DISCRIMINATION OF HONEY OF DIFFERENT BOTANICAL ORIGINS USING AN UNTARGETED HIGH-DEFINITION METABOLOMIC WORKFLOW

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INTRODUCTION

Recent scandals have highlighted that food fraud can also result in major food safety issues. Food fraud is a collective term which describes a substitution, addition, alteration or a misrepresentation, deliberate and intentional, of food ingredients or of food packaging, or false or misleading statements formulated concerning a product for economic gain [1].

For example, in Europe foods labelled as beef were found to contain undeclared horse meat whereas in China, milk and infant formula were adulterated with nitrogen rich melamine, added to food products to increase their apparent protein content; both for monetary gain.

Adulteration of honey by sugars, syrups or flavour enhancers can make it cheaper to produce or extend shelf life. The purity of the honey can be deceptive. Isotope ratio mass spectrometry can be used to detect as little as 7% addition of corn syrup and cane sugar [2]. However, fraud due to mislabeling or false declaration of botanical origin can be more difficult to detect.

In a high profile case in 2008 [3], the German food ingredients conglomerate ALW took Chinese-origin honey, repackaged and mislabelled it and shipped it to the U.S. via intermediaries (Figure 1). Country of origin certificates were falsified. The honey was stripped of indicators which could be used to trace shipments back to their true origin and adulterated with foreign sugars. In some cases, the honey was found to be contaminated with the residue of antibiotics banned in the U.S.

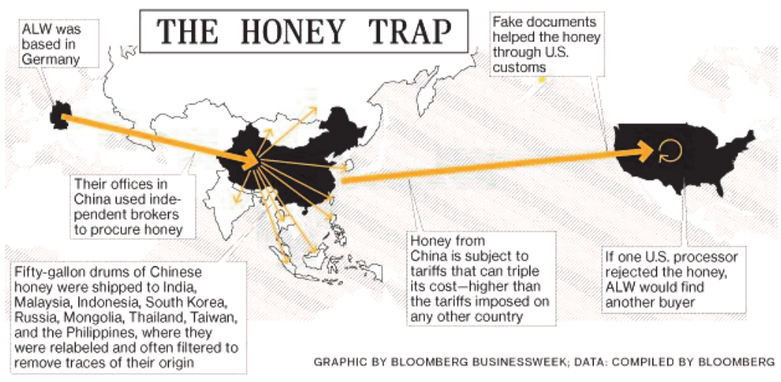

Figure 1. Map showing illicit honey laundering scheme (3)

Pollen analysis is used for determination of botanical origin of honey but this can fail to detect closely related species (*e.g.* Manuka and Kanuka). NMR has also been used to distinguish between different honey types [4]. In this study we aimed to investigate whether untargeted metabolomics, using UPLC-HDMS and multivariate statistics, could differentiate honeys of different floral origin.

METHODS

Authentic samples of rape (3), heather (9), buckwheat (5) and Manuka (8) honeys were obtained from indisputable sources. Each floral class contained separate samples from different countries (Norways, Denmark, Lithuania, Poland and New Zealand) and years of collection giving multiple biological replicates per floral class. Honey samples were subjected to minimal sample preparation: honey (0.5g) was diluted (10mL methanol/1% formic acid in water, 50:50), shaken, sonicated (20 min) and centrifuged.

Samples were analyzed in triplicate using a generic UPLC-HDMS^E acquisition technique. The complex mixtures of metabolites were separated using one dimensional reversed phase chromatography on an ACQUITY I-Class, using a gradient (5 to 90% acetonitrile over 90 minutes) and ACQUITY BEH C18 column (2.1 x 100 mm, 1.7µm), operated at 0.5 mL/min. Metabolites were analyzed by HDMS^E acquisition on a SYNAPT G2-Si mass spectrometer in electrospray positive and negative ion modes (Figure 2). The use of ion mobility was employed in order to maximize peak capacity when analyzing highly complex mixtures. Samples were analyzed in a randomized order including interspersed pooled honey samples for QC purposes. HDMS^E data-independent analysis provides accurate mass measurements of all detectable fragment ions from every detectable precursor ion. Chromatographic and drift time alignment of precursor and fragment ion data reduces miss-assignment of fragment ions to parent ions of similar mass or retention time

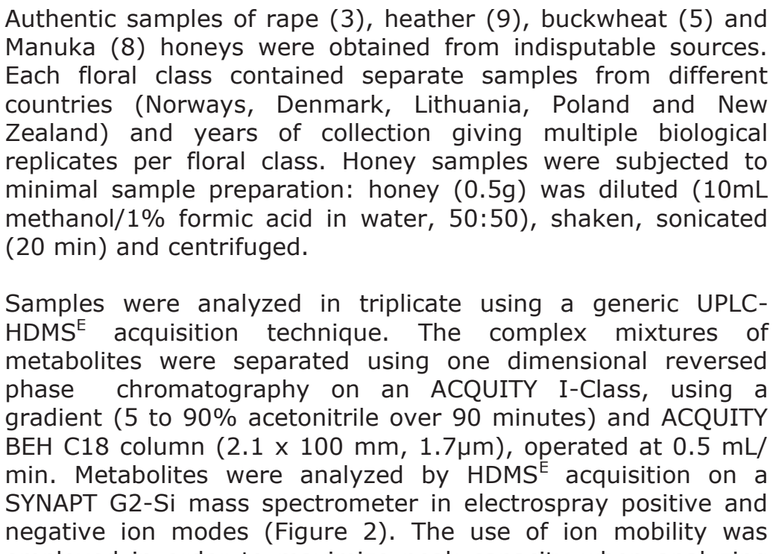

Figure 2. Schematic of the SYNAPT G2-Si HDMS with inset showing separation of isobaric ions in the Triwave mobility device in HDMS^E acquisition mode

The data produced were examined using Progenesis QI and EZinfo (MKS Data Analytics Solutions) software including evaluation using both principal component analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA). Feature annotation or identification, quantification and comparative analyses were extracted using Progenesis QI. Prior to annotation features were subject to filtering based on a P value <0.0001 and a mean abundance change of >5 fold. Annotation and identification was achieved by searching multiple contained databases (HMDB, Metlin and MassBank).

RESULTS AND DISCUSSION

Figure 3 shows chromatograms of the authentic honey samples analyzed in the experiment. It is clear by eye that there are discernible differences between the metabolite profiles of the different honeys under examination.

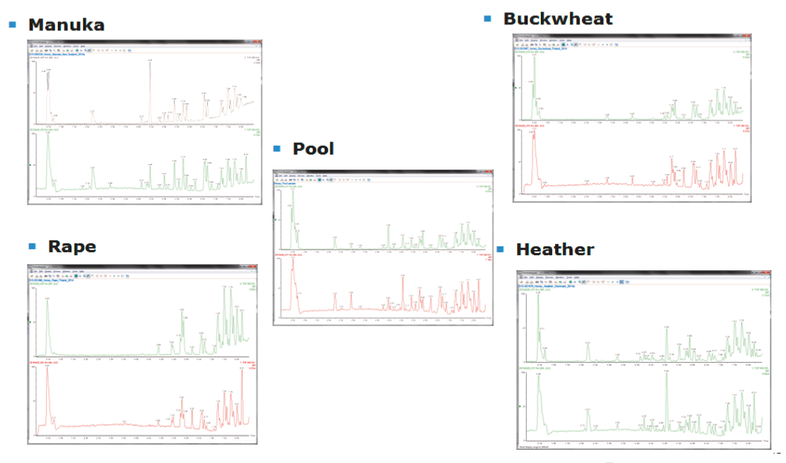

Figure 3. Example of chromatograms (ESI, HDMS^E, negative ion mode) of analyzed honey samples (low energy traces in green, elevated energy traces in red)

The raw data acquired was loaded into Progenesis QI. The experiment then moves to searching for markers of floral origin with Progenesis QI. With this software we can look for significant differences by comparing multiple samples using an all in one high-throughput guided workflow. During the data analysis the HDMS^E data is imported and RT aligned using an auto-wizard workflow. Feature detection of all raw data follows using co-detection which ensures no missing values for downstream quantification. Abundance profiles for all features across specified groups can be viewed. Multivariate statistical analysis is employed to allow differentiation of the features belonging to each analytical condition. Filtering of differentiating features is possible prior to automated metabolite identification using incorporated tools.

From the triplicate technical replicates of the 5 honey types >9000 features were identified in total over the experiment. A thorough statistical evaluation of the data was made at the MS1 level. Figure 4 shows the unsupervised principal component analysis (PCA) of the 5 different conditions under investigation during the analysis. Each condition is shown to be discriminated discretely from the others with technical replication displayed.

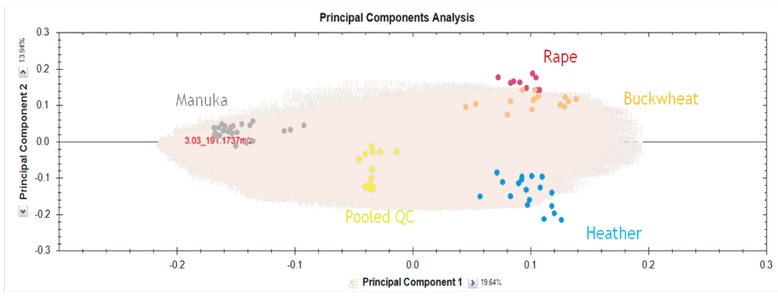

Figure. 4 PCA analysis of the authentic honey samples showing differentiation of the different groups (pool QC, buckwheat, heather, rape and Manuka)

Further investigation was made into the specific differences between the honeys by binary comparison. The differentiation between the two botanical origins is investigated using OPLS-DA by an integrated export into EZinfo software (Figure 5).

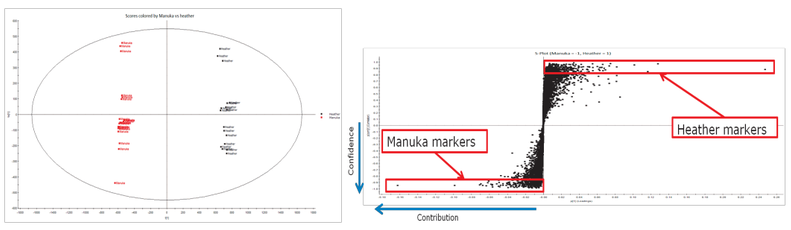

Figure. 5 OPLS-DA supervised analysis of Manuka and heather honey showing differentiation of the respective botanical origins and an S-plot highlighting the features responsible

An S-plot can also be created as shown to quickly highlight those features responsible for the difference between the conditions with the highest confidence and contribution. Selected features from the S-plot can then be imported back into Progenesis QI and tagged allowing them to be filtered and viewed independently. Prior to annotation features were subject to filtering based on significance. Annotation and identification were afforded by multiple iterations of database searches. Searches can use a combination of mass accuracy tolerance, RT tolerance, exact mass of precursor and fragment ions and, where appropriate, collisional cross section values obtained through the use of ion mobility.

The effect of the addition of ion mobility into the experimental workflow, particularly with respect to spectral cleanup, can be seen in Figure 6. Without ion mobility the high energy mass spectra from data-independent analysis can be complex and interpretation may be ambiguous. However, once ion mobility is employed we can use the apex drift time alignment of precursor and fragment ions to produce much cleaner mass spectra which are therefore much easier to interpret. Feature identification is much more easily facilitated with this type of data.

Figure 6 – Advantages of ion mobility for spectral clean-up

Some of the metabolites identified were highlighted as being able to differentiate the Manuka honey samples (Figure 7). These metabolites have been verified as Manuka markers previously [5]. The identification of other markers is ongoing.

Tentative ID	m/z	Formula	Mass error (ppm)	RT (mins)	Ion	P value	Fold change (mean)
Methyl syringate	211.0611	C10H12O5	-0.4	2.9	[M-H]⁻	<1.1E-16	2090
Leptosin	535.1657	C22H32O15	-2.1	4.7	[M-H]⁻	<1.1E-16	771
Leptosperin	581.1725	C23H34O17	0.4	4.4	[M-H]⁻	<1.1E-16	11000

Figure 7. Two markers for Manuka honey and their normalized abundance profiles compared with honeys from other origins

Independent verification of the identification of leptosperin as a marker of Manuka honey in the UPLC-HDMS experiment was afforded by analysis using targeted LC-MS/MS in MRM mode using ACQUITY I-Class with Xevo TQ-S (Figure 8).

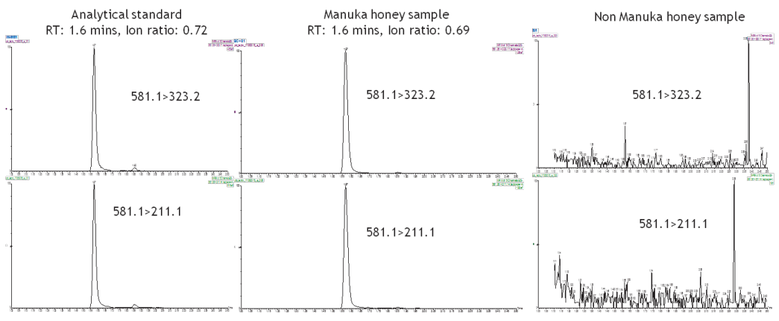

Figure 8. Chromatograms showing leptosperin content in various types of honey using LC-MS/MS with MRM

In a parallel experiment, Rapid Evaporative Ionization Mass Spectrometry (REIMS) was used to analyze the honeys directly, without sample preparation or chromatography (Figure 9). The resulting MS spectra were evaluated in the same fashion as the UPLC-HDMS data within Progenesis QI.

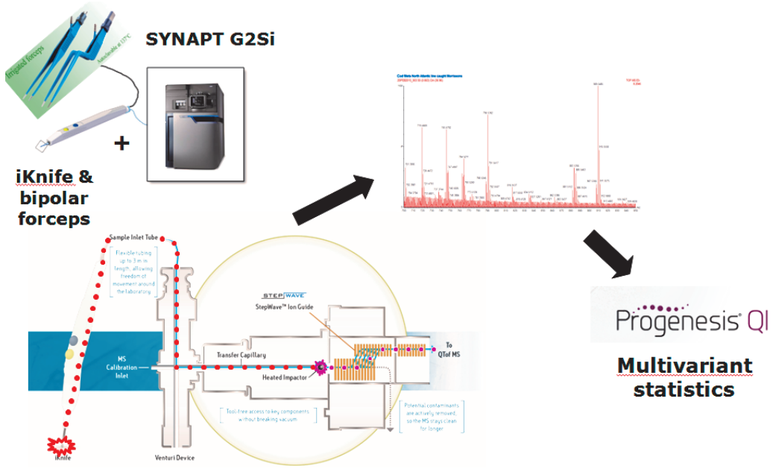

Figure 9. REIMS workflow

Data was examined to see if the REIMS data could provide discrimination between honeys of different botanical origins and to discover whether the same markers were present in the REIMS data as detected by UPLC-HDMS. Figure 10 shows that botanical discrimination between heather and orange honeys was possible using both strategies.

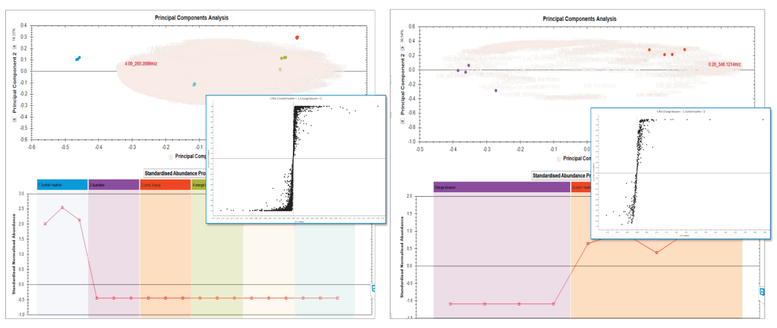

Figure 10. Comparison of data from UPLC-HDMS (left) and REIMS (right) experiments for discrimination of botanical origin

The total number of features identified by UPLC-HDMS was much greater, which also afforded identification from fragment ion information. REIMS was much quicker with no sample preparation or chromatography required. The two techniques are complimentary. UPLC-HDMS could be used for in-depth characterisation coupled with REIMS for simple point of origin testing. Alternatively REIMS could be used for rapid screening with only suspect samples submitted for further investigation by UPLC-HDMS. Twelve unique markers were common to the two workflows.

CONCLUSION

- **Food fraud is a major concern across the globe**
- **A non-targeted, profiling approach, using UPLC-HDMS combined with multivariate statistics, has been shown to be capable of differentiating samples of honey from different botanical origins**
- **Biologically significant information is obtained by comparing multiple samples using an all in one high-throughput guided workflow in Progenesis QI**
- **The Progenesis QI facile database search engine allows both confident and putative assignment of markers**
- **Making use of ion mobility gives "cleaner" mass spectra facilitating easier identification of markers**
- **Verification of markers is important using complimentary technology**
- **Direct analysis using REIMS could provide opportunities for testing at "point of entry"**

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