripened when it reached approximately 80 °Brix, and the final honey product was extracted by manual scraping off the wax containers. All nectar and honey samples were stored at -20 °C until analysis.

Leptosperin and lepteridine analysis

Leptosperin and lepteridine concentrations were quantified on a Dionex Ultimate^{TM} 3000 reversed-phase high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, New Zealand) with diode-array detection (DAD) based on known methods in the art.

Honey and nectar samples were diluted in 0.1% v/v formic acid to a final sugar concentration in the range of 1 to 2 °Brix. The injection volume was 3 µl. Separation was carried out on a Hypersil GOLD column (150 × 2.1 mm; 3 µm particle size) by gradient elution at a constant flow rate of 0.200 ml/min. The binary mobile phase consisted of 0.1% v/v aqueous formic acid (Solvent A) and 80:20 acetonitrile:Solvent A (Solvent B). A 30 min gradient elution programmed was employed: initial (5% B, held 2 min), 14 min (50% B), 16 min (100% B, held 3 min), 20 min (5% B, held 10 min). The column was thermostatically controlled at 25 °C. Leptosperin and lepteridine were monitored at 262, and 320 nm, respectively. Identification of these compounds were based on retention time. Under the specified

chromatographic conditions, leptosperin has a retention time of 14.1 min, and lepteridine 12.9 min at the respective detection wavelengths.

Data acquisition and peak integration were performed with Thermo Fisher Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS) software. The compounds of interests were quantified using external calibration curves of respective chemical standards in 0.1% v/v formic acid (leptosperin, 0.0625–0.5 mg/ml; lepteridine 0.5625–50 µg/ml) based on integrated measurement of peak area.

Effects of enzymes on leptosperin and lepteridine contents

An artificial nectar (20 °Brix) was supplemented with leptosperin and lepteridine chemical standards at a concentration equivalent to 250 mg/kg and 15 mg/kg in honey, respectively. The solution was incubated at 37 °C for two hours in the presence glucose oxidase (0.1 mg/ml), α-glucosidase (0.0005 mg/ml), and β–glucosidase (0.0005 mg/ml). A control with no enzyme addition was included. All samples were subjected to HPLC analysis following incubation.

Effects of proteins on leptosperin and lepteridine contents

An artificial nectar (20 °Brix) with supplemented leptosperin and lepteridine at 2500 mg/kg and 150 mg/kg honey equivalent, respectively, was doped with bovine albumin at concentrations ranging from 0.02 to 0.1 % w/v concentrations. The samples were incubated at 37 °C, and quantified for leptosperin and lepteridine concentrations following incubation for 18 and 36 hours.

Expression of results for leptosperin, lepteridine, DHA, and MGO content

The concentrations of leptosperin and lepteridine are expressed as a weight ratio of the compound of interest/80 °Brix sugar solution in mg/kg. The normalisation by sugar content to 80 °Brix eliminates variance due to differences in sugar content, and therefore allows fair comparison between all samples.

Results of Leptosperin and lepteridine

Leptosperin and lepteridine concentrations were measured by HPLC-DAD with monitoring at 262 nm and 320 nm, respectively. Figure 11A and B illustrate independently the HPLC chromatograms for leptosperin (262 nm) and lepteridine (320 nm) in the undiluted *L. scoparium* nectar used during this analysis. The major peak with a retention time of 14.1 min (Figure 11A) corresponds to leptosperin, whereas the peak at 12.9 min (Figure 11B) corresponds to lepteridine. This nectar carried 9966 ± 13 mg/kg leptosperin and 212 ± 0.4 mg/kg lepteridine.

During the nectar dehydration process, water molecules were lost by evaporation thereby increasing sugar content in the ripening nectar. Determination of total sugar content was carried out by means of

refractometry. One °Brix, by strict definition, represents 1g of sucrose in 100g of aqueous solution. The method also provides a good approximation of the total sugar content as °Brix is directly related to the amount of sugar as known in the art. Interference from other solutes was negligible in the context of this study as sugar constitutes by far the principal dissolved components in nectar and honey. In addition, refractometry-derived measurement of sugar content is not sensitive to changes in carbohydrate composition as known in the art. The shift in sugar profile during the honey ripening process was therefore considered to have no significant influence on sensitivity of the method employed.

Figure 11C shows the relationships between sugar (x-axis) and both leptosperin (—; left y-axis) and lepteridine (---; right y-axis) concentrations in *L. scoparium* nectar during conversion to honey. The data plotted encompassed all three floral dilution groups at 100%, 66%, and 33% *L. scoparium* nectar content subsampled at various time points during the dehydration process. The increases in both leptosperin and lepteridine concentrations were non-linear with respect to sugar concentration. The individual correlations were best-fitted to a second-order polynomial function, and were consistent irrespective of the *L. scoparium* nectar content. This departure from linearity indicates that the concentration change of sugars in nectar does not equate with the concentration change of leptosperin and lepteridine. As nectar dehydrates into honey, the increase in concentrations of both leptosperin and lepteridine were fractionally reduced, suggesting that some of these compounds were lost during the process.

Normalisation of the nectar concentrations to 80 °Brix followed by two-tailed paired *t*-test analysis showed significantly reduced concentrations in both leptosperin and lepteridine in the fully ripened honey (t_3) compared to the corresponding nectar at start of the dehydration process (t_0) (leptosperin, $p<0.0001$; lepteridine, $p<0.001$) (Figure 12A and B). One-way ANOVA analysis revealed no significant effect of enzyme treatment on both leptosperin and lepteridine losses ($p>0.05$), suggesting that the process was most likely non-enzymatic. In addition, nectars ripened in the presence of glucose oxidase (Group 1, 2, and 3) behaved similarly to the nectar control without glucose oxidase ($p>0.05$). Therefore acidification was unlikely to account for the loss of these compounds.

To validate this observation, an artificial nectar (20 °Brix) with supplemented leptosperin and lepteridine at 2500 mg/kg and 150 mg/kg concentration equivalent in honey, respectively, was incubated independently with glucose oxidase, α-glucosidase, and β-glucosidase at concentrations similar to the nectar dehydration experiment. Analysis by HPLC revealed no significant changes in both compound concentrations following a two-hour incubation at 37 °C ($p>0.05$), thereby confirming that the observed loss of leptosperin and lepteridine during the nectar dehydration process was not due to hydrolysis by these enzymes.

Figure 12C illustrates the distribution of leptosperin and lepteridine loss as a mean percentage of the initial concentration in the nectar. Overall, there was a mean percentage loss of 13.66 ± 0.77% for leptosperin and 9.62 ± 1.03% for lepteridine, which were considerably less compared to the

concentration differences in nectar and honey previously reported.

The effect of floral dilution was subsequently examined. Figure 12D and E illustrate, respectively, the normalised concentration changes of leptosperin and lepteridine over time at 100% (●), 66% (■), and 33% (▲) *L. scoparium* nectar content. The datasets plotted represent combined mean value from all enzyme treatment groups and control. Whilst the concentrations of both compounds at t_3 were significantly reduced (leptosperin, $p<0.0001$; lepteridine, $p<0.001$), one-way ANOVA comparison of the % compound loss between the individual nectar groups revealed no significant differences between the 100%, 66%, and 33% *L. scoparium* nectar groups for both leptosperin (shaded bars) and lepteridine (unshaded bars) (Figure 12F, $p>0.05$), suggesting that the process occurred independent of the extent of floral dilution. As the initial concentrations of these compounds in nectar is directly correlated to the extent of floral dilution, it would appear that the observed loss of leptosperin and lepteridine were not concentration-driven. In other words, a constant proportion of these compounds was lost during the dehydration process. Accordingly, the concentration of leptosperin and lepteridine in honey would be expected to correlate with the inherent quantities present in the bulk nectar incorporated into the beehive, thus reinforcing the use of leptosperin and lepteridine as an indicator for florality status of manuka honey.

The lack of floral dilution effect also suggests that the mechanism of loss for leptosperin and lepteridine are not driven by specific compounds present in *L. scoparium* nectars. Further analysis of methyl syringate content showed no apparent trend associated with leptosperin loss, thereby indicating that leptosperin loss was not due to hydrolysis into the corresponding gentiobiose and aglycone methyl syringate.

The effect of proteins was also examined by incubation of artificial nectars (20 °Brix) containing supplemented leptosperin and lepteridine with bovine albumin at concentrations ranging from 0.02 to 0.10% w/v. This nectar concentration range was chosen based on previous publications that honey may contain up to 1% proteins and amino acids by weight. The results of HPLC analysis showed no significant decrease in both leptosperin and lepteridine concentrations following incubation ($p>0.05$). Accordingly, the observed losses of these compounds during the dehydration process (Figure 12F) was most likely not due to protein fractionation or cross-linking.

Materials and Methods

HPLC Analysis of Honey Samples: Raw honey was subjected to SPE as described above. Honey extracts were analyses by HPLC prior to concentration. Samples were analysed in duplicate.

LC-MS/MS quantification: HPLC-grade acetonitrile and formic acid were purchased from Merck. Water was purified using the Barnstead Nanopure Diamond laboratory water system. A 10ul injection was made of each sample directly onto a 0.3 x 100 mm Zorbax 300SB- C18 column (Agilent, Santa Clara, CA,

USA) at 12 ul/min for 6 minutes. The HPLC gradient between Buffer A (0.1% formic acid in water) and Buffer B (0.1% formic acid in acetonitrile) was formed at 6 ul/min as follows: 10% B for the first 3 min, increasing to 25% B by 18 min, increasing to 97% B by 21 min, held at 97% until 24 min, back to 10% B at 25.5min and held there until 30 min. The LC effluent was directed into the Ionspray source of QSTAR XL hybrid Quadrupole-Time-of-Flight mass spectrometer (Applied Biosystems, Foster City, CA, USA) scanning from 150-800 m/z. Two product ions were selected for MS/MS analysis (m/z 207 and 210) over the mass range m/z 70-210 with a collision energy of 35 V. The mass spectrometer and HPLC system were under the control of the Analyst QS 2.0 software package (Applied Biosystems).

Data Analysis: Chromatographic peaks and mass spectrums were analysed by MultiQuant v3.0 (Sciex). Statistical data analysis were performed using Graphpad Prism software (Version 6.01). All correlations in this study were determined by regression analysis and compared by slope analysis. Differences between group means were determined by one-tailed Student's t-tests.

6-Amino-3-methyluracil (6): 6-Aminouracil (**5**) (5.18 g, 40.7 mmol) was suspended in HMDS (25 mL) and H_2SO_4 (0.1 mL) was added. The mixture was heated at reflux for 3 h then concentrated *in vacuo*. The residue was dissolved in DMF (30 mL), MeI (8.5 mL, 136.5 mmol) was added, and stirring was continued for 72 h at room temperature. The reaction was cooled to 0 °C and $NaHCO_3$ was carefully added. The mixture was stirred at 0 °C until no more bubbling was observed. The precipitate was filtered, washed with MeOH and H_2O and dried to give the *title compound* **6** (4.49 g, 78%) as a yellow solid which was used without further purification.

R_f 0.23 (EtOAc-MeOH-NH_4OH, 7:2.7:0.3);

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.36 (1H, s, N*H*), 6.16 (2H, s, NH_2), 4.56 (1H, s, *H*-5); 3.04 (3H, s, NCH_3);

^{13}C NMR (100 MHz, DMSO-d_6) δ 163.3 (C-4), 153.5 (C-6), 151.1 (C-2), 74.0 (C-5), 25.9 (NCH_3);

MP Lit: 327 °C[19], found: 320-323 °C;

Spectroscopic data and melting point were in good agreement with those previously reported[43].

6-Amino-3-methyl-5-nitrosouracil: 6-Amino-3-methyluracil (**6**) (1.04 g, 7.40 mmol) was suspended in H_2O (10 mL). The suspension was heated at reflux for 2 h then cooled to room temperature. AcOH (4.20 g, 69.9 mmol) was added. A solution of $NaNO_2$ (1.04 g, 15.1 mmol) in H_2O (7 mL) was then added dropwise over 5 min, during which the pale yellow suspension became violet. The mixture was stirred for 30 min before the precipitate was filtered, washed with MeOH and H_2O and dried to give the *title compound* (1.07 g, 85%) as a violet solid which was used without further purification.

R_f 0.12 (EtOAc-MeOH-NH_4OH, 7:2.7:0.3);

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.41 (1H br, s, N*H*), 7.97 (2H br, s, NH_2), 3.21 (3H, s, NCH_3);

^{13}C NMR (100 MHz, DMSO-d_6) δ 161.4 (C-4), 149.3 (C-2), 144.4 (C-6), 139.7 (C-5), 26.7 (NCH_3);

MP Lit: >350 °C[44], found: >400 °C (decomp);

Spectroscopic and physical data were in good agreement with those previously reported[44].

5,6-Diamino-3-methyluracil (7): 6-Amino-3-methyl-5-nitrosouracil (0.50 g, 2.96 mmol) was suspended in a mixture of NH_4OH (7.5 mL, 28-30%) and H_2O (7.5 mL). The suspension was heated to 70 °C and $Na_2S_2O_4$ (1.10 g, 6.33 mmol) was added portionwise over 30 min. The mixture was stirred at 70 °C for 1 h before being concentrated *in vacuo* until a red precipitate formed. The suspension was cooled to room temperature and the precipitate was filtered and washed with cold H_2O (5 mL) to give the *title compound* **7** (0.17 g, 37%) as a red solid which was used without further purification.

R_f 0 (EtOAc-MeOH-NH_4OH, 7:2.7:0.3);

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.69 (1H, s, N*H*), 7.07 (2H, s, NH_2), 3.32 (2H, s, NH_2) 3.12 (3H, s, NCH_3);

^{13}C NMR (100 MHz, DMSO-d_6) δ 164.2 (C-4), 156.8 (C-6), 153.5 (C-2), 141.3 (C-5), 26.7 (NCH_3);

MP Lit: >340 °C[43], found: >400 °C;

Spectroscopic and physical data were in good agreement with those previously reported[43].

3,6,7-trimethyllumazine (3): 5,6-Diamino-3-methyl uracil **7** (0.48 g, 3.05 mmol) was suspended in EtOH (10 mL). 2,3-Butandione **8** (0.29 g, 3.42 mmol) and AcOH (0.94 g, 15.7 mmol) were added. The mixture was heated at reflux for 24 h before being cooled to r.t. and concentrated *in vacuo*. The crude product was purified by flash chromatography (pet. ether-EtOAc 1:4) to give the *title compound* **3** (0.40 g, 63%) as a colourless solid.

R_f 0.27 (pet. ether-EtOAc, 1:4);

IR (film) ν_{max} 2955, 1728, 1664, 1437, 1399, 1368, 1285, 1046 cm^{-1};

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.54 (1H, s, N*H*), 3.50 (3H, s, N-CH_3), 2.66 (3H, s, C7-CH_3), 2.65 (3H, s, C6-CH_3);

^{13}C NMR (100 MHz, $CDCl_3$) δ 161.0 (C-4), 158.8 (C-6), 150.5 (C-7), 150.4 (C-2), 144.9 (C-8a), 123.6 (C-4a), 28.3 (N-CH_3), 22.6 (C6-CH_3), 21.8 (C7-CH_3);

UV-vis (H_2O) λ_{max} (log ε) 208 (0.54), 234 (0.45), 329 (0.35);

HRMS (ESI^+) calculated for $C_9H_{10}N_4O_2Na^+$ $[M+Na]^+$: 229.0689, found: 229.0696; and

MP Lit: 271-272 °C[26], found: 268-269 °C.

Native 3,6,7-trimethyllumazine (3):

R_f 0.27 (pet. ether-EtOAc, 1:4);

IR (film) ν_{max} 2921, 1723, 1667, 1435, 1393, 1363, 1281, 1119 cm^{-1};

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (1H, s, N*H*), 3.50 (3H, s, N-CH_3), 2.67 (3H, s, C7-CH_3), 2.63 (3H, s, C6-CH_3);

^{13}C NMR (100 MHz, $CDCl_3$) δ 161.1 (C-4), 158.9 (C-6), 150.6 (C-7), 149.9 (C-2), 144.8 (C-8a), 123.7 (C-4a), 28.5 (N-CH_3), 22.8 (C6-CH_3), 21.9 (C7-CH_3);

UV-vis (H_2O) λ_{max} (log ε) 211 (2.77), 231 (2.36), 329 (1.42);

HRMS (ESI^+) calculated for $C_9H_{10}N_4O_2Na^+$ ($M+Na^+$): 229.0689, found: 229.0696;

MP Lit: 271-272 °C[26], found: 270-271 °C.

6-Amino-3-($^2H_3$)methyluracil: 6-Aminouracil (1.05 g, 8.29 mmol) was suspended in hexamethyldisilazane (5 mL) and sulfuric acid (0.02 mL) was added. The mixture was heated at reflux for 1.5 h then concentrated *in vacuo*. The residue was dissolved in dimethylformamide (6 mL) and iodomethane-d_3 (0.8 mL, 12.9 mmol) was added, and stirring was continued for 72 h at room temperature. The reaction was cooled to 0 °C and sodium bicarbonate (15 mL) was carefully added. The mixture was stirred at 0 °C until no further bubbling was observed. The precipitate was filtered, washed with methanol and water and dried to give the *title compound* (0.43 g, 36 %) as a yellow solid which was used without further purification.

IR (film) ν_{max} 3417, 3193, 1632, 1587, 1434, 1237, 788 cm^{-1};

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.35 (1H, s, H-1), 6.15 (2H, s, NH_2), 4.56 (1H, s, H-5);

^{13}C NMR (75 MHz, DMSO-d_6) δ 163.2 (C-4), 153.5 (C-6), 151.1 (C-2), 74.0 (d, J = 168.1 Hz, C-4);

HRMS (ESI^+) calculated for $C_5H_4D_3N_3O_2Na^+$ [M+Na^+]: 167.0619, found: 167.0618;

MP 340-345 °C (decomp);

6-Amino-3-($^2H_3$)methyl-5-nitrosouracil: 6-Amino-3-($^2H_3$)methyluracil (**9**) (0.40 g, 2.76 mmol) was suspended in water (5 mL). The suspension was heated at reflux for 2.5 h then cooled to room temperature. Acetic acid (1.68 g, 28.0 mmol) was added. A solution of sodium nitrite (0.46 g, 6.70 mmol) in water (4 mL) was then added dropwise over 5 min, during which the pale yellow suspension became grey. The mixture was stirred for 5 min before the precipitate was filtered, washed with methanol and water and dried to give the *title compound* (0.39 g, 81%) as a grey solid which was used without further purification;

IR (film) ν_{max} 3207, 3012, 1719, 1649, 1517, 1430, 1251, 1045, 768 cm^{-1};

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.37 (1H br, s, H-1), 7.98 (2H br, s, NH_2);

^{13}C NMR (100 MHz, DMSO-d_6) δ 161.4 (C-4), 149.4 (C-2), 144.5 (C-6), 139.7 (C-5);

HRMS (ESI^+) calculated for $C_5H_3D_3N_4O_3Na^+$ [M+Na^+]: 196.0520, found: 196.0527;

MP >350 °C

5,6-Diamino-3-($^2H_3$)methyluracil: 6-Amino-3-($^2H_3$)methyl-5-nitrosouracil (0.12 g, 0.71 mmol) was suspended in a mixture of ammonium hydroxide (1.5 mL, 28-30%) and water (1.75 mL). The suspension was heated to 70 °C and sodium dithionite (0.51 g, 2.93 mmol) was added portionwise over 25 min until the red solution became pale yellow. The mixture was stirred at 70 °C for 1 h before being concentrated *in vacuo*. The crude solid was then continuously extracted with refluxing ethanol (70 mL) for 3 h and the extract was concentrated *in vacuo* to give the *title compound* **10** (0.04 g, 37%) as a yellow solid.

IR (film) ν_{max} 3330, 2919, 1701, 1595, 1459, 1171, 962 cm^{-1}

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.69 (1H br, s, H-1), 6.93 (2H br, s, NH_2), 3.33 (2H br, s, NH_2);

^{13}C NMR (100 MHz, DMSO-d_6) δ 164.2 (C-4), 156.9 (C-6), 153.5 (C-2), 141.3 (C-5);

HRMS (ESI^+) calculated for $C_5H_5D_3N_4O_2Na^+$ [M+Na^+]: 160.0908, found: 160.0918;

MP 222-225 °C decomp

3,6,7-(3-$^{2}H_3$)Trimethyllumazine (11): 5,6-Diamino-3-($^{2}H_3$)methyl uracil **10** (0.04 g, 0.26 mmol) was suspended in ethanol (2 mL). 2,3-Butandione **8** (0.03 g, 0.33 mmol) and acetic acid (0.07 g, 1.22 mmol) were added. The mixture was heated at reflux for 24 h before being cooled to r.t. and concentrated *in vacuo*. The crude product was purified by flash chromatography (pet. ether-EtOAc 1:4) to give the *title compound* **11** (0.02 g, 36%) as a colourless solid. An analytical sample was recrystallized from a mixture of chloroform and ethanol (1:1).

IR (film) ν_{max} 2920, 1724, 1662, 1561, 1353, 1274, 940 cm^{-1}

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.53 (1H, s, H-1), 2.66 (3H, s, C7-CH_3), 2.65 (3H, s, C6-CH_3);

^{13}C NMR (100 MHz, $CDCl_3$) δ 161.1 (C-4), 158.9 (C-6), 150.6 (C-7),* 150.4 (C-2),* 145.0 (C-8a), 123.7 (C-4a), 22.8 (C6-*C*H_3), 21.9 (C7-*C*H_3); *assignments are interchangeable.

HRMS (ESI^+) calculated for $C_9H_7D_3N_4O_2Na^+$ [$M+Na^+$]: 232.0888, found: 232.0888.

MP 274-277 °C

Aspects of the present invention have been described by way of example only and it should be appreciated that modifications and additions may be made there to without departing from the scope thereof, as defined in the appended claims.

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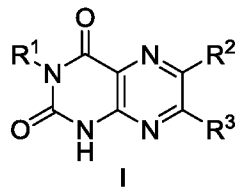
What is Claimed is:

1. A marker compound of Type I isolated from a *Leptospermum* honey and represented by the following structural formula **I**:



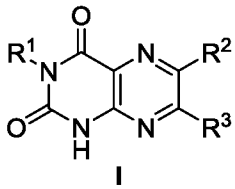
I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

2. The marker compound as claimed in claim 1 wherein, R^1, R^2, and R^3 are $-CH_3$.

3. The marker compound as claimed in claims 1 and 2 wherein, the Leptospermum honey is selected from the flower group comprising: *Leptospermum scoparium, Leptospermum scoparium var. exinium, Leptospermum polygalifolium, Leptospermum subtenue.*

4. A marker compound of Type II represented by the following structural formula **I**:



I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group where the marker compound of Type II contains at least one isotope not having an identical atomic mass to that of the most abundantly occurring isotope of that element in nature.

5. A method of isolation of at least one compound of Type I as claimed in any one of claims 1 to 4, comprising the following step:
   a. a chromatography step in which a honey fraction is collected by eluting a honey derived from nectar collected from a *Leptospermum* flower with at least one elution solvent.

6. The method of isolation of at least one compound of Type I as claimed in claim 5, wherein the at least one compound of Type I can be further purified with a second and subsequent chromatography or other purification steps.

7. The method of isolation of the at least one compound of Type I as claimed in claim 5 or claim 6, wherein the organic elution solvent is a solution selected from the group comprising: acetic acid or acetonitrile.

8. A method of assaying and quantifying a *Leptospermum* honey comprising the following steps:
   a. deriving a calibration curve for the concentration of at least one compound of Type II using mass spectrometry of the *Leptospermum* honey;
   b. generating a calibration curve mass spectrum of a *Leptospermum* honey that contains a known concentration of the at least one compound of Type II as an internal standard; and
   c. deriving the concentration of the at least one compound of Type I native to the *Leptospermum* honey via interpolation using the calibration curve generated in step (a) and the mass spectrum generated in step (b).

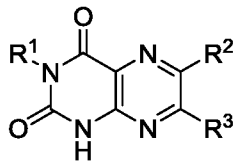
9. A method of assaying and quantifying a Leptospermum honey, comprising the following steps:
   a. subjecting a Leptospernum honey to a stimulus sufficient to cause fluorescence of at least one compound of Type I as claimed in any one of claims 1 to 4 present in the Leptospernum honey; and
   b. measuring an amount of fluorescence of the at least one compound of the Type I in the Leptospernum honey.

10. The method of assaying and quantifying a *Leptospermum* honey as claimed in claim 9, wherein at least one compound of Type I is at least two compounds.

11. The method of assaying and quantifying a *Leptospermum* honey as claimed in claim 9 or claim 10, wherein the method of assaying and quantifying would also include the method step c) of determining the authenticity of the a *Leptospermum* honey based on the measured amount of the at least one compound represented by the formula I.

12. A test kit for testing the purity of a *Leptospermum* honey, the kit comprising at least one compound of the Type I or Type II as claimed in any one of claims 1 to 4.

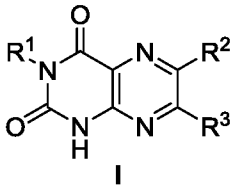
13. The use of at least one compound of Type I isolated from a *Leptospermum* honey and represented by the following structural formula I:



I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group, in the manufacture of a medicament for the treatment of disease.

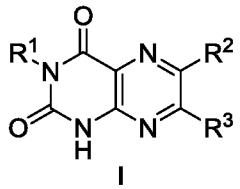
14. A composition comprising of at least one compound of Type I isolated from a *Leptospermum* honey and represented by the following structural formula I:



I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group for use in the treatment of wounds.

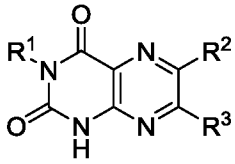
15. A food supplement to improve physiological oxidative stress comprising at least one compound of Type I isolated from a *Leptospermum* honey and represented by the following structural formula I:



I

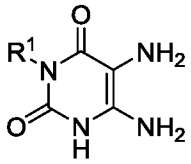
or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a -H, $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

16. The manufacture of at least one compound represented by the following structural formula I:



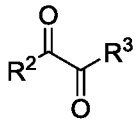
I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group produced via a condensation of an intermediate of the following structural formula **II**



II

wherein R^1 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group with a compound of the following structural formula **III**

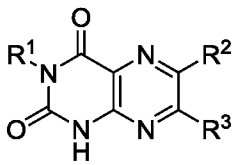


III

wherein R^2 and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

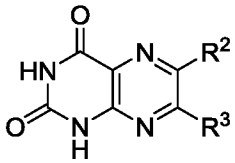
17. The manufacture of at least one compound represented by the following structural formula **I** as claimed in claim 16, wherein the compound of the structural formula **III** is in the presence of an acid in a liquid carrier.

18. The manufacture of at least one compound represented by the following structural formula **I**:



I

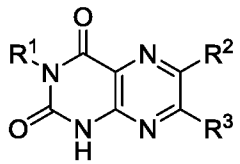
or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group produced via an alkylation of the compound represented by the following structural formula **IV** at position N-3



IV

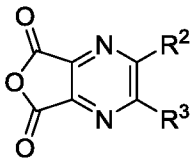
wherein R^2 and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

19. The manufacture of at least one compound represented by the following structural formula I:



I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group produced via the generation of a transient isocyanate species from a compound of the following structural formula **V**:

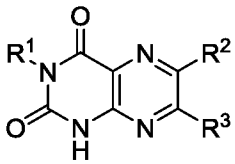


V

wherein R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

20. The manufacture of at least one compound represented by the following structural formula I as claimed in claim 19, wherein the transient isocyanate species is that generated by a Curtius, Hofmann, Lossen or Schmidt rearrangement.

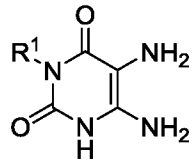
21. The manufacture of at least one compound of Type II represented by the following structural formula I:



I

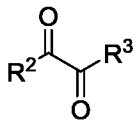
or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group, where the compound of Type II contains at least one isotope

produced via a condensation of an intermediate of the following structural formula **II**



II

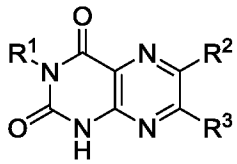
wherein R^1 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group with compound of the following structural formula **III**



III

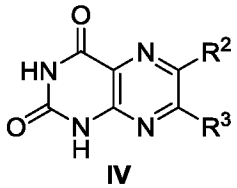
wherein R^2 and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

22. The manufacture of at least one compound of Type II represented by the following structural formula I:



I

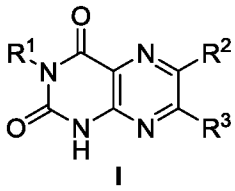
or a tautomer thereof, wherein R^1, R^2, and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group, produced via an alkylation of the compound represented by the following structural formula IV at position N-3:



IV

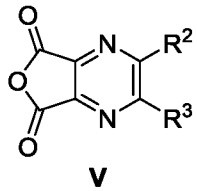
wherein R^2 and R^3 independently represents either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

23. The manufacture of at least one compound represented by the following structural formula I:



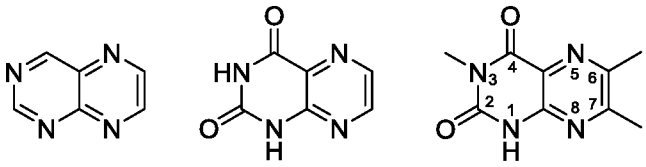
I

or a tautomer thereof, wherein R^1, R^2, and R^3 independently represent either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group produced via the generation of a transient isocyanate species from a compound of the following structural formula V:

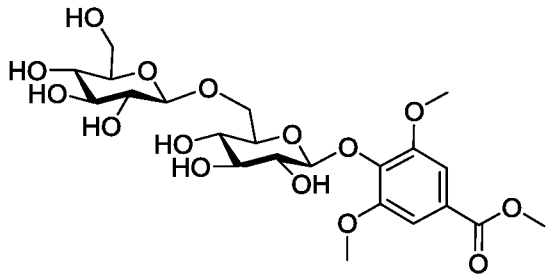


V

wherein R^2, and R^3 independently represent either a $-CH_3$, $-C_2H_5$, $-C_3H_7$ or $-C_4H_9$ alkyl group.

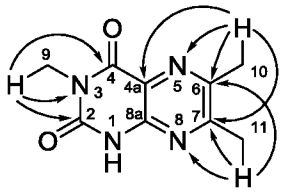


1 2 3



4

FIGURE 1



3

FIGURE 2

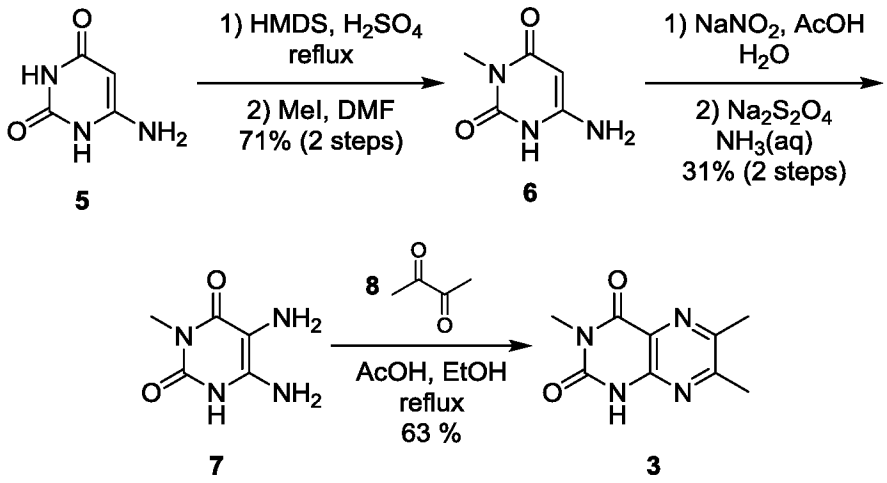

1) HMDS, H2SO4
reflux
2) MeI, DMF
71% (2 steps)
1) NaNO2, AcOH
H2O
2) Na2S2O4
NH3(aq)
31% (2 steps)
8
AcOH, EtOH
reflux
63 %
5
6
7
3

FIGURE 3

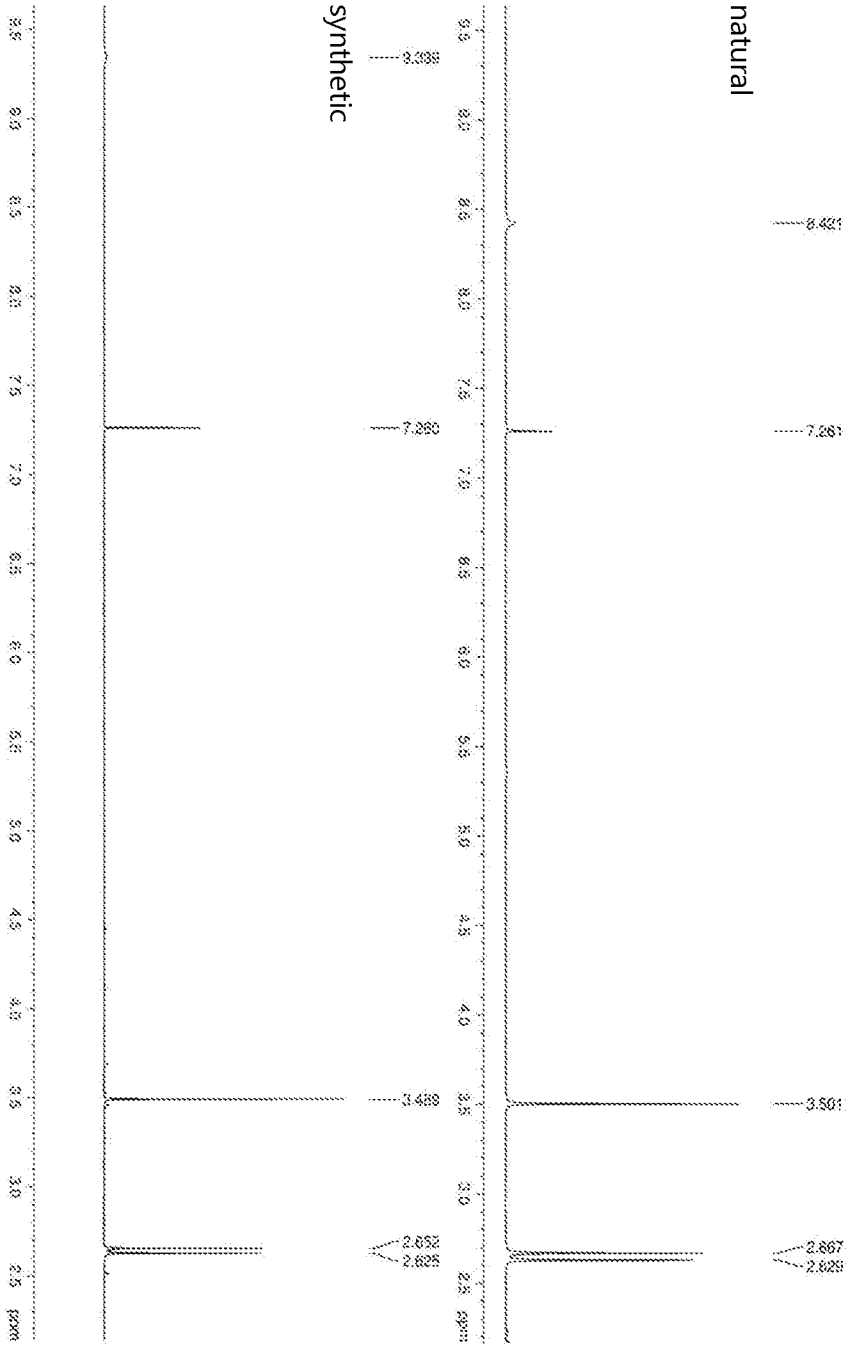

synthetic
9.038
7.280
3.489
2.652
2.625
natural
8.421
7.281
3.501
2.867
2.629
ppm

FIGURE 4/12

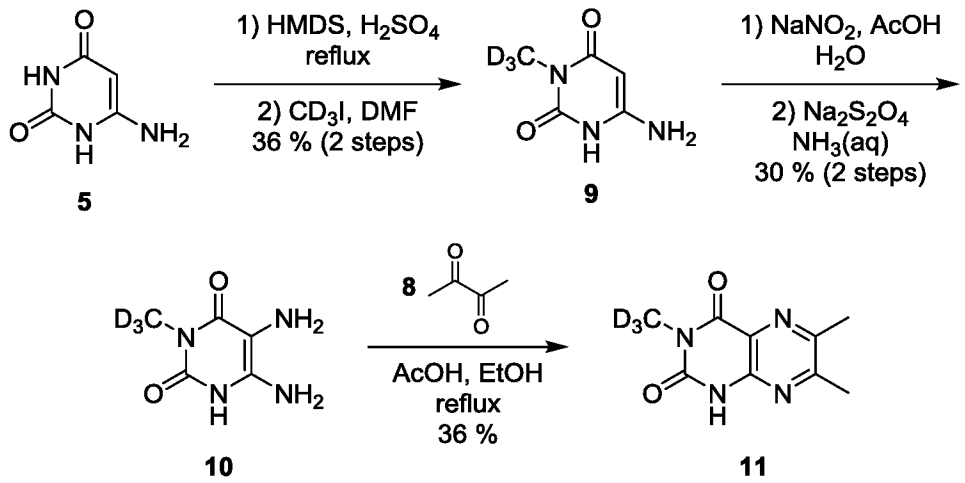

1) HMDS, H2SO4
reflux
2) CD3I, DMF
36 % (2 steps)
1) NaNO2, AcOH
H2O
2) Na2S2O4
NH3(aq)
30 % (2 steps)
8
AcOH, EtOH
reflux
36 %
5
9
10
11

FIGURE 5

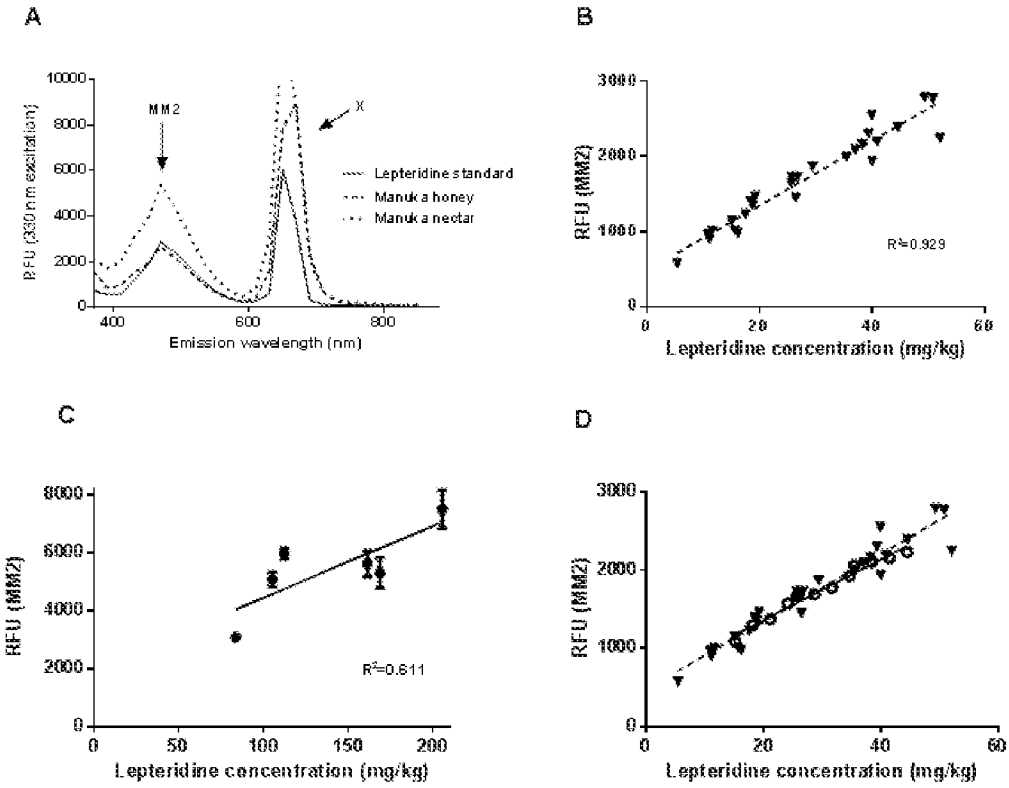

A
B
C
D
MM2
X
Lepteridine standard
Manuka honey
Manuka nectar
RFU (330 nm excitation)
Emission wavelength (nm)
RFU (MM2)
Lepteridine concentration (mg/kg)
R²=0.929
R²=0.611

FIGURE 6

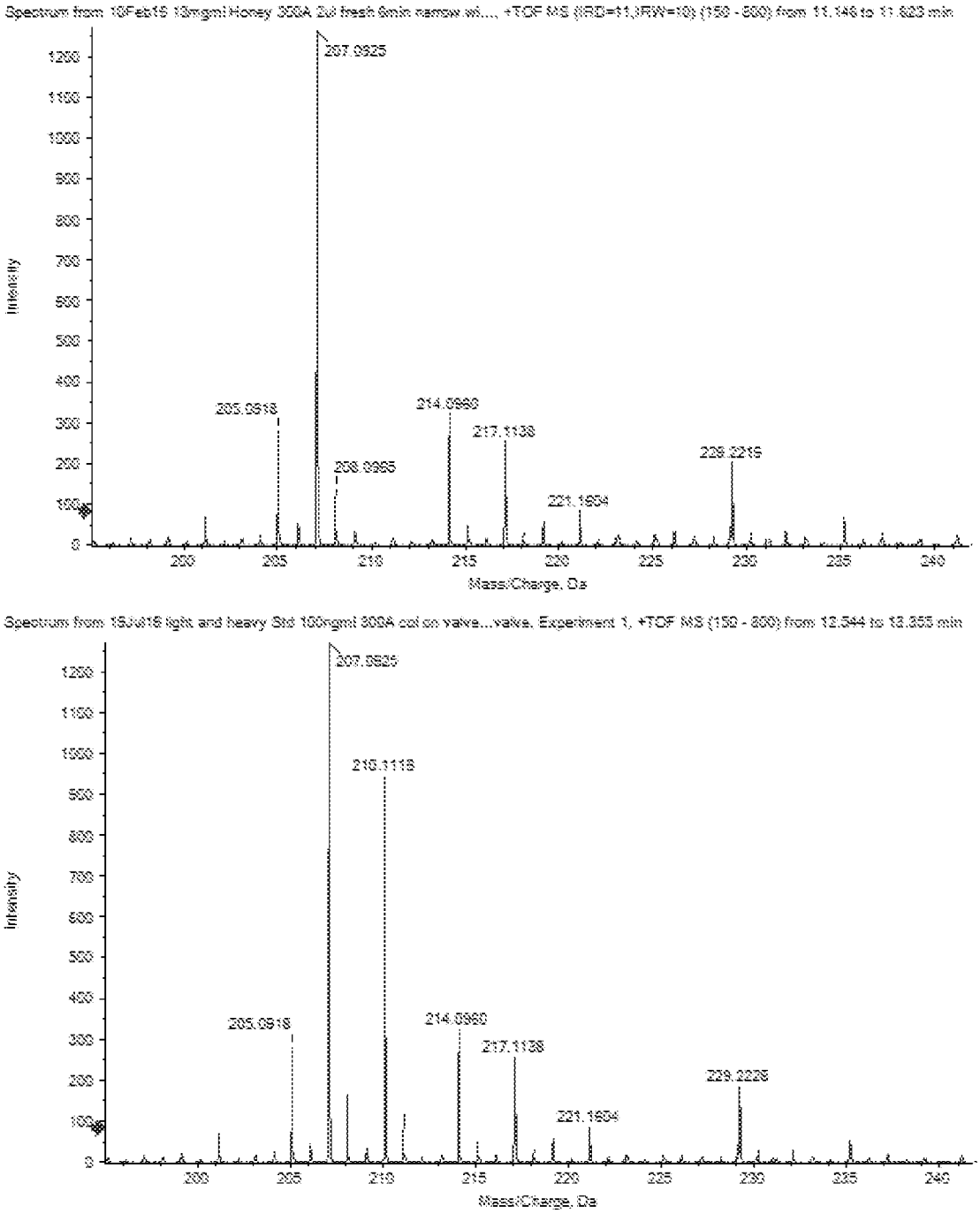

Spectrum from 18Feb16 13mgml Honey 300A 2ul fresh 6min narrow wi..., +TOF MS (IRD=11,IRW=10) (150 - 600) from 11.146 to 11.823 min
Intensity
Mass/Charge, Da
207.0825
205.0918
214.0960
217.1138
208.0965
221.1604
229.2228
Spectrum from 18Jul16 light and heavy Std 100ngml 300A col on valve...valve, Experiment 1, +TOF MS (150 - 600) from 12.344 to 13.355 min
Intensity
Mass/Charge, Da
207.0825
210.1118
205.0918
214.0960
217.1138
229.2228
221.1604

FIGURE 7

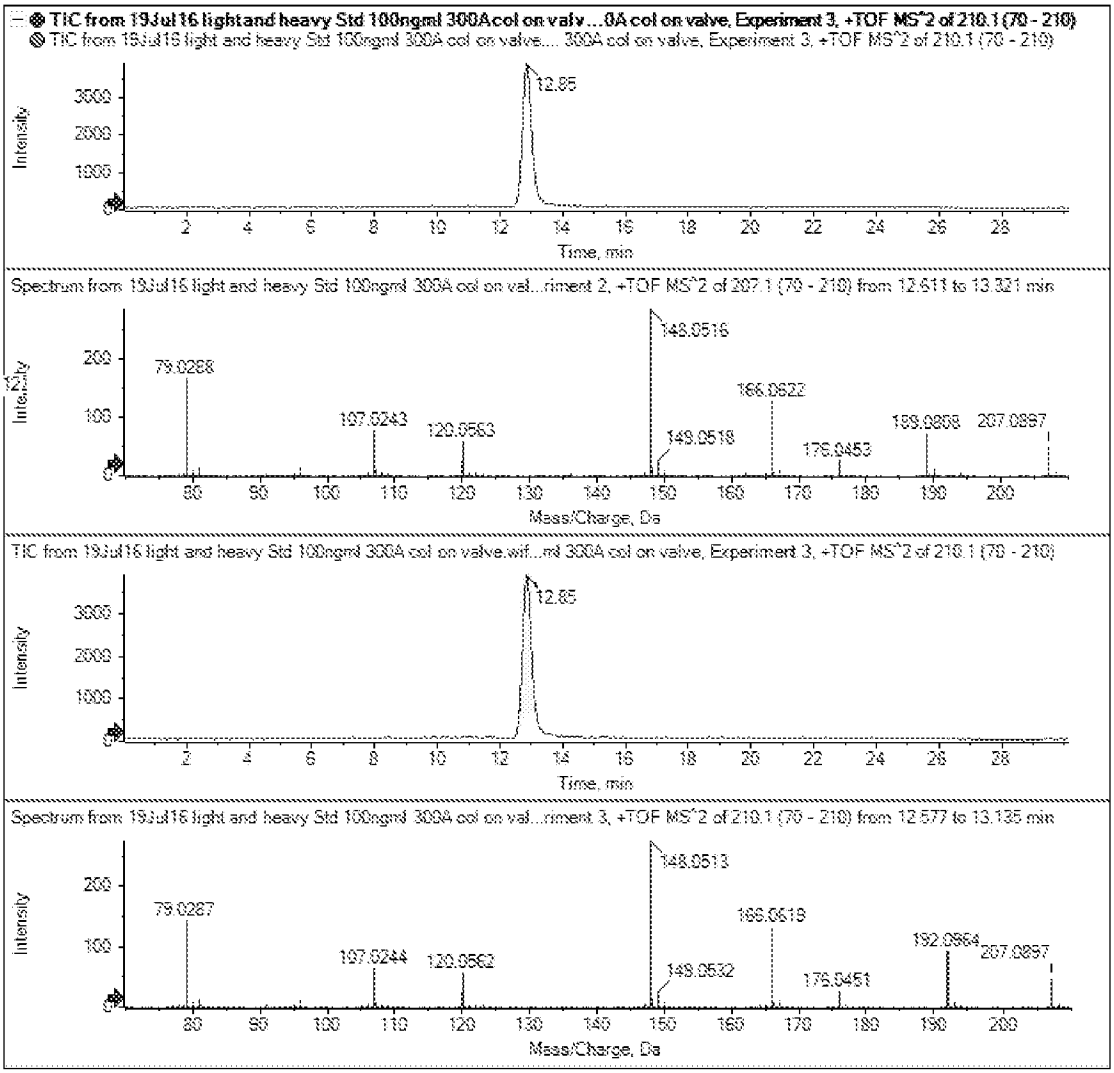

TIC from 19Jul16 light and heavy Std 100ngml 300A col on valv...0A col on valve, Experiment 3, +TOF MS^2 of 219.1 (70 - 219)
TIC from 19Jul16 light and heavy Std 100ngml 300A col on valve.... 300A col on valve, Experiment 3, +TOF MS^2 of 219.1 (70 - 219)
Intensity
12.85
Time, min
Spectrum from 19Jul16 light and heavy Std 100ngml 300A col on val...riment 2, +TOF MS^2 of 207.1 (70 - 219) from 12.611 to 13.321 min
79.0288
197.0243
120.0563
148.0516
149.0518
166.0622
176.0453
189.0808
207.0897
Mass/Charge, Da
TIC from 19Jul16 light and heavy Std 100ngml 300A col on valve.wif...ml 300A col on valve, Experiment 3, +TOF MS^2 of 219.1 (70 - 219)
Spectrum from 19Jul16 light and heavy Std 100ngml 300A col on val...riment 3, +TOF MS^2 of 219.1 (70 - 219) from 12.577 to 13.135 min
79.0287
197.0244
120.0562
148.0513
149.0532
166.0619
176.0451
192.0864
207.0897

FIGURE 8

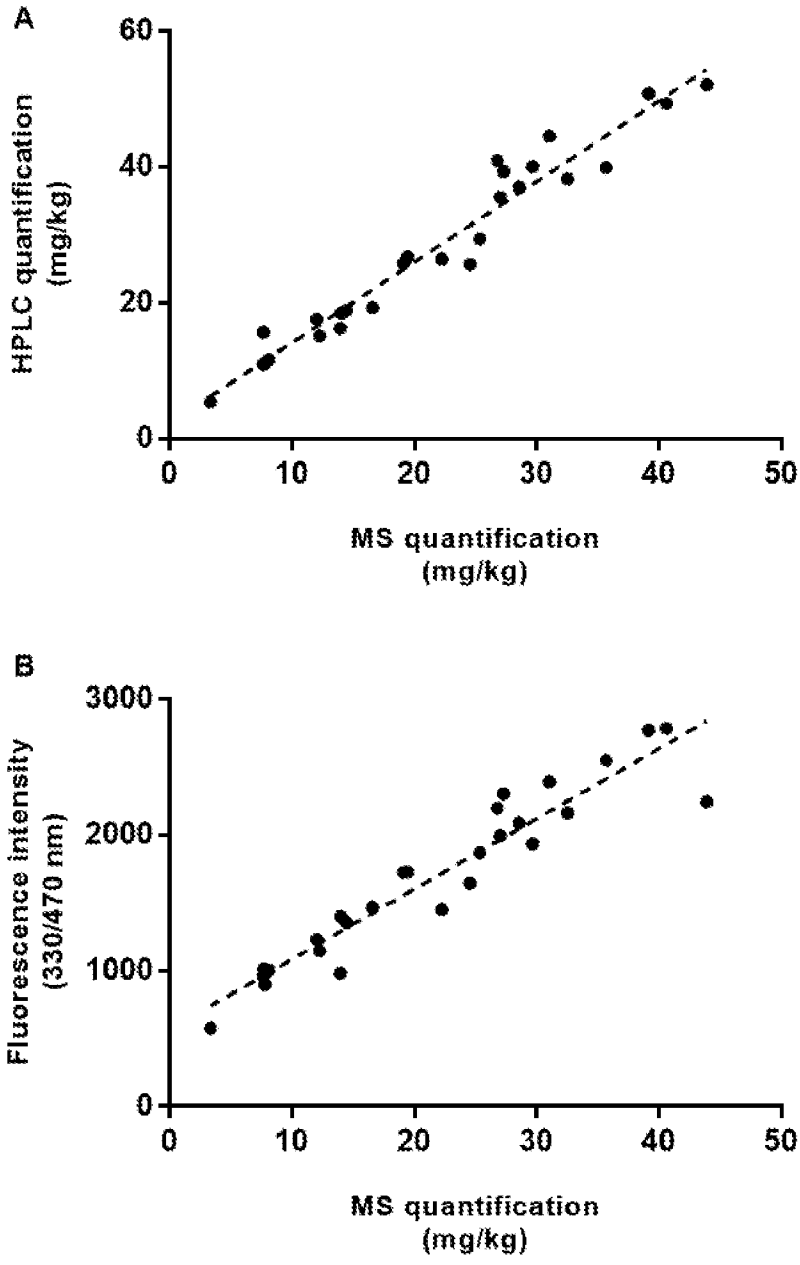

A
HPLC quantification (mg/kg)
60
40
20
0
0
10
20
30
40
50
MS quantification (mg/kg)
B
Fluorescence intensity (330/470 nm)
3000
2000
1000
0
0
10
20
30
40
50
MS quantification (mg/kg)

FIGURE 9

L. scoparium nectar content	Enzyme treatment groups			
	Control	**Group 1**	**Group 2**	**Group 3**
100% 66% 33%	Nectar only	Nectar + Glucose oxidase	Nectar + Glucose oxidase + α-glucosidase	Nectar + Glucose oxidase + α-glucosidase + β-glucosidase

FIGURE 10

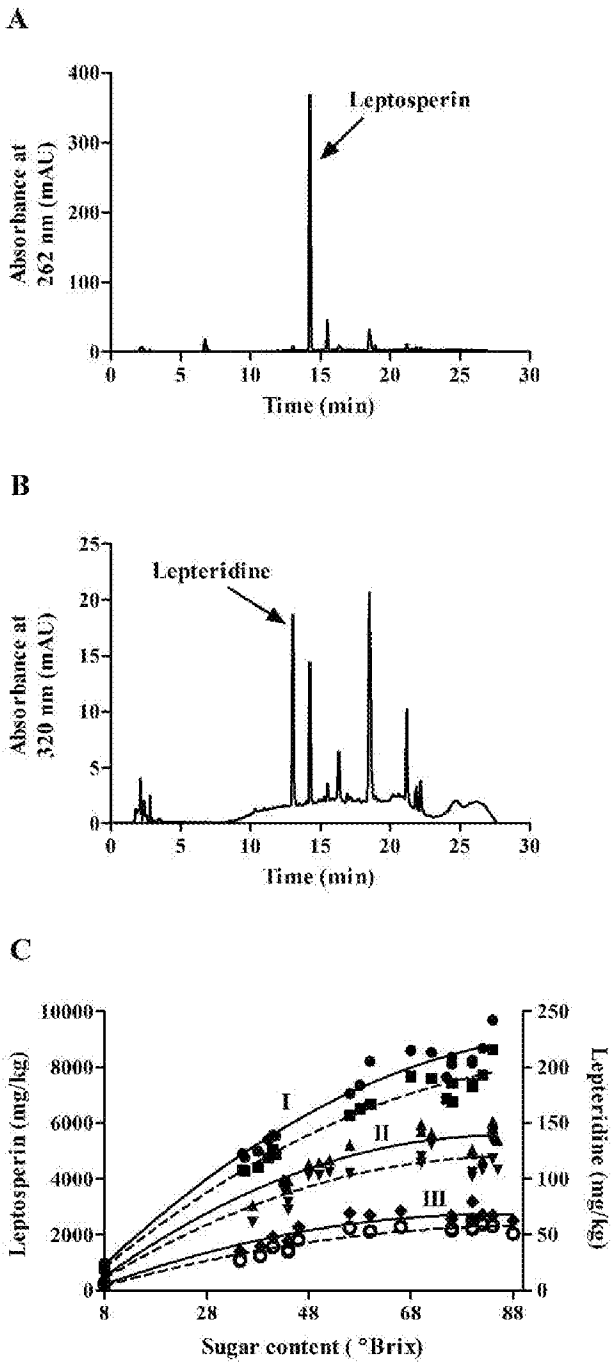

A
Leptosperin
Absorbance at 262 nm (mAU)
Time (min)
B
Lepteridine
Absorbance at 320 nm (mAU)
Time (min)
C
I
II
III
Leptosperin (mg/kg)
Lepteridine (mg/kg)
Sugar content (°Brix)

FIGURE 11

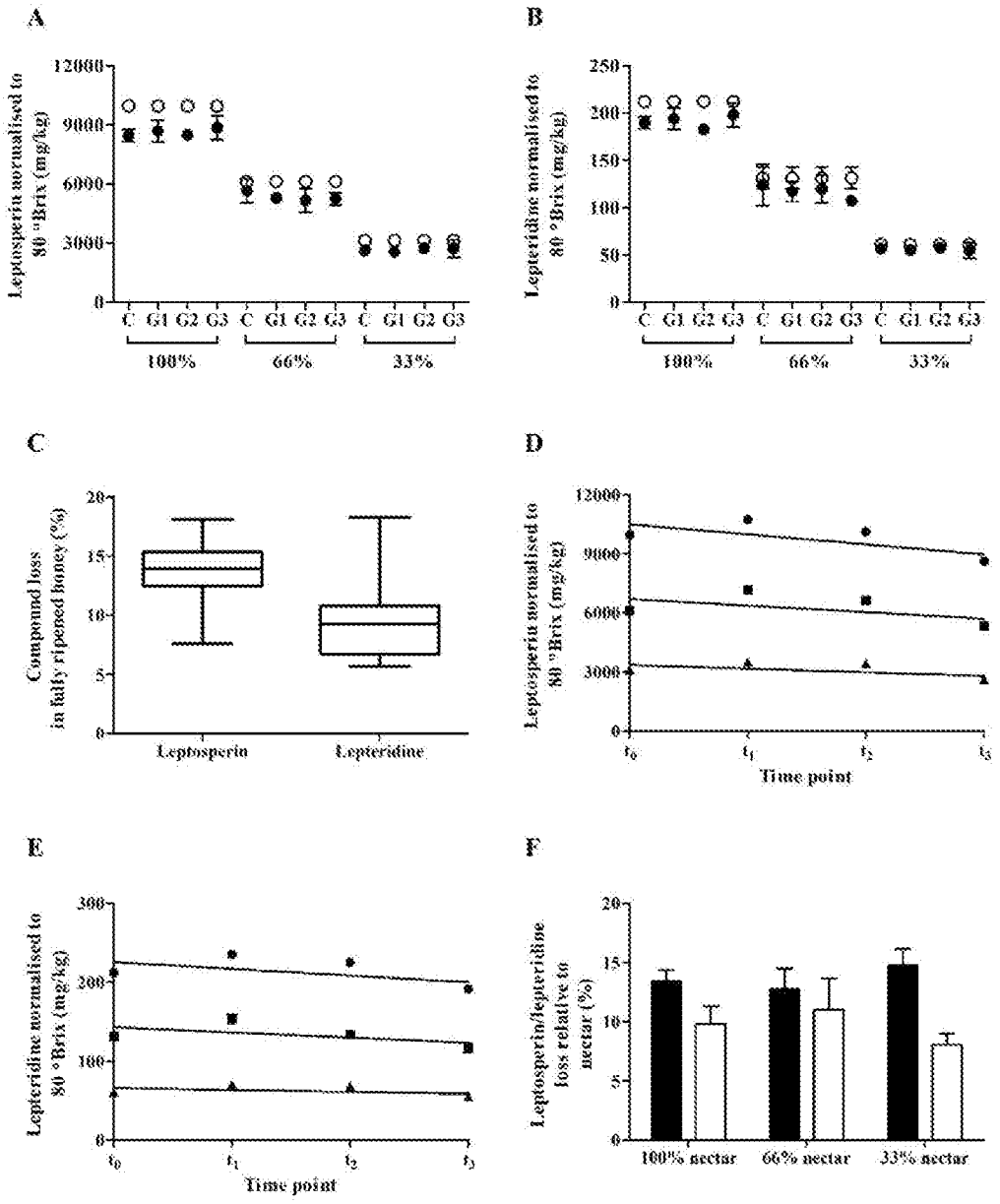

A
Leptosperin normalised to 80 °Brix (mg/kg)
12000
9000
6000
3000
0
C G1 G2 G3 C G1 G2 G3 C G1 G2 G3
100% 66% 33%
B
Lepteridine normalised to 80 °Brix (mg/kg)
250
200
150
100
50
0
C G1 G2 G3 C G1 G2 G3 C G1 G2 G3
100% 66% 33%
C
Compound loss in fully ripened honey (%)
20
15
10
5
0
Leptosperin
Lepteridine
D
Leptosperin normalised to 80 °Brix (mg/kg)
12000
9000
6000
3000
0
t_0 t_1 t_2 t_3
Time point
E
Lepteridine normalised to 80 °Brix (mg/kg)
300
200
100
0
t_0 t_1 t_2 t_3
Time point
F
Leptosperin/lepteridine loss relative to nectar (%)
20
15
10
5
0
100% nectar
66% nectar
33% nectar

FIGURE 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2016/050195

A. CLASSIFICATION OF SUBJECT MATTER

C07D 487/04 (2006.01) A61K 31/519 (2006.01) A61P 17/02 (2006.01) A61K 31/33 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Invention Search: Database- STN Registry and CAPlus: Based upon substructure searches based on compounds of Formula I and Keywords 'Manuka', 'Honey', 'LEPTOSPERMUM HONEY' and similar terms. Additionally, Preparation of compounds using 'Prep' role in CAPlus.

Database- Google Scholar: Keywords 'Compounds' with 'Honey', 'Lepteridine', 'Medical uses' and 'Honey'.

Applicant/Inventor Search: Database- Patenscope: Keywords 'COMVITA LIMITED' or 'Gordana PRIJIC' or 'Margaret Anne BRIMBLE or 'Ralf Christian SCHLOTHAUER' or 'Jonathan STEPHENS' or 'Benjamin DANIELS'

Applicant(s)/Inventor(s) name searched in internal databases provided by IP Australia.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 May 2017	15 May 2017
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Will Findlay AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262832018

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	**PCT/NZ2016/050195**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Erejuwa O.O. et al., Honey: A Novel Antioxidant, Molecules, 2012, 17, pages 4400-4423	
A	Abstract, Section 3 (pages 4404 -4411) and Figure 2a and b (page 4403)	1-23
	Alvarez-Suarez J.M. et al., The Composition and Biological Activity of Honey: A Focus on Manuka Honey, Foods, 2014, 3, pages 420-432	
X	Sections 3-5 (pages 423-428) and Table 1 (page 423)	13-15
A	Abstract and whole document (pages 420-431)	1-12, 16-23
	Eteraf-Oskouei T. et al, Traditional and Modern Uses of Natural Honey in Human Diseases: A Review, Iranian Journal of Basic Medical Sciences, 2013, 16(6), pages 731-742	
X	Abstract, 'Place of honey in modern medicine' (pages 733-738) and 'Chemical composition of natural honey' (pages 731-732)	13-15
A	Abstract and whole document (pages 731-742)	1-12, 16-23
	Rückriemen J. et al, Identification and Quantitation of 2-Acetyl-1-pyrroline in Manuka Honey (*Leptospermum scoparium*), Journal of Agricultural and Food Chemistry, 2015, 63, pages 8488−8492	
X	Materials and Methods, pages 8488-8489	8-12
A	Abstract	1-7, 13-23
	US 8759774 B2 (Aitkenhead et al.) 24 June 2014	
X	Examples 1-8	8-12
A	Summary	1-7, 13-23
	Adams C.J. et al., Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (*Leptospermum scoparium*) honey, Carbohydrate Research, 2008, 343(3), pages 651-659	
X	Results and discussion (pages 654-658)	5-7
A	Abstract	1-4, 8-23
	Daniels B.J. et al, Isolation, Structural Elucidation, and Synthesis of Lepteridine From Mānuka (*Leptospermum scoparium*) Honey, Journal of Agricultural and Food Chemistry, 2016, 64, pages 5079−5084	
P,X	Abstract and whole document (pages 5079-5084) and Supporting document (pages 1-17)	1-23

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/NZ2016/050195**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
US 8759774 B2	24 June 2014	US 2013284945 A1	31 Oct 2013
		US 8759774 B2	24 Jun 2014
		AU 2011337271 A1	30 May 2013
		AU 2011337271 B2	24 Jul 2014
		CA 2818493 A1	07 Jun 2012
		EP 2646805 A1	09 Oct 2013
		NZ 589582 A	28 Mar 2013
		WO 2012074413 A1	07 Jun 2012

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.