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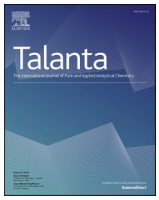

Identification of novel unique mānuka honey markers using high-resolution mass spectrometry-based metabolomics

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ABSTRACT

Mānuka honey is a valuable commodity produced by bees foraging the flowers of *Leptospermum scoparium*, a bush native to New Zealand and Australia. Due to its high value and proven health benefits, authenticity fraud in the sale of this food is a significant risk, as recounted in the literature. Four compulsory natural products must be present at minimum concentrations to authenticate mānuka honey (3-phenyllactic acid, 2′-methoxyacetophenone, 2-methoxybenzoic acid, and 4-hydroxyphenyllactic acid). However, spiking other kinds of honey with these compounds and/or the dilution of mānuka honey with other varieties may result in fraud going undetected. In this work, liquid chromatography coupled with high-resolution mass spectrometry and a metabolomics-based strategy has allowed us to tentatively identify 19 natural products –putative mānuka honey markers–, nine of which are reported for the first time. Chemometric models applied to these markers allowed the detection of both spiking and dilution fraud attempts of mānuka honey, even at 75% mānuka honey purity. Thus, the herein-reported methodology can be employed in the prevention and detection of mānuka honey adulteration even at low levels, and the tentatively identified markers presented in this work proved valuable for mānuka honey authentication procedures.

1. Introduction

Mānuka honey, which is produced by bees foraging on the mānuka tree (*Leptospermum scoparium*), a tree from New Zealand –that also occurs in Australia– is the highest-priced monofloral honey in the honey world market [1]. This fact stems from the well-known antibacterial properties, high antioxidant capacity, and antibacterial properties of mānuka honey [2].

Fraud in valuable commodities such as mānuka honey results in both economic and health repercussions to manufacturers and consumers, respectively, hence why the relevant authorities attempt to prevent it with various legislative bodies and control policies. Due to its high value, many attempts to commercialise other kinds of honey as mānuka honey have been reported, which consequently has sparked the interest of the scientific community [3–6]. During an investigation led by The Independent newspaper journalists in 2014, it was found that approximately 83% of honey sold as mānuka honey was counterfeit: that year, 1700 tonnes were produced in New Zealand, but global consumption sat at 10,000 tonnes—1800 of which in the United Kingdom only– [7]. To hamper this fraud, the New Zealand legislation approved in 2017 a precise legal definition for mānuka honey authenticity that requires the presence of 3-phenyllactic acid at a level ≥400 mg/kg, 2′-methoxyacetophenone at a level ≥5 mg/kg, 2-methoxybenzoic acid at a level ≥1 mg/kg, and 4-hydroxyphenyllactic acid at a level ≥1 mg/kg –for

Abbreviations: **^{1}H-NMR**, proton magnetic nuclear resonance; **DDA**, data dependent acquisition; **ESI**, electrospray ionisation; **FS**, full scan; **HRMS**, high-resolution mass spectrometry; **IDA**, information-dependent acquisition; ***m/z***, mass-to-charge ratio; **MeOH**, methanol; **MS^2**, tandem mass spectrometry; **NMR**, nuclear magnetic resonance; OPLS-DA, orthogonal partial least squares-discriminant analysis; **PC**, principal component; **PCA**, principal component analysis; **PDA**, photodiode array; **ppm**, parts-per-million; **QqQ-MS^2**, triple quadrupole tandem mass spectrometry; **QToF**, quadrupole-time-of-flight; **SPME-GC/MS**, solid-phase microextraction coupled with gas chromatography mass spectrometry; **TIC**, total ion chromatogram; **t_R**, retention time; **UHPLC**, ultra-high-pressure liquid chromatography; **UHPLC-QToF-HRMS**, UHPLC coupled with of quadrupole-time-of-flight high-resolution mass spectrometry; **UMF**, Unique Mānuka Factor; **XIC**, extracted ion chromatogram.

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monofloral mānuka honey– [8]. Additionally, the legislation also requires the presence of a specific DNA marker from mānuka pollen. The concentration of phytochemicals (particularly 2′-methoxyacetophenone and 3-phenyllactic acid) was set high to prevent the mixture of monofloral mānuka honey with multifloral honey of lower price that could be further sold as genuine mānuka honey. Nevertheless, mānuka honey's chemical composition is far more complex than the four chemicals listed in the regulation, which also does not include some characteristic components of mānuka honey such as methylglyoxal, leptosperin, or fluorescent lepteridines [9,10].

Methylglyoxal is one of the main mānuka honey constituents responsible for the beneficial properties of this commodity –alongside its precursor, dihydroxyacetone, which is formed when the nectar of plants is converted into honey by the enzymes in the bees' stomachs [9,11]. Methylglyoxal is present in small amounts in many types of honey. Methylglyoxal has been shown to have antibacterial properties and is thought to contribute to the unique medicinal properties of mānuka honey. New Zealand mānuka honey bee producers developed an index called Unique Mānuka Factor (UMF), graded from UMF5+ to UMF25+, based on the concentration of four chemicals additional to those legally controlled: methylglyoxal, leptosperin, dihydroxyacetone, and hydroxymethylfurfural [12]. The higher the UMF, the higher the price of mānuka honey. The concentration of methylglyoxal in mānuka honey can vary widely, with higher levels generally indicating a higher quality product. Unfortunately, methylglyoxal concentration can deplete upon mānuka honey manufacture and storage, as it can react with proteins and sugars, yielding complex chemicals that make the analysis and characterisation of this commodity even more complicated [13]. Moreover, flavonoid content in mānuka honey is the highest among kinds of honey, luteolin being the majoritarian flavone. Other polyphenols, such as syringic acid derivatives and benzophenones, have been described in mānuka honey [6]. Given all these possible variables in the chemical composition, it is hard to find a comprehensive and, at the same time, unequivocal authenticity test for mānuka honey, as the four legal compounds which must be present in mānuka honey can be added by manufacturers to accomplish the regulation requirements [8]. Furthermore, as methylglyoxal is a cheap chemical, fraud attempts achieving higher UMF values by spiking it to lower UMF-value mānuka honey have been reported [14].

In recent years, coupling advanced chemometric tools based on statistical multivariate analysis with powerful, sensitive instrumentation capable of performing either targeted or non-targeted evaluations has resulted in great tools to respond to this analytical challenge posed by mānuka honey characterisation. The discovered differences can then be used as chemical markers of a given condition of the commodity or organism, an approach that has been successfully implemented by our research groups in previous works [15–17]. To extract information on potential markers and/or classify different sample groups, the acquired data must be processed using chemometric models. These models may be descriptive, such as principal component analysis (PCA), or classificatory, like orthogonal partial least squares-discriminant analysis (OPLS-DA). The former is typically employed to determine the variance between two or more sample groups, whereas the latter prioritises separation between two groups. Borràs et al. provide an extensive overview of the existing models, the data processing strategies for chemometric analysis, and possible data fusion strategies [18].

In a recent study, the uniqueness of mānuka honey, compared against kanuka and jelly bush honey, was assessed via the presence of some sesquiterpenoids such as unedone among the volatiles analysed by solid-phase microextraction coupled with gas chromatography-mass spectrometry (SPME-GC/MS) [19]. The authors also disclosed some polyphenols as possible markers, such as those already included in the legislation, and they identified other new substances (e.g., methyl syringate, kojic acid, or leptosperin) employing photodiode array (PDA) coupled with triple quadrupole tandem mass spectrometry (QqQ-MS^2) and ultra-high-pressure liquid chromatography (UHPLC). Proton nuclear magnetic resonance (^{1}H NMR) metabolomics in combination with chemometric tools has proven useful in distinguishing mānuka honey from other floral types [1].

The "gold standard" in the identification and evaluation of new secondary metabolites, however, is the combination of UHPLC-HRMS with multivariate analysis, in which chemometrics plays a key role [20]. Mass spectrometry instruments performing accurate exact mass measurements offer the opportunity to identify compounds according to their exact masses. Within this frame, Kasiotis et al. recently employed this combination to characterise high-value monofloral honey, such as Ikaria heather or "Anama" honey, made by bees foraging the nectar of *Erica manipulifora*, a plant natural to the Greek island of Ikaria. The authors determined some characteristic metabolites of Anama honey using a UHPLC coupled with a quadrupole-Orbitrap (UHPLC-Q-Orbitrap) instrument working in data-dependant acquisition (DDA) mode, also known as information-dependant acquisition (IDA). The data were processed using both PCA and OPLS-DA. The former showed some overlapping between the compared groups, however, the OPLS-DA models allowed the authors to differentiate Anama honey from thyme or pine honey. Kasiotis et al. identified 32 possible markers of Anama honey authenticity using this approach [21]. Regarding mānuka honey, Jandrić et al. reported the use of UHPLC coupled with quadrupole-time-of-flight (UHPLC-QToF) to study this commodity and to distinguish it from other varieties. Initially, many of the detected compounds were classified as unknowns; however, in a later work, the same group was able to identify some common polyphenols such as pinobanksin or gallic acid together with already known mānuka honey components. Again, the use of chemometrics (PCA only in this case) was critical in the characterisation of the honey types in terms of botanical and geographical origin. The models employed by the authors allowed them to differentiate even closely related honey types, such as UMF6+ and UMF10+ mānuka honey [22,23]. More successful examples of HRMS-based analyses and chemometric models are found in the literature. Koulis et al. for instance, evaluated the metabolic differences between Greek and Polish honey employing a Q-ToF HRMS instrument, and then classified honey samples according to their geographical origin using PCA and partial least squares-discriminant analysis (PLS-DA) chemometric models [24]. A different authentication approach was undertaken by Yan et al. who used a UHPLC-Q-ToF instrument to differentiate naturally and artificially matured acacia honey, which they achieved with the use of PCA models [25].

However, the standardisation of HRMS analyses is still a pending task, even if harmonisation in data reporting has improved [26]. Unlike targeted analyses, for which the legislation body offers precise and extensive guidelines –such as for the assessment of mānuka honey authenticity [8]–, no such thorough and standardised directives exist for HRMS analyses. This situation is worrisome insomuch as it has been reported to result in false positives in terms of honey adulteration, as recently reported by the Danish Beekeeper Association [27]. On a related note, some honey commodities –including mānuka honey– are known to fail the C-4 sugar method utilised to determine adulteration via sugar addition [13], hence, the inclusion of new secondary metabolites for specific commodities is essential in the assessment of their authenticity.

In this work, we describe the use of UHPLC-QToF-HRMS combined with multivariate analysis to find and elucidate unique chemical markers of mānuka honey which permit its univocal differentiation from other monofloral kinds of honey. The potential of the metabolomics-based tools herein developed to identify manuka honey among other varieties has been evaluated as well. With this purpose, several mānuka honey adulteration strategies were essayed, including its dilution with other honey varieties and/or via the spiking of natural mānuka honey markers.

2. Material and methods

2.1. Reagents

The standards 2,4-D (CAS 94-75-7), carbendazim-D_3 (CAS 1255507-88-0), dichlorvos-D_6 (CAS 203645-53-8), dimethoate-D_6 (1,219,794-81-6), malathion-D_{10} (CAS 121-75-5), 3-phenyllactic acid (CAS 828-01-3), 2′-methoxyacetophenone (CAS 579-74-8), 2-methoxybenzoic acid (CAS 579-75-9), *p*-hydroxyphenyllactic acid (CAS 306-23-0), and 4-hydroxyphenylacetic acid (CAS 156-38-7) were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). Individual stock standard solutions were prepared at 1 mg/mL in acetonitrile and stored in amber screw-capped glass vials in the dark at −30 °C. A mix solution of 2,4-D and dimethoate-D_6 (injection standards) and a mix containing carbendazim-D_3, dichlorvos-D_6, and malathion-D_{10} (procedural internal standards) prepared in methanol (MeOH) were used as internal standards in order to ensure quality measurements.

LC-MS grade Water was obtained from Fisher Scientific (Fair Lawn, New Jersey, USA) and LC-MS grade methanol from Fluka Analytical (Steinheim, Germany). Ammonium formate was purchased from Sigma-Aldrich (Steinheim, Germany). Formic acid (99% purity) was acquired from Fluka (Buchs, Switzerland).

2.2. Experimental setup and sample preparation

To ascertain the unique natural food components within mānuka honey as tentative markers against fraud, an initial experiment was planned: 11 monofloral mānuka honey samples of different UMF values (UMF5+, UMF10+, and UMF15+) were extracted alongside four monofloral avocado honey samples, three monofloral chestnut honey samples, and four monofloral eucalyptus honey samples. Mānuka honey sample brands were New Zealand Honey Co. (UMF5+), Egmont Honey, Happy Belly, Happy Valley, Steens Honey, Swisse, WildCape (UMF10+), New Zealand Honey Co., Kiva raw mānuka Honey, mānuka honey (off-brand), and Steens Honey (UMF15+) –i.e., 1 UMF5+ sample, 6 UMF10+ samples, and 4 UMF15+ samples–. The non-mānuka monofloral honey samples were obtained from local honey producer beekeepers in the Andalusia region (Málaga, Spain; and Córdoba, Spain). At this point, the main objective was to find exclusive mānuka markers, hence the need for well-characterised honey from beekeepers instead of commercial samples. One of these monofloral types was eucalyptus honey due to its antibacterial activity similar to that of mānuka honey reported in the literature [28]. It was hypothesised that the chemical profile may be similar as well, thus aiding in the identification of exclusive mānuka honey markers. Sample extraction was performed as follows: 2 g of honey were weighed in 50 mL PTFE tubes, then, 20 mL of a MeOH:H_2O (2:3, V/V) mixture were added alongside 20 μL of a 10 mg/L mixture of the procedural internal standards. Then, the samples were automatically shaken at 40 °C for 5 min, centrifuged at 4000 rpm for another 5 min to precipitate solid matter, and the solution was transferred to 7 mL amber glass vials. Before injection, the extracts were diluted 2-fold with LC-MS grade water containing the injection standards at 0.1 mg/L.

Monofloral mānuka honey must contain the following natural products: 3-phenyllactic acid (≥400 mg/kg), 2′-methoxyacetophenone (≥5 mg/kg), 2-methoxybenzoic acid (≥1 mg/kg), and 4-hydroxyphenyllactic acid (≥1 mg/kg) [8]. To test the viability of fraud via spiking, the four natural products were spiked to various honey samples: almond, avocado, chestnut, eucalyptus, mixed-flower, mānuka, orange blossom, and rosemary honey. For this second round of experiments, honey samples were purchased from a local supermarket (Andalusia, Spain), i.e., none of the honey samples from the first experiment were used, except for mānuka honey. Four 2 g aliquots of each sample were extracted with a methanol:water mixture as aforementioned. One of the aliquots was extracted as is, and for the remaining three, one was spiked at 50% of the legally required concentrations of each natural product, another one at 100% of the required concentration, and the last one at 200% of the required concentration. In summary, 8 single-origin samples and 24 adulterated samples were analysed. In the PCA model each sample (authentic or not) was included in the class corresponding to the honey type, e.g., all authentic and spiked chestnut samples were classified as “chestnut”.

To further test the robustness of the identified natural mānuka products as markers against fraud over the legally required analytes, two additional tests were performed: in one case, the supermarket-acquired eucalyptus sample and a UMF10+ mānuka honey sample were mixed in a 1:1 (mānuka:eucalyptus, m/m) ratio, and in the second case, in a 3:1 (mānuka:eucalyptus, m/m) ratio. Eucalyptus honey was selected for this adulteration study because of its similar metabolomic profile with mānuka honey, as previously mentioned, which makes it the most challenging matrix to distinguish from pure mānuka variety. In both cases, the resulting mixed samples were also spiked with the four legally required natural products as previously described. This resulted in 8 additional adulterated samples, which were classified as the corresponding mānuka:eucalyptus mixtures in the PCA model.

In each batch, during extraction, a reagent blank sample was included in which the 2 g of honey were substituted by 2 g of LC-MS grade water. Furthermore, as a quality control (QC) measure, identical aliquots of all sample extracts were mixed and analysed together with the real samples. QC samples were prepared for each experiment (marker identification and fraud evaluation) in the following way: three QC samples were prepared by pooling identical aliquots of all sample extracts. Then, the three pooled QC samples were subsequently mixed and analysed at the beginning, in the middle, and at the end of the sequence. Both in the marker identification experiment and fraud evaluation experiment, samples were injected in triplicate.

2.3. UHPLC-HRMS analyses

An ExionLC™ (AB SCIEX™ Framingham, Massachusetts, USA) UHPLC instrument was coupled with an X500R tandem quadrupole time-of-flight (QToF) high-resolution mass spectrometry (HRMS) instrument equipped with a Turbo V™ Source with a TwinSprayer probe (AB SCIEX™) and using an electrospray ionisation source (ESI).

Liquid chromatography separation was performed using an Agilent Zorbax® Eclipse Plus C8 column (100 × 2.1 mm, 1.8 μm particle size) by Agilent Technologies (Santa Clara, California, USA). The flow rate was set at 0.35 mL/min, the column temperature at 35 °C, and the injection volume was 5 μL. The mobile phase composition was H_2O:MeOH (98:2, V/V) for solvent A and MeOH:H_2O (98:2, V/V) for solvent B. In the case of ESI+ analyses, both solvents contained 0.1% (V/V) of formic acid and 5 mM of ammonium formate, whereas in the case of ESI– analyses, the ammonium formate modifier was omitted. The gradient was identical for both polarity mode analyses: UHPLC separation began at 0% (V/V) solvent A, from 0 to 1 min, 100% A; 70% A at 3 min, 50% A at 6 min, 0% A at 16 min, which was maintained for 4 min. The initial conditions were run for 4 min with 100% A before the following injection.

HRMS instrument configuration was as follows: all analyses were performed in full scan (FS) mode combined with IDA scans, with data acquisition first in the positive mode and, in a second analysis, in the negative mode (ESI+ and ESI–, respectively). The total method duration was 23 min with a scan time of 1.311 s for both polarities. The instrument’s resolving power (FWHM) was 32,000 for a mass-to-charge ratio (m/z) of 200. For the ESI+ and ESI– analyses, acquisition parameters were: ion source gas 1, 40 psi; ion source gas 2, 50 psi; curtain gas, 25 (arbitrary units); CAD gas, 7 (arbitrary units); temperature, 450 °C; polarity for ESI+, positive; polarity for ESI–, negative; spray voltage for ESI+, 5500 V; spray voltage for ESI–, −4500. The FS mode parameters were: TOF start mass, 80 Da and TOF stop mass, 950 Da (scan range m/z 80–950); accumulation time, 0.25 s; declustering potential for ESI+, 80 V; declustering potential for ESI–, −80 V; DP spread, 0 V; collision energy in ESI+, 10 V; collision energy in ESI–, −10 V; CE spread, 0 V. The MS/MS parameters were: TOF start mass, 50 Da and TOF stop mass, 950

Da (scan range *m/z* 50–950); declustering potential for ESI+, 80 V; declustering potential for ESI–, −80 V; DP spread, 0 V; accumulation time, 0.1 s; collision energy for ESI+, 35 V; collision energy for ESI–, −35 V; CE spread, 15 V. The IDA criteria (configured for small molecule) were: maximum candidate ions, 10; intensity threshold exceeds, 200 cps; dynamic background subtraction, checked; exclude former candidate ions for 6 s after 1 occurrences, checked.

2.4. Data processing

Potential markers exclusive to mānuka honey were identified using MarkerView™ Software version 1.3.1 (AB SCIEX™). First, a peak list was created from the acquired files. The minimum retention time (t_R) was set at 1 min, and the maximum at 21 min. The 'Enhance' peak finding option was used: subtraction offset 10 scans, checked; minimum spectral peak width, 5 parts-per-million (ppm); subtraction mult. factor, 1.2; minimum t_R peak width, 2 scans; noise threshold, 80 cps; and assign charge states was checked. Then, the peak list was processed from the newly created peak list file. The samples were classified into groups according to the honey variety. Peak alignment employed a retention time tolerance of 0.1 min and |5| ppm mass accuracy. A maximum number of 5000 peaks was evaluated, with an intensity threshold of 80 cps, and peaks not present in at least 5 samples were filtered out. Retention times were corrected based on those of the injection standards (6.28 min and 9.43 min for dimethoate-D_6 in ESI+ and 2,4-D in ESI–, respectively).

Additionally, qualitative analyses were performed using SCIEX OS version 3.0.0.3339 (AB SCIEX™). Mass Frontier 8.0 (Thermo Scientific) software was employed to produce *in silico* fragmentation patterns and structures against which to compare the experimental IDA MS spectra. Venn diagrams were created using the free tool provided by the University of Gent (Belgium) [29]. A PCA was performed using MarkerView™ Software for the non-supervised evaluations, whereas R software stable version 4.3.1 was used in the supervised PCA of the tentatively identified mānuka honey markers. The data were pre-processed with a square root function before creating the PCA models. The PCA model used to differentiate authentic mānuka honey from adulterated honey was validated via *k*-fold cross-validation (*k*, 10). The relative root mean squared error (RRMSE), the relative root mean square error of prediction (RRMSEP), the fit of the model (R^2) and the predictive ability of the model (Q^2) were evaluated. To select which ions to evaluate in detail within the MarkerView™ Software, a *t*-test was performed to compare all honey varieties except mānuka honey with mānuka honey itself, and only those ions with *p*-values $\leq$0.05 were considered.

3. Results and discussion

To authenticate mānuka honey and ensure its genuineness, a metabolomic workflow based on HRMS has been successfully employed to analyse the honey and compare it to other monofloral kinds of honey. Instrumental development with new and interesting features, that allows HRMS instruments to perform simultaneously mass spectrometric experiments such as the MS and MS^2 level, provided enormous possibilities to detect and elucidate new marker compounds in natural matrices such as mānuka honey.

3.1. Identification of mānuka markers

To find specific and unique mānuka honey markers, the UHPLC-ESI-HRMS secondary metabolite profiles of monofloral honey from mānuka (UMF5+, UMF10+, and UMF15+ grade), avocado, chestnut, and eucalyptus were analysed through ESI+ and ESI–, using an IDA acquisition mode in combination with FS data. This data-dependent acquisition (DDA) approach permits the obtention of HRMS-MS^2 spectra in which the quadrupole isolates a mass of interest after automatic evaluation of the most intense ions within the full scan data.

Once sample profiles were filtered and aligned as discussed in subsection 2.4, peaks appearing only in all the mānuka honey samples were selected and further studied to assess their usefulness as chemical markers. A Venn diagram was created using retention times and monoisotopic masses from the MarkerView™ Software peak list to visualise how many ions appeared in the different mānuka samples, and which of them were potentially exclusive to mānuka honey. In the case of ESI+ data, for instance, up to 2131 ions were found to be potentially exclusive to mānuka honey (Supplementary Fig. S1). This is a stark difference compared to avocado, chestnut, and eucalyptus honey varieties, for which only 189, 236, and 159 ions were potentially unique –respectively–. No attempt was made to identify the latter ions, as the focus of the present work was on mānuka honey. Differences between samples can also be observed in the non-supervised PCAs obtained with the MarkerView™ Software (Supplementary Figs. S2–S5). The structures of some of these ions exclusively detected in mānuka honey were then elucidated, using a combination of isotopic abundance and the rationalisation of their fragmentation using the MS^2 data, applying the concepts from the work by Demarque et al. and the help of Mass Frontier software [30].

Out of the aforestated thousands of potentially exclusive mānuka natural products, 19 could be tentatively identified at level 2b [26]. Although HRMS fragmentation data coupled with chromatographic t_R determinations is a powerful tool for the identification of new compounds, it nevertheless lacks either further spectral characterisation –e.g., NMR or X-ray crystallography data– or the comparison against a known analytical standard –which, for analytes identified through non-target analysis, may not be available–. These mānuka honey markers, summarised in Fig. 1 and Table 1, are methyl syringate (**1**), leptosperin (**2**), lepteridine (**3**), leptosperin free acid –whose suggested name is *leptosperinic acid*– (**4**), leptosperin triglycoside (**5**), methyl syringate dimer (**6**), methyl syringate trimer (**7**), acetosyringone (**8**), 3,4,5-trimethoxybenzoic acid (**9**), lumichrome (**10**), 3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione (**11**), α-hydroxy-2-methoxy-γ-oxobenzene butanoic acid (**12**), 2′-hydroxyacetophenone (**13**), paeonol (**14**), kojic acid (**15**), unedone (**16**), 3,4-dihydroxyphenylacetic acid (DOPAC) (**17**), 3-(3,4-dihydroxyphenyl)-4-phenyldihydrofuran-2(3*H*)-one (**18**), and *p*-hydroxyphenylacetic acid (**19**). Out of these 19 compounds, nine of them have been identified in mānuka honey for the first time –to the best of our knowledge– in this work: **compounds 4**–**8**, **12**, **14**, **17** and **18**, with no previous records existing for **compounds 4**–**7** and **18** at all before this work. An identification process example is depicted in Fig. 2 for **compound 12** showing the total ion chromatogram (TIC), the extracted ion chromatogram (XIC), the FS spectrum, and the MS^2 spectrum. The four legally regulated natural mānuka products –i.e., 3-phenyllactic acid, 2′-methoxyacetophenone, 2-methoxybenzoic acid and 4-hydroxyphenyllactic acid– were also characterised using analytical standards: three of them were analysed in the ESI– mode, while one was analysed in the ESI+ mode. The three compounds analysed in the ESI– mode were 3-phenyllactic acid (t_R, 6.2 min, $[M-H]^-$ at *m/z* 165.0557), 2-methoxybenzoic acid (t_R, 5.0 min, $[M-H]^-$ at *m/z* 151.0400), and 4-hydroxyphenyllactic acid (t_R, 4.4 min, $[M-H]^-$ at *m/z* 181.0506). The compound analysed in the ESI+ mode was 2′-methoxyacetophenone (t_R, 8.1 min, $[M+H]^+$ at *m/z* 151.0754). The complete identification of all fragment ions within the experimental MS^2 spectra, the mass accuracy in ppm, fragment ions' chemical formulae, and proposed structures for each fragment are provided in **Supplementary File S1** for the 19 tentatively identified compounds and the four legally controlled natural markers.

Except for methyl syringate (**1**), leptosperin (**2**), and lepteridine (**3**), which are amongst the most frequently reported mānuka honey natural products, all 19 potential markers were identified *ex novo* and, then, reports of their occurrence in mānuka honey were searched for using the CAS SciFinder^n database. The elucidation of some of these structures was hence based on previous reports on different natural products in mānuka honey, such as leptosperin (**2**), for which several glycosylated

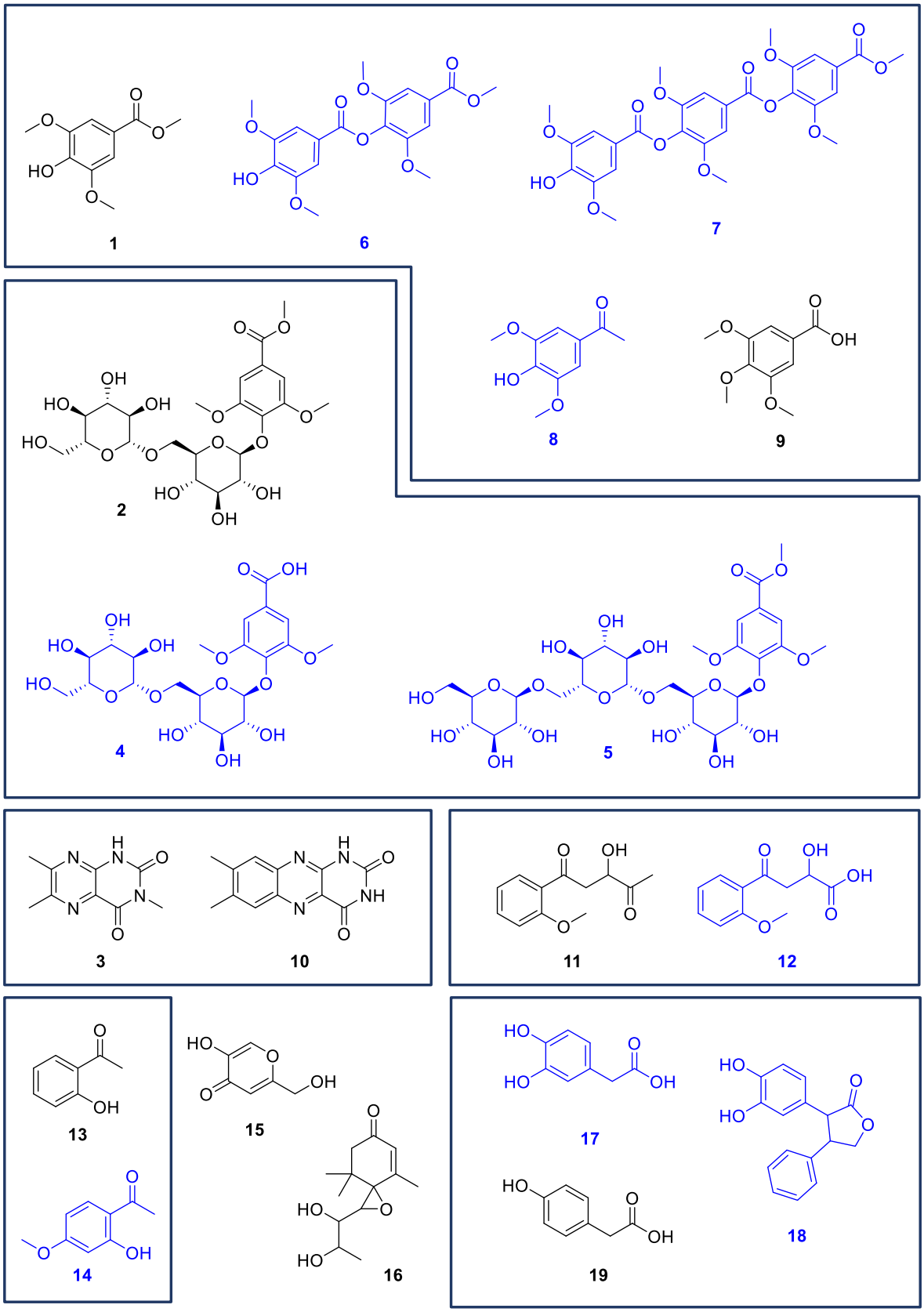

Fig. 1. Chemical structures for the 19 known and potential mānuka markers evaluated in this work (in blue, the structures of those markers tentatively identified for the first time in mānuka).

Table 1
Compounds unique to mānuka honey or significantly more abundant compared to other honey varieties. Only the most significant fragment ions are given here, for all MS and MS^2 data, see **Supplementary File S1**.

Assigned compound (No.)	Chemical class	CAS No.	Chemical formula	Adduct	Precursor ion adduct exact mass[a] (*m/z*)	MS^2 fragment ions[a] (*m/z*)	No. of MS/MS fragment ions identified ≤ | 5| ppm	t_R (min)	First report in mānuka honey	References
Methyl syringate[b] (**1**)	Phenol	884-35-5	$C_{10}H_{12}O_5$	$[M+H]^+$	213.0758	139.0392 154.0629 181.0503[e]	66	7.17	1990	Russell et al., 1990
Leptosperin[b] (**2**)	Glycosilated phenol	1431845-42-9	$C_{22}H_{32}O_{15}$	$[M+NH_4]^+$	554.2079	154.0625 181.0495 213.0758[e]	14	5.22	2012	Kato et al., 2012
Lepteridine[b] (**3**)	Pteridine	50,256-22-9	$C_9H_{10}N_4O_2$	$[M+H]^+$	207.0867[e]	148.0505 176.0455 189.0771	21	4.93	2016	Daniels et al., 2016
Leptosperinic acid[b] (**4**)	Glycosilated phenolic acid	–	$C_{21}H_{30}O_{15}$	$[M+NH_4]^+$	540.1923	155.0703 199.0601[e] 343.1024	10	4.02	–	–
Leptosperin triglycoside[b] (**5**)	Glycosilated phenol	–	$C_{28}H_{42}O_{20}$	$[M+NH_4]^+$	716.2608	163.0601 213.0758[e] 325.1129	21	4.98	–	-
Methyl syringate dimer[b] (**6**)	Methoxylated phenol	–	$C_{19}H_{20}O_9$	$[M+NH_4]^+$	410.1451	197.0808 315.0863 361.0918[e]	14	7.50	–	–
Methyl syringate trimer (**7**)	Methoxylated phenol	–	$C_{28}H_{28}O_{13}$	$[M+H]^+$	573.1603	197.0808 211.0601 287.0550[e]	19	9.42	–	–
Acetosyringone[b] (**8**)	Acetophenone	2478-38-8	$C_{10}H_{12}O_4$	$[M+H]^+$	197.0808	77.0386 91.0542[e] 137.0597	17	6.36	–	–
3,4,5-Trimethoxybenzoic acid[c] (**9**)	Phenolic acid	118-41-2	$C_{10}H_{12}O_5$	$[M-H]^-$	211.0612	153.0193 181.0142[e] 196.0377	4	5.21	1990	Russell et al., 1990
Lumichrome (**10**)	Pteridine	1086-80-2	$C_{12}H_{10}N_4O_2$	$[M+H]^+$	243.0877[e]	145.0760 172.0869 198.0662	11	7.28	2014	Beitlich et al., 2014
3-Hydroxy-1-(2-methoxyphenyl)penta-1,4-dione[b] (**11**)	Aromatic ketone	1388209-20-8	$C_{12}H_{14}O_4$	$[M+H]^+$	223.0965	97.0284 135.0441[e] 205.0859	36	6.74	2012	Oelschlaegel et al., 2012
α-Hydroxy-2-methoxy-γ-oxobenzenebutanoic acid[b] (**12**)	Acetophenone	1464879-24-0	$C_{11}H_{12}O_5$	$[M+H]^+$	225.0758	135.0441[e] 161.0597 189.0546	23	6.18	–	–
2′-Hydroxyacetophenone (**13**)	Phenol	118-93-4	$C_8H_8O_2$	$[M+H]^+$	137.0597	77.0386[e] 107.0491 121.0284	5	6.67	2014	Beitlich et al., 2014
Paeonol[b] (**14**)	Acetophenone	552-41-0	$C_9H_{10}O_3$	$[M+H]^+$	167.0703	77.0386[e] 121.0649 149.0596	13	6.71	–	–
Kojic acid (**15**)	Pyranone	501-30-4	$C_6H_6O_4$	$[M+H]^+$	143.0339[e]	69.0335 97.0284 125.0233	10	2.32	2012	Oelschlaegel et al., 2012
Unedone (**16**)	Norisoprenoid	1199815-09-2	$C_{13}H_{20}O_4$	$[M+H]^+$	241.1434	67.0542 109.1012[e] 137.0961	43	5.92	2012	Oelschlaegel et al., 2012
DOPAC[c] (**17**)	Phenolic acid	102-32-9	$C_8H_8O_4$	$[M-H]^-$	167.0350	77.0397 93.0346[e] 121.0295	7	3.82	–	–
3-(3,4-Dihydroxyphenyl)-4-phenyldihydrofuran-2(3*H*)-one (**18**)	Phenol	–	$C_{16}H_{14}O_4$	$[M-H]^-$	269.0818[e]	79.0187 165.0192 227.0713	26	11.01	–	–
p-Hydroxyphenylacetic acid[b] (**19**)	Phenolic acid	156-38-7	$C_8H_8O_3$	$[M]^+$	135.0441[d]	51.0229 77.0386[e] 92.0257	9	6.38	2021	McLoone et al., 2021

[a] *Theoretical* m/z *values*.
[b] Supplementary File S1.
[c] *Also amenable by ESI+ ionisation, see* ***Supplementary File S1***.
[d] *Actual precursor ion:* m/z, *153.0546, but in-source fragmentation results in* H_2O *loss into a more abundant precursor,* m/z *135.0441*.
[e] *Most abundant ion in the* MS^2 *experiment.*

and demethylated analogues were sought, resulting in the identification of **compound 4** and **compound 5**; or methyl syringate (**1**), condensation products of which were searched for, from which the dimer (**6**) and the trimer (**7**) emerged, as well as other analogues such as **compound 8** and **compound 9**. The newly tentatively identified markers **compounds 4**, **5**, **6**, and **7** had been previously reported neither in food products nor in any other application and are lacking a CAS number at the time of writing this work. In the case of the methyl syringate dimer (**6**), the coelution of another analyte ($C_{21}H_{29}O_7^+$) resulted in a mixed IDA spectrum for the $[M+H]^+$ spectrum that could not be successfully employed

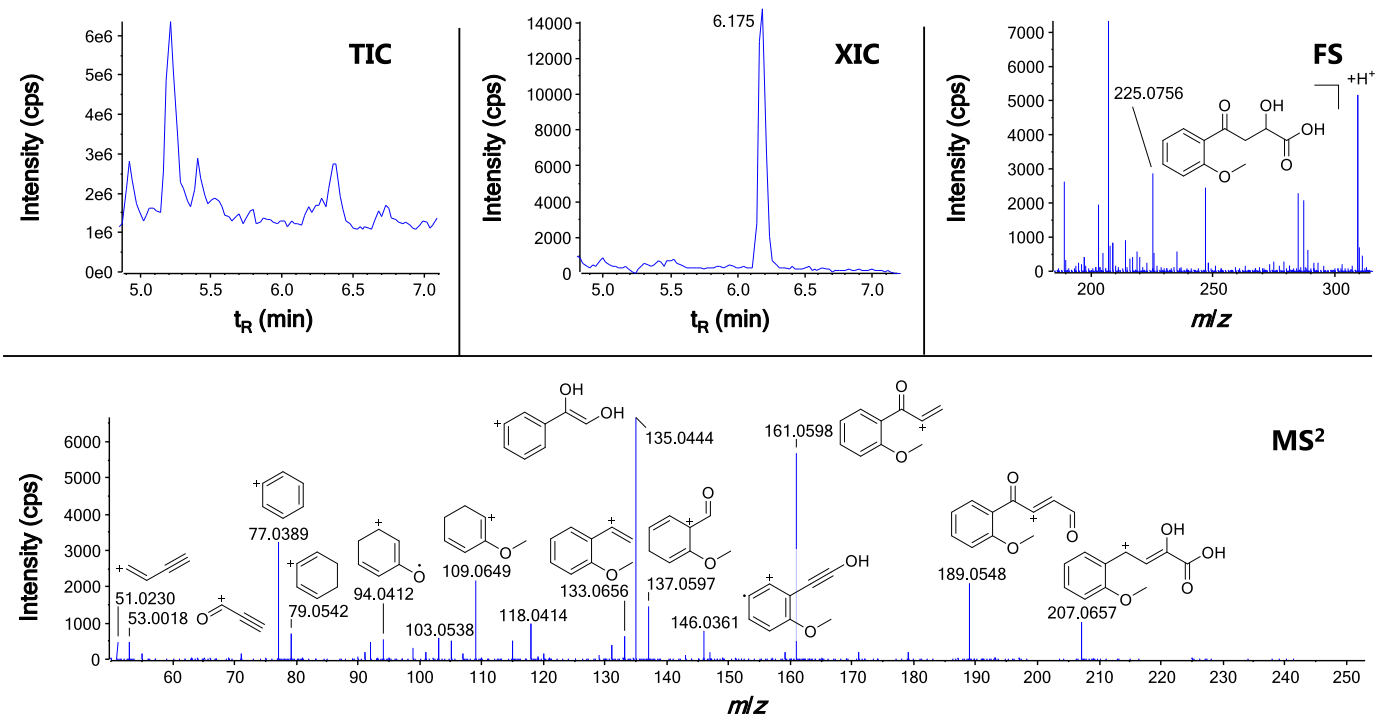

Fig. 2. Mānuka honey TIC (*top left*), XIC for *m/z* 225.0758 ± 0.01 (*top centre*), FS spectrum in the *m/z* 200–300 range (*top right*), and IDA MS^2 spectrum of precursor *m/z* 225.1 at 6.2 min with proposed structures for the observed fragment ions (*bottom*) depicting the tentative identification of **compound 12**.

to identify **compound 6** univocally. The issue was solved by evaluating its ammonium adduct instead, which was not formed for the interfering analyte. Conversely, both acetosyringone (**8**) and the gallic acid derivative (**9**) possess a CAS number. The gallic acid derivative (**9**) was identified for the first time in mānuka honey alongside one of its signature markers, methyl syringate (**1**), in 1990. These two compounds were identified as providing mānuka honey with part of its antibacterial properties [31].

As for lepteridine (**3**) and lumichrome (**10**), these two pteridines are involved in vitamin B_2 biosynthesis [32,33]. Lepteridine (**3**) is the deribitylated analogue of a riboflavin biosynthesis intermediate product, while lumichrome (**10**) is a deribitylated riboflavin analogue. Riboflavin itself has been recently described in other honey varieties, alongside lumichrome (**10**) [21]. Regarding their presence in mānuka honey, lepteridine has been known since 2016, in a work in which the authors also provided a total synthesis procedure for this natural compound [34]. Lumichrome (**10**), for its part, was first reported in mānuka honey in 2014 and has been employed in novel mānuka authentication strategies, such as fluorescence-based characterisation [10,19].

In the case of **compounds 11** and **12**, their identification was based on the hypothesis that well-known natural products occurring in mānuka honey, such as 3-phenyllactic acid, 2′-methoxyacetophenone, 2-methoxybenzoic acid, 4-hydroxyphenyllactic acid, and methylglyoxal, may react together to give rise to new mānuka honey markers. **Compound 11** can be viewed as a reaction product between 2′-methoxyacetophenone and methylglyoxal, whereas **compound 12** can be similarly attributed to a 2′-methoxyacetophenone derivative with glyoxylic acid, or as an oxidation product of **compound 11**. Oelschlaegel et al. reported in 2012 the presence of **compound 11** in mānuka honey –but not its carboxylic acid analogue, **compound 12**, and without a discussion on its possible biosynthetic [11]. The proposed synthetic mechanisms for these two compounds are shown in Supplementary Scheme S1.

Structure proposals for 2′-hydroxyacetophenone (**13**) and paenolol (**14**) were similarly based on the evaluation of *in silico* spectra for the well-known mānuka markers. These two substances can be regarded as demethylated 2′-methoxyacetophenone analogues. While the proposed structures with the given substitution patterns were those whose *in silico* fragmentation patterns better matched the experimental MS^2 spectra, final confirmation of the benzene ring substitution positions can only be achieved via the analysis of analytical standards. There were, nevertheless, diagnostic ions based on the *in silico* experiments that pointed towards a given substitution pattern. In the case of paenolol (**14**), the ESI– MS^2 spectrum contains an *m/z* 135.0088 for which the *in silico* fragmentation software was only able to provide a fragmentation path for the 2′-hydroxy-4′-methoxyacetophenone substitution pattern. One of these two substances, paenolol (**14**), is reported for the first time in mānuka honey in this work, while the presence of **compound 13** had already been described in the literature [19].

Kojic acid (**15**) was found as a possible structure for the very early eluting *m/z* 143.0339, whose theoretical chemical formula indicated a high number of oxygen atoms. After an expeditious literature search, reports of kojic acid (**15**) in mānuka honey dating back to 2012 were found [11], and the *in silico* fragmentation of its structure matched the observed fragment ions. Similarly, a literature search of the precursor *m/z* 241.1434 quickly offered unedone (**16**) as a possible natural product. The *in silico* evaluation of unedone (**16**) allowed the matching of several dozen fragment ions. Oelschlaegel et al. were also the first to report unedone (**16**) in mānuka honey alongside kojic acid (**15**). Unedone (**16**), a putative abscisic acid derivative, had been previously described as a strawberry tree honey marker [35], and has been proposed as a mānuka honey marker [11]; however, as will be discussed in later sections, neither kojic acid (**15**) nor unedone (**16**) are exclusive mānuka honey natural products as per the findings herein described.

The two remaining analytes, **compound 17** and **compound 18**, were elucidated based on the thorough study of the experimental MS^2 spectra and the *in silico* evaluation of possible chemical structures for the observed ions. First, an MS^2 spectrum was observed to match that of a γ-lactone, with the well-known losses of CO and CO_2 groups assisted by neighbouring aromatic moieties. The proposed structure, 3-(3,4-dihydroxyphenyl)-4-phenyldihydrofuran-2(3*H*)-one (**18**), can be viewed as the condensation of two shikimate pathway intermediates: 2-

phenylethan-1-ol and DOPAC (**17**). The proposed synthetic mechanism is shown in Supplementary Scheme S2. This chemical structure has been described, to the best of our knowledge, for the first time in this work, as no previous reports could be found in the CAS SciFinder^n database and no CAS number exists for it. Once **compound 18** was identified, with 26 fragment ions matching the *in silico* fragmentation pattern with < |5| ppm, the two reacting molecules were also searched for in the ESI+ and ESI– mānuka honey profiles. While 2-phenylethanol could not be identified, DOPAC (**17**) was found eluting at t_R, 3.8 min. As previously discussed, the chemical structure for DOPAC (**17**) was the one better matching the experimental MS^2 spectrum; however, technical limitations of the *in silico* fragmentation tools can mean that a different substitution pattern may be found if comparing the spectra against analytical standards. This work constitutes the first report of DOPAC (**17**) and **compound 18** in mānuka honey. While no reports of DOPAC (**17**) presence in honey were found, its presence in eucalyptus bark has been previously reported [20].

Finally, after the tentative identification of DOPAC (**17**), the rest of its phenolic analogues –i.e., mono-, di-, tri-, and tetrahydroxylated phenylacetic acids– were searched for in the UHPLC-QToF-HRMS mānuka honey metabolic profile. Only *p*-hydroxyphenylacetic acid (19) was found, a compound which has been very recently reported for the first time in mānuka honey [36]. Interestingly, the search for DOPAC (**17**) analogues prevented the misidentification of *m/z* 135.0441 as 3-coumaranone: **compound 19** and the preliminarily identified 3-coumaranone presented identical coelution, and after the evaluation of the MS^2 spectra and XICs, the conclusion was that *m/z* 135.0441 was the result of the in-source fragmentation of *p*-hydroxyphenylacetic acid (**19**). Similarly, an *m/z* 181.0495, whose experimental MS^2 spectrum partially matched the *in silico* fragmentation pattern of caffeic acid, was found to be the result of methyl syringate (**1**) in-source fragmentation instead.

As aforementioned, amongst the previously reported natural products, methyl syringate (**1**), leptosperin (**2**), lepteridine (**3**), and lumichrome (**10**), are the ones most frequently reported in the literature on mānuka honey, and their roles in the beneficial properties of this commodity have been thoroughly discussed in the past, as recently summarised by other authors [20]. Over half of the herein-identified structures are phenols or phenolic derivatives (Fig. 1 and Table 1). The presence of these compounds has been related to the antibacterial mānuka honey properties, as it has been suggested that free radical production of phenols may exert an inhibitory effect on microbial proteins [2,36].

3.2. Evaluation of the tentatively identified natural products in other honey varieties

The chromatographic areas of the 19 tentatively identified compounds were first obtained using SCIEX OS. The abundance of these natural products in almond, avocado, chestnut, eucalyptus, mixed-flower, orange blossom, and rosemary honey relative to that in mānuka honey is summarised in Fig. 3. In the case of **compounds 2–9** and **11–14** (12 compounds), these were found to be exclusive markers of mānuka honey amongst the evaluated honey varieties. This list includes both previously reported natural products as well as newly elucidated structures, particularly, **compounds 4–8**, **12** and **14** (seven compounds) of the markers exclusive to mānuka honey are being reported for the first time in this work, while **compounds 2**, **3**, **9**, **11** and **13** (five compounds) had already been reported in this commodity. It is worth noting that most of these exclusive mānuka markers are methyl syringate (**1**) and/or leptosperin (**2**) derivatives or analogues, well-known natural mānuka markers. Others, such as **compound 12** (and, in a lesser manner, **compound 4**), may be viewed as reaction products of other well-known mānuka natural products. It is plausible that further research on possible combinations of these secondary metabolites will yield additional mānuka markers of interest in its characterisation and authenticity assessment. Instead of the current four chemical markers evaluated in the authenticity assessment of mānuka honey –i.e., 3-phenyllactic acid, 4-hydroxyphenyllactic acid, 2-methoxybenzoic acid, and 2′-methoxyacetophenone–, some authors support the substitution of 2-methoxybenzoic acid and 2′-methoxyacetophenone for leptosperin (**2**) and lepteridine (**3**), as the former two are not chemically stable over mid and long-term storage, whereas the latter two are stable [10]. The results derived from this work in terms of the uniqueness of leptosperin (**2**) and lepteridine (**3**) in mānuka honey support this stance.

As per the remaining seven tentatively identified natural products, **compounds 1**, **10** and **15–19** were also detected in other honey varieties albeit at far lower abundances, save for kojic acid (**15**) and unedone (**16**). Regarding kojic acid (**15**), it was found at abundances exceeding those of mānuka honey (+236%) in eucalyptus honey (Fig. 3). Other varieties, such as mixed-flower, almond, and orange blossom honey were also found to contain kojic acid (**15**), but at abundances below 40% of that of mānuka honey for mixed-flower honey and below 20% for almond and orange blossom honey. Kojic acid (**15**) is a known carbohydrate degradation product, so its presence in other honey varieties is unsurprising considering the nature of these matrices [11].

As observed in Fig. 3, the remaining natural product whose abundance was greater in honey other than mānuka honey was unedone (**16**). As previously discussed, this analyte had been suggested as a strawberry honey marker [35], and in light of these results and those previously reported in the literature, its presence amongst various honey varieties seems fairly commonplace. Two of its features are worth noting: one, its absence in most of the evaluated honey varieties; two, it may behave as an almond honey natural marker, given its +15,000% abundance over both mānuka and orange blossom honey. Nevertheless, to confirm this hypothesis, further studies are necessary, e.g., to concurrently analyse

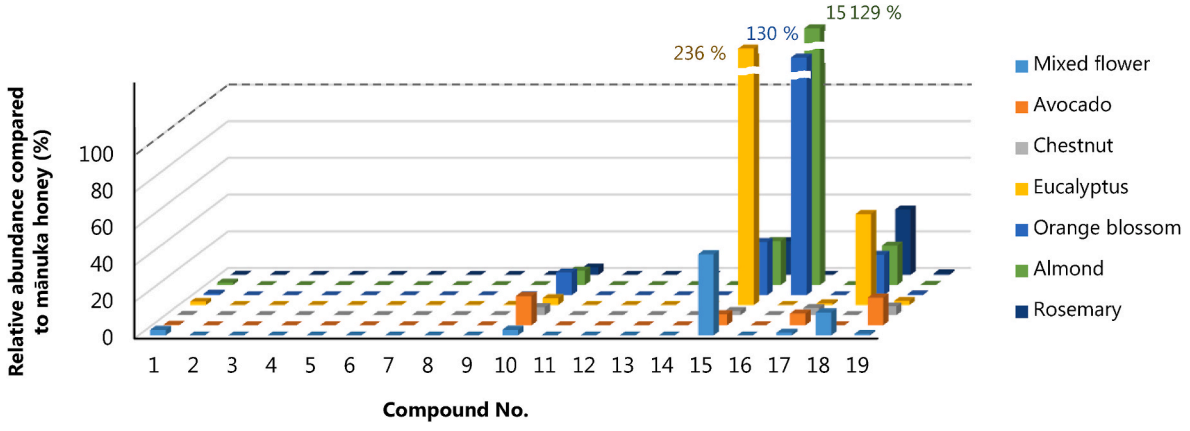

Fig. 3. Average areas for the 19 tentatively identified mānuka markers relative to their respective abundance in mānuka honey (dotted line).

almond and strawberry tree honey and compare the unedone (**16**) levels.

Within those analytes found in other honey varieties, none besides kojic acid (**15**) and unedone (**16**) exceed the abundance levels found in mānuka honey. Lumichrome (**10**), however, is an interesting analyte due to its nature as a riboflavin metabolite. While no honey variety contains lumichrome (**10**) at levels over 15% of those in mānuka honey, it is the only other natural product –besides kojic acid (**15**)– found in every single honey variety evaluated. This is in accordance with previous reports in the literature indicating that riboflavin and its metabolite, lumichrome (**10**), are typical natural products of most honey varieties [37]. Another compound found in all of the evaluated honey –except for avocado and chestnut varieties– was **compound 18**. It was previously hypothesised in this work that this pentacyclic lactone may be the result of the reaction between DOPAC (**17**) (or a differently substituted polyphenol) and 2-phenylethanol, and while the former had never been previously described in honey, it is known to be present in eucalyptus bark and bees [38,39]. For its part, 2-phenylethanol has been extensively reported in most honey varieties both in Europe and in Australia, so the presence of this moiety as part of other natural products is feasible [40,41]. Interestingly, while **compound 18** was not found in avocado nor chestnut honey, DOPAC (**17**) was present in these two varieties; and *vice versa*, DOPAC (**17**) was absent from all honey varieties except for avocado and chestnut honey –and, of course, mānuka honey– (Fig. 3).

3.3. Application of the identified markers in mānuka authenticity assessment

As described in section 2.2, two different approaches to assess fraud in mānuka honey were evaluated: (*i*) the addition of the four legally regulated natural products at various concentrations, i.e., at 50% of the minimum concentration for monofloral mānuka honey, 100%, and 200% of said value; and (*ii*) the dilution or adulteration of monofloral mānuka honey with other honey, in this case, 1:1 and 3:1 (m/m) mānuka:eucalyptus honey mixtures. The spiking described in (*i*) was also applied to the adulterated mānuka honey from (*ii*). Alongside the original avocado, chestnut, and eucalyptus honey, mixed-flower honey and monofloral almond, orange blossom, and rosemary honey were included in the evaluation of the 19 natural mānuka honey products identified in section 3.1.

Once all chromatographic peaks in all samples were manually revised, the robustness of the tentatively identified mānuka markers was evaluated in terms of the authenticity assessment of mānuka honey. First, non-supervised PCAs were created using MarkerView™ Software. These PCAs demonstrated that the amount and levels of natural mānuka honey markers are so significant compared to other honey that neither the fraud attempt at artificially spiking 3-phenyllactic acid, 2′-methoxyacetophenone, 2-methoxybenzoic acid, and 4-hydroxyphenyllactic acid, nor the adulteration of mānuka honey with other honey varieties, nor the combination of these two fraud strategies, resulted in mānuka honey samples grouped with any other honey sample (Supplementary Fig. S6 and Supplementary Fig. S7). The next question to answer was whether the supervised PCA of the 19 tentatively identified markers plus the four legally controlled ones would similarly result in a model in which mānuka honey could be differentiated from other honey samples no matter the fraud strategy attempted.

The results for the supervised PCA of both spiking and adulteration fraud attempts are shown in Fig. 4. The results of the *k*-fold cross-validation yielded an RRMSE of 0.185% and an RRMSEP of 0.227% relative to the average value, and R^2 and Q^2 values of 0.991 and 0.898, respectively. The R code used in the creation of this PCA model is provided in Supplementary File S2. Five distinct sample groups can be observed: from left to right, all honey samples except for eucalyptus honey, the mānuka:eucalyptus 1:1 (m/m) adulterated honey, the mānuka:eucalyptus 3:1 (m/m) adulterated honey, and mānuka honey. The distinct elongated shapes each sample group presents are due to the

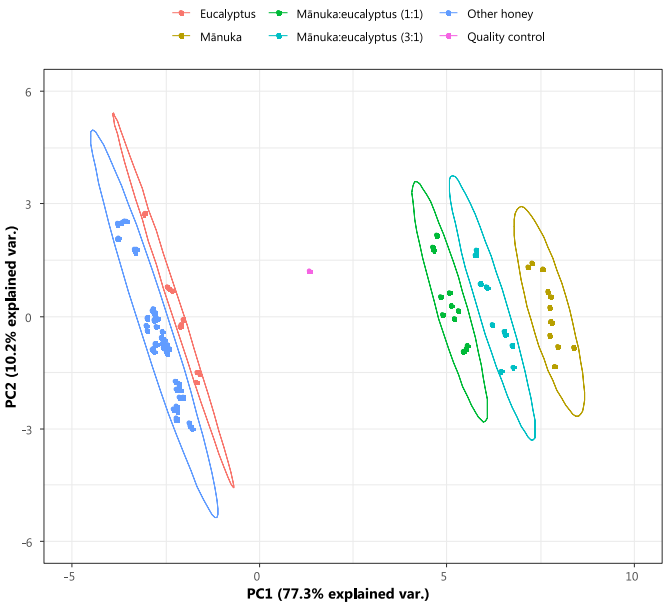

Fig. 4. Targeted PCA based on the 19 evaluated mānuka markers plus the four compulsory analytes. All honey varieties were spiked with 0%, 50%, 100%, and 200% of the minimum target concentration of the four compulsory analytes. Mānuka:eucalyptus mixtures are samples mixed (m/m) prior to extraction (ellipses drawn at 99% significance).

increasing concentration levels of the four compulsory analytes spiked at 0%, 50%, 100%, and 200% concentration levels relative to the minimum legally required amounts. The 77.3% explained variance that the first principal component (PC) provides is evidence of the significance of the tentatively identified mānuka Markers: by itself, it could be employed to distinguish mānuka honey from any other of the evaluated samples, no matter the fraud attempt –albeit in the case of mānuka: eucalyptus 3:1 (m/m) adulterated honey, this sample group falls extremely close to unadulterated mānuka honey–. An evaluation of the PC1 loadings in Supplementary File S2 shows that the model did not employ unedone (**16**) levels in the creation of this parameter, which is largely unsurprising given the observed relative abundances discussed in section 3.2 and shown in Fig. 3. However, even if unedone (**16**) is not a definite mānuka marker, its presence at exceedingly high levels in this commodity could also be indicative of adulteration via mixture with other honey. Together with PC2, the first two principal components explain 87.5% of the observed variance between groups. While authenticity assessment could potentially fail at mānuka honey mixtures above 3:1 (m/m), significantly more profitable adulteration strategies such as 1:1 (m/m) ratios, which the herein developed model can detect, and any lower mānuka ratio can be prevented via the evaluation of these 19 natural mānuka honey markers. Eucalyptus honey, the one employed in mānuka honey adulteration, is the sample group falling closer to mānuka honey than any other honey variety (Fig. 4). Hence, 3:1 (m/m) mānuka adulteration ratios with almond, avocado, chestnut, mixed-flower, orange blossom, nor rosemary honey would not go undetected either by the developed model.

Finally, supervised PCA models were also created for the unadulterated honey samples, i.e., pure almond, avocado, chestnut, eucalyptus, mānuka, orange blossom, and rosemary honey samples (Fig. 5). In these supervised PCA models, including both the 19 tentatively identified mānuka markers plus the four compulsory analytes (Fig. 5a) and the 19 tentatively identified mānuka markers only (Fig. 5b), mānuka honey differentiation from the remaining honey varieties is even more evident than in Fig. 4. The first PC explains 86.7% and 84.8% of the variance between groups in Fig. 5a and b, respectively. Together with the PC2 of each model, the explained variance rises to 92.9% in Fig. 5a and to

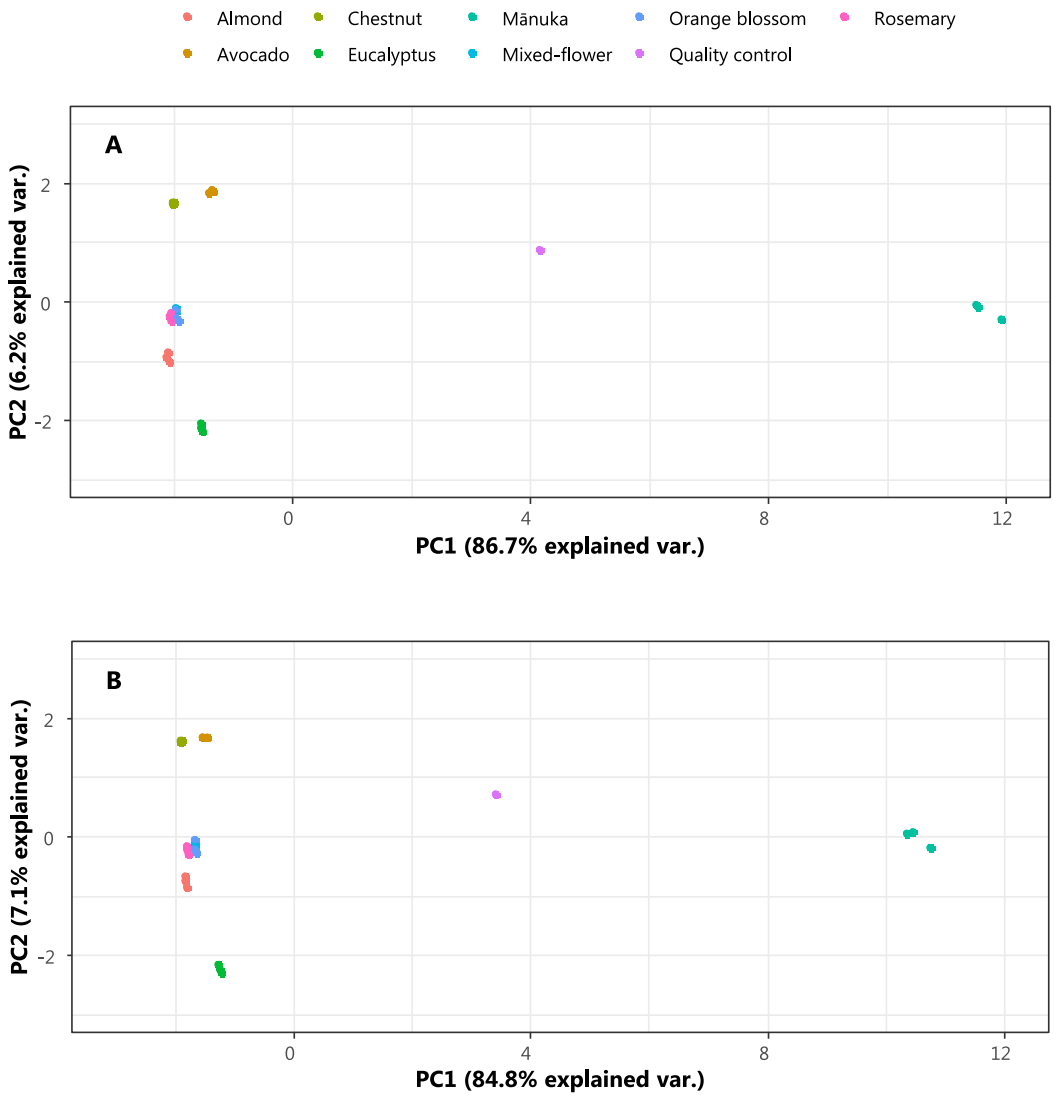

Fig. 5. (a) Supervised PCA based on the 19 evaluated mānuka markers plus the four compulsory analytes, and (b) Supervised PCA based on the 19 evaluated mānuka markers. Only non-spiked data points were employed.

91.9% in Fig. 5b. These values are greater than that of PC1 in Fig. 4 due to the removal of adulterated samples and the uniqueness of the natural products to mānuka honey –except for kojic acid (**15**) and unedone (**16**), which the model does not employ for either PC1 (Supplementary File S2)–. While long-term studies on the stability of these 19 natural mānuka markers and additional analyses of other monofloral honey varieties are needed, these results further support the proposals made in the literature to increase the number of legally required markers for the authentication of mānuka honey [10].

As a final note regarding the adulteration experiments, the levels of the four spiked substances follow a linear trend. An example for 2′-methoxyacetophenone and eucalyptus honey is shown in Fig. 6. Similar behaviour is observed in other honey kinds, as also shown in Supplementary Fig. S8. Additionally, Fig. 6e–g shows how very similar levels of 2′-methoxyacetophenone are achieved in mānuka honey mixed with eucalyptus honey (mānuka:eucalyptus (1:1, m/m) +100% of the required level in Fig. 6f, and mānuka:eucalyptus (3:1, m/m) +50% in Fig. 6g) compared to authentic monofloral mānuka honey (Fig. 6e).

4. Conclusions

The well-known beneficial health effects and antibacterial properties associated with mānuka honey result in fraud galore. Due to the vast secondary metabolic profile of mānuka honey compared to other varieties, high-resolution mass spectrometry tools can provide methods and models to identify new natural products exclusive to this commodity to be employed against fraud attempts.

Four compounds are required to be present at specific concentrations to prove mānuka honey authenticity according to New Zealand legislation, however, not all of them seem to be appropriate in long-term evaluations and may even result in wrongly classifying unadulterated mānuka honey due to their instability. The herein-developed methodology, using metabolomics-based high-resolution mass spectrometry, allowed us to tentatively identify 19 natural products present in mānuka honey exclusively or at far higher abundance compared to monofloral avocado, chestnut, and eucalyptus honey. The method allowed us to identify compounds previously reported in mānuka honey of great importance, such as leptosperin, lepteridine, or methyl syringate. Furthermore, it permitted us to identify nine compounds never before reported in mānuka honey, of which five had never been reported before

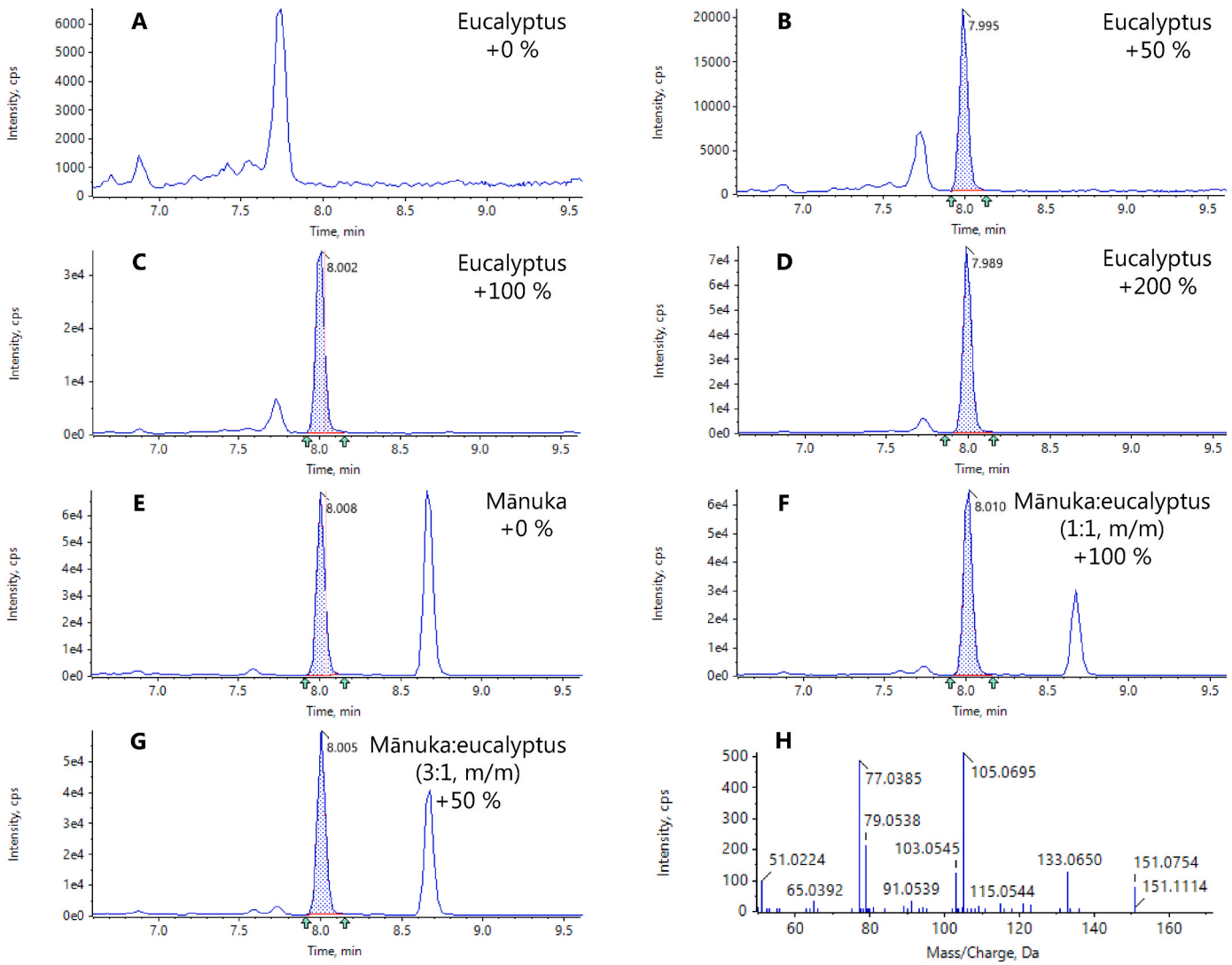

Fig. 6. XIC of 2′-methoxyacetophenone in (**a**) eucalyptus honey, (**b**) eucalyptus honey +50% of the minimum legally required concentration, (**c**) eucalyptus honey +100% of the required concentration, (**d**) eucalyptus honey +200% of the required concentration, (**e**) mānuka honey, (**f**) mānuka:eucalyptus (1:1) +100% of the required concentration, (**g**) mānuka:eucalyptus (3:1) +50% of the required concentration, and (**h**) MS^2 spectrum of 2′-methoxyacetophenone.

in any other honey variety or commodity to the best of our knowledge. Except for kojic acid and unedone, these compounds were still found to be exclusive or extremely more abundant in mānuka honey than in other honey varieties when analysing almond, mixed-flower, orange blossom, and rosemary honey. These constitute potentially new, exclusive mānuka honey markers which can aid in its authenticity assessment and fraud prevention.

The supervised principal component analysis models created with the 19 newly identified mānuka markers plus the four compulsory ones allowed us to differentiate unadulterated mānuka honey from adulteration methods, either by spiking with the compulsory mānuka analytes and/or by dilution of mānuka honey with other varieties. The usefulness of the 19 tentatively identified markers was further proved when supervised principal component analysis models of the unadulterated honey varieties with and without the four compulsory analytes were compared, showcasing minute differences only. That these two models were barely indistinguishable from one another demonstrates that the importance of the four legally controlled analytes is significantly lesser compared to the 19 tentatively identified markers presented in this work, and supports the inclusion of these –or some of these– in future mānuka honey authentication procedures.

The workflow described in the present work is not exclusive to mānuka honey authentication; on the contrary, with the appropriate selection of initial samples and variables, it can be employed to characterise various matrices or commodities. Specialised software also allows the integration of multiple multivariate statistical tests simultaneously which are an open door to face the study of very complex matrices.

Credit author statement

Francisco J. Díaz-Galiano: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization; **Horacio Heinzen**: Validation, Formal analysis, Investigation, Writing – review & editing; **María J. Gómez-Ramos**: Conceptualization, Methodology, Resources, Writing – review & editing, Funding acquisition; **María Murcia-Morales**: Formal analysis, Writing – review & editing, Visualization; **Amadeo R. Fernández-Alba**: Conceptualization, Resources, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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