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

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OVERVIEW

Honey is a natural product which possesses therapeutic, nutritional and industrial value. It can be a high value food commodity especially in the case of certain unifloral honeys used in apitherapy due to their healing and antibacterial properties. Honey can also be the subject of fraud, commonly by adulteration by the addition of sugars but also by mislabeling or false declaration of botanical origin. In recent years there has been growing interest in verifying floral origin to ensure traceability and fraud reduction. Here the feasibility of applying an untargeted high-definition metabolomic approach, in combination with multivariate statistics was evaluated. This approach was applied to a variety of genuine honeys of different floral origin to assess if floral differentiation was possible.

INTRODUCTION

Recent scandals have highlighted that food fraud can represent major food safety issues. These include the European horse meat scandal where foods advertised as beef were found to contain undeclared horse meat of unknown origin. Horsemeat itself is as safe to eat as beef. However, veterinary drugs such as human hazardous phenylbutazone had illegally entered the human food chain. A second severe case can be highlighted in the 2008 Chinese milk scandal. The case involved milk and infant formula being adulterated with nitrogen rich melamine (a flame retardant resin), illegally added to food products to increase their apparent protein content.

Product adulteration is widespread and is undertaken mainly for financial gain, product extension, substitution or reduced manufacturing costs. Horsemeat is much cheaper than beef to procure. The fraudulent dilution of milk with water could be concealed with the addition of melamine as the demand for milk far outstrips supplies due to poor animal husbandry, production and storage (1).

It is clear from serious incidents such as these that by controlling food fraud, many food safety issues will be controlled too. Understanding the ingredient supply chain and what's "normal" is the basis for food fraud prevention. This applies to the animal feed markets in much the same way as for the human food market.

In the case of honey adulteration of honey by sugars, syrups or flavour enhancers can make it production cheaper or inhibit crystallization. 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 the German food ingredients conglomerate ALW took Chinese-origin honey repackaged, mislabelled and shipped it to the U.S. via intermediaries. 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.

Worryingly, in some cases, it was found to be contaminated with the residue of antibiotics banned in the U.S (figure 1).



Fig. 1 - The Honey Launderers: Uncovering the Largest Food Fraud in U.S. History. Bloomberg business week. Susan Berfield September 23, 2013 (5)

Classically melissopalynology (pollen analysis) is used for honey analysis (3). However, this can fail to detect closely related species substitutions for instance with Manuka (NPA active) and Kanuka (NPA inactive) honeys. Typically NMR is used commercially to distinguish between different honey types (4). In this study we aimed to investigate whether an untargeted high-definition metabolomic approach, in combination with multivariate statistics could differentiate genuine honeys of different floral origin.

METHODS

Authentic honeys of rape, heather, buckwheat and manuka origins were obtained from indisputable sources (6,7). Each floral origin contained separate samples from different countries and years of collection giving multiple biological replicates per floral origin (Figure 2).

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Fig. 2- Table of genuine honey samples used in study

Honey samples were subjected to minimal sample preparation 0.5 g of each honey was diluted in 10 mL in 50:50 methanol/ water 1% of formic acid. The samples were shaken, sonicated for 20 min and then centrifuged using a high speed centrifuge.

Complex metabolite mixtures were separated using UPLC performed using an ACQUITY I-Class. One dimensional reversed phase (RP) experiments were performed using a 9 minute gradient (5 to 90% acetonitrile over 90 minutes) utilizing a 2.1 x 100 mM, 1.7µM C18 ACQUITY BEH column operated at 0.5 mL/min.

Each sample was then analysed in triplicate by dataindependent UPLC-HDMS^E (10) on a SYNAPT G2Si mass spectrometer (Waters, Manchester UK) (Figure 3). The use of ion mobility was employed in order to maximise peak capacity when analyzing highly complex mixtures (Figure 3 inset). Samples were analysed in ESI +tive and -tive modes a randomized order including interspersed pooled honey samples for QC purposes. HDMS^E data-independent analysis provides accurate mass measurements of all detectable precursor and product ions. Chromatographic and drift time alignment of precursor and product ion data reduces miss-assignment of product ions to parent ions of similar mass or retention time.

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The data produced were analyzed using Progenesis QI software with EZinfo including 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 (8). 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).



Figure 3. Schematic of the SYNAPT G2Si HDMS- inset shows Triwave mobility device in HDMS^E analysis mode

RESULTS

Figure 4 shows example HDMS^E 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 4 - Example HDMS^E Chromatograms (ESI -tive) of analysed honey samples. Low energy traces in green, elevated energy traces in red

The acquired raw data was loaded into Progenesis OI. The experiment then moves to searching for markers of floral origin with Progenesis QI. With this software we can obtain biologically meaningful information 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.



Figure. 5 PCA analysis of 5 separate conditions of authentic honey HDMS^E analyses showing differentiation of the respective phenotypes (pool QC, buckwheat, heather, rape and manuka

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 5 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.

Further investigation was made into the specific differences between the honeys by binary comparison. An example showing manuka vs. heather honeys is shown in figure 6.



Figure. 6 a) OPLS-DA supervised analysis of manuka and heather honey HDMS^E analyses showing differentiation of the respective botanical origins and b) S-plot highlighting the features responsible for the effect

The differentiation between the two botanical origins is investigated using OPLS-DA by an integrated export into EZinfo software (9). 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 a P value < 0.0001 and a mean abundance change of > 5 fold. Annotation and identification were afforded by multiple iterations of database searches. Searches can use a combination of PPM mass accuracy tolerance, RT tolerance, Exact mass of precursor and fragments and where appropriate collisional cross section of the molecule obtained through ion mobility.



Figure 7 – Advantages of Ion mobility activation on the clean-up of fragmentation spectra which aids metabolite identification

The effect of the addition of ion mobility into the experimental workflow particularly with respect to fragmentation spectra can be seen in figure 7. Without ion mobility the high energy fragmentation 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 parent and fragment ions to produce much cleaner fragmentation spectra which are therefore much easier to interpret. Feature identification is much more easily facilitated with this type of data.

Some of the metabolites identified and highlighted as being uniquely up regulated in the manuka honey samples are shown in figure 8 (Methyl syringate, leptosin and leptosperin). These metabolites have been verified as manuka up-regulated in other peer reviewed publications (11). The identification of other unique markers are the subject of further work.

Independent validation of the manuka up-regulated marker leptosperin was afforded by a targeted MRM experiment on a triple quadrupole instrument (I-Class UPLC with Waters TQS 000). The results of two MRM transitions monitored for the compound can be seen in figure 9. The results highlight the specific nature of the marker discovered in the OMICS experiment.



In a parallel experiment the novel ionization source Rapid Evaporative Ionization was used to analyze honey directly. It was not necessary to perform any sample preparation or UPLC separation on the honey prior to ionization (Figure 10). The resulting MS1 level data was processed in the same fashion as the OMICS data within Progenesis OI.

In the first instance the data was assessed to see if the REIMS data could also provide discrimination between the honeys of different botanical origins. Then the data was investigated to cross compare the uniquely identifying metabolites across the two experiments. Figure 11 shows that botanical discrimination between heather and orange honeys was possible using both strategies. The total number of features identified was much higher in the OMICS experiment—which also afforded feature identification due to the MS2 information obtained in the HDMS^L experiment. However, the REIMS experiment was less complicated with no sample preparation or chromatography required. A combination of the two techniques, an initial OMICS experiment for in-depth characterisation and REIMS for simple point of origin testing at an MS level would allow confident discrimination of botanical origin. Twelve uniquely identifying markers were common to the two workflows.

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Figure 8 – a table detailing a selection of identified metabolites uniquely up regulated in the Manuka samples vs. the rest of the botanical origins. Also showing molecular structure and normalized abundance pro-

Figure 9 - Validation experiment for Leptosperin content of Manuka vs. non-manuka honeys. Left to right; analytical standard, Manuka honey and non-manuka honey conducted on UPLC I-Class with Waters Xevo TQ-S



Figure 10 – Rapid Evaporative Electrospray Ionosation Mass Spectromtry (REIMS) analytical workflow



Figure 11 – Comparison of OMICS and REIMS experiments for discrimination of botanical origin

CONCLUSIONS

- Food authenticity, adulteration and safety is a major concern across the globe
- A non-targeted high resolution MS OMICS approach combined with multivariate data analysis can identify 'normal' profiles of foodstuff allowing detection of fraud during the investigative stages
- · Can obtain biologically meaningful information by comparing multiple samples using an all in one highthroughput guided workflow in Progenesis QI
- Confident structural assignment in Progenesis QI enables markers to be annotated and identified by databases of user selection
- Combining ion mobility with MS gives "cleaner" fragmentation data allowing easier identification of markers
- Validation of markers is important using complimentary technology for confirmation
- New innovative rapid evaporative techniques will open new doors into authentication techniques at "point of entry"

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