An LC-Orbitrap-MS Based Non-targeted Metabolomics Approach for Investigation of Honey Adulteration

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ABSTRACT

Purpose: Create qualitative screening methods for honey by using a simple dilute-and-shoot methodology and analysis by high resolution accurate mass (HRAM) with the Thermo Scientific™ Exploris Orbitrap™ 240 mass spectrometer.

Methods: Six honey samples were collected from a local market. The diluted samples were analyzed by two chromatography methods: Reverse phase and hydrophilic interaction liquid chromatography (HILIC) with a data dependent MS2 acquisition strategy to enable detection and confirmation of markers of honey adulteration. These included polysaccharides,5 di-fructose anhydrides (DFAs), and 2-acetyl furan-3-glucopyranoside (AFGP) [1]. The acquired data (n=5) was processed by using Thermo Scientific™ Compound Discoverer software. For identification and confirmation, the precursor ion and product ion mass error was kept less than 5 ppm as per the SANTE guideline [2].

Results: The automated screening methods provided clear identification of compounds in comparison with databases (m/z Cloud™, ChemSpider, etc). The two methods were able to identify more than 200 metabolites in honey samples by following stringent mass accuracy (<5 ppm) criteria. Further, the six samples were differentiated through statistical analysis using Principal Component Analysis (PCA) with score plots. The PCA results showed that four samples were potentially adulterated with excess amounts of added sugars.

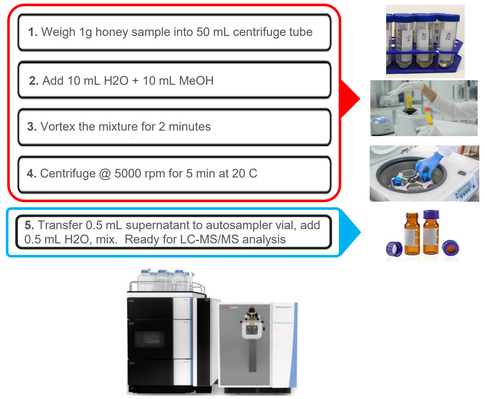
INTRODUCTION

Honey, a sweet, flavorful, and nutritious food produced by honeybees is widely consumed by humans as it has several therapeutic effects due to its antioxidant, antimicrobial, and anti-inflammatory properties [3]. Also, honey has been used in the treatment of wounds, burns, and gastric ulcers. [4]. The high price, low production, and complex nature of honey have attracted more attention towards adulteration which can adversely affect consumer health. Common honey adulterants are sugar syrups, such as corn syrup, high-fructose corn syrup, inverted syrup, and rice syrup added to replace pure honey.

There are currently no simple and available methods that can simultaneously detect all the common sugar syrups present in the adulterated honey samples. Therefore, there is a need to develop simple, rapid, and sensitive detection methods that simultaneously detects sugar syrups. To identify oligosaccharides, polysaccharides, and phytochemicals, a uniquely featured accurate, sensitive mass spectrometer for data acquisition and data processing is required, followed by retrospective data mining.

MATERIALS AND METHODS

Sample Preparation



MATERIALS AND METHODS- cont.

Liquid Chromatography and Mass Spectrometry Conditions

The LC system comprised a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system. Two methods were created, one for the reverse phase and the other a HILIC analysis of the honey samples. Table 1 and Figure 1 describe the basic gradient profiles and conditions used in this study.

Parameter	Method 1 (Reverse Phase)	Method 2 (HILIC)
Column	Accucore aQ (100 X 2.1 mm X 2.6 um) (Thermo Scientific™, P/N.27926-102130)	Accucore amide HILIC (150 X 4.6 mm X 2.6 um) (Thermo Scientific™, P/N. 16726-154630)
Mobile phase A	Water + 0.1% HCOOH	Water
Mobile phase B	Methanol + 0.1% HCOOH	Acetonitrile
Gradient	0-1 min, 5% B; 1-4min, 5-45% B; 4-11.5 min, 45-95% B; 11.5-15 min, 95% B; 15-15.5 min, 95-5% B; 15.5-20 min, 5% B.	0-1.5 min, 95% B; 1.5-11min, 95-30%B; 11-17 min, 30% B; 17-17.5 min, 30-95% B; 17.5-22min, 95% B
Flow rate	0.3 mL/min	0.6 mL/min
Column Oven	40 ° C	40 ° C
Total run time	20 min	22 min

Table 1: LC conditions for reverse phase (Method 1) and HILIC (Method 2) methods.

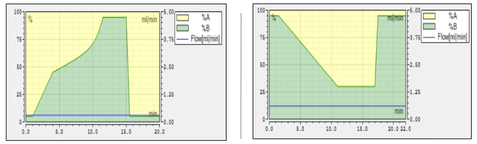


Figure 1: Gradient profiles for reverse phase (left) and HILIC method (right).

The Exploris 240 mass spectrometer settings for both methods are described in Table 2. The data acquisition was performed in a full MS-ddMS2 mode without an inclusion list. In this mode, the instrument measures one full MS scan (survey scan) with the HRMS spectral information. After triggering the required threshold in the quadrupole, the precursor ion was fragmented in the higher-energy collision dissociation (HCD) cell. The resulting fragments are transferred into Orbitrap where they are measured.

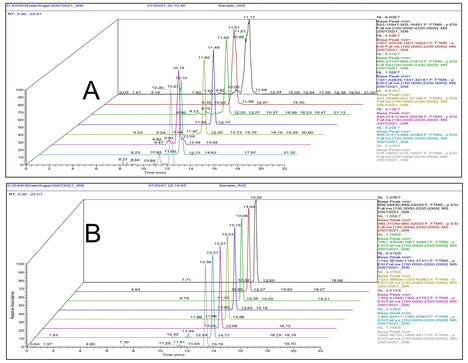
Parameter	Method 1 (Reverse Phase)	Method 2 (HILIC)
Acquisition	Full scan-ddMS2	Full scan-ddMS2
Full Scan mass range	m/z 100–1000	m/z 200–2000
Full Scan resolution	R=240,000	R=240,000
ddMS2 resolution	R=15000	R=15000
NCE	Stepped (10, 30, 55)	Stepped (10, 30, 55)
Ion Spray voltage	3.5 kV (Pos) / 2.5 K (Neg)	3.5 kV (Pos) / 2.5 K (Neg)
Sheath gas	40.0 arb	50.0 arb
Aux gas	10.0 arb	10.0 arb
Sweep gas flow rate	1.0	1.0
Capillary temp	300 ° C	300 ° C
Aux gas Heater temp.	320 ° C	350 ° C
RF-lens (%)	70	70

Table 2: Orbitrap Exploris 240 settings for each analysis method

RESULTS

Chromatography and impact of resolving power

For polar analytes, the Accucore amide HILIC chemistry offered excellent retention and resolution along with symmetrical peak shapes with better sensitivity and selectivity for oligosaccharides and polysaccharides (Figures 2). Also, the reverse phase chemistry was used for the metabolomics approach to screen for the natural components that are present in honey samples.



Figures 2: A (top)- Representative EIC for different oligosaccharides (DP 3-10) in honey and B-(bottom) representative EIC for different polysaccharides (DP 11-18) in honey

The data was acquired on high resolving power (R=240000) and evaluated for the back-calculated (measured) resolution over the wide mass range used. Three different representative masses were selected which cover the wide mass range: maltooctadecaose, m/z 2934.9614 (high); maltotriose; m/z 503.1690 (middle); and 2-coumaric acid m/z 163.0401 (low). The observed resolution for the masses in negative polarity in honey samples were R= 93003 for Maltooctadecaose m/z = 1466.4746 [M-2H]2-, R= 159107 for maltotriose m/z = =503.1619 [M-H]-, and R= 286206 for 3-coumaric acid m/z 163.04007 [M-H]- respectively (Figure 3).

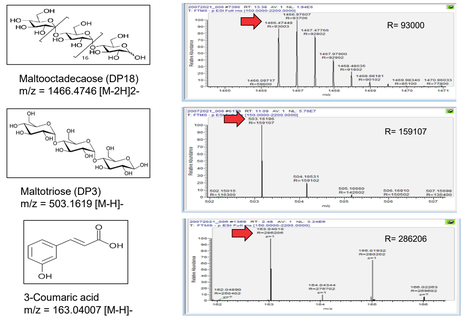


Figure 3: Comparison of theoretical (R= 240000) vs observed resolution for a wide mass range in a honey sample. Excellent mass accuracy and resolution adds more confidence to the identification of potential adulterants such as added sugars.

RESULTS- cont.

Identification of compounds

Data processing was performed using Thermo Scientific™ Compound Discoverer™ via a non-target screening approach. The software enables peak picking based on accurate mass, retention time alignment, full scan MS, and MS/MS spectral match with comparison to online databases such as ChemSpider and mzCloud. A total of 97 compounds were identified using HILIC in honey samples, including higher sugars (oligosaccharides and polysaccharides) and other polar metabolites. 75 compounds in positive mode (+ve) and 46 compounds in negative mode (-ve), including polyphenols and amino acids were detected using the reverse phase method. The identified compounds were confirmed based on a library score (>65%), and parent ion with one fragment ion mass accuracy (<5 ppm). The identification of naringenin was based on the protonated ion m/z 273.0753 with 0.43 ppm mass error which was further confirmed through the MS/MS spectral interpretation (Figure 4). .

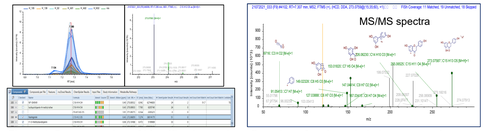


Figure 4: Demonstration of identification and confirmation of naringenin in honey samples through compound discoverer

Six samples were compared and differentiated through statistical analysis using Principal Component Analysis (PCA) within the Compound Discoverer software. The PCA clearly differentiated both compliant and non-compliant honey samples as per EU regulations. The box and whisker plots clearly indicated that DP 6 and DP 16 sugars had elevated concentration levels in samples A02, A01, H8, and 139 (Figure 5).

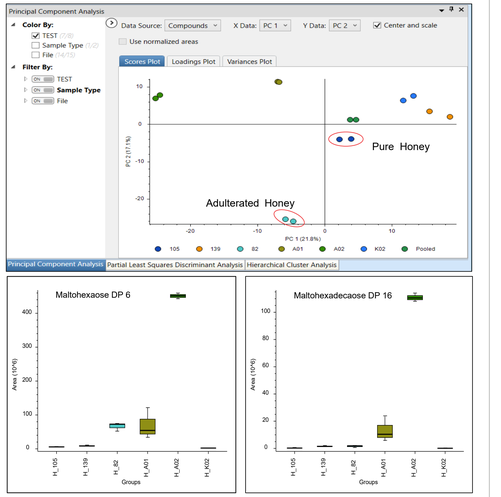


Figure 5: Principal Component Analysis (PCA) for honey samples, with box whisker plots (bottom) for maltohexaose (DP6), and maltohexadecaose (DP16). Sample A02 was further analyzed by NMR and IRMS and found to be non-compliant compared to the other samples.

RESULTS- cont.

Impact of resolution and polarity switching on sensitivity

The impact of polarity switching as well as resolving power on sensitivity was evaluated for Naringenin, which is an analyte that occurs naturally in the honey samples. The negative polarity offered a better signal (4X) for Naringenin over positive polarity. The area variation observed at three different resolutions was < 8.35% in negative and 3.66% in positive polarity mode which indicates that there is no significant change observed in the sensitivity of Naringenin as a function of resolution (Figure 6A and 6B). In addition to this, the impact of the resolution was evaluated for the peak quality in terms of scans/peak (data points). The data acquired in high resolution (R=240000) showed >15 scans/peak (Figure 6 C). The high-resolution data therefore provides sufficient scans/peak which enables accurate quantitation and reproducibility, which are critical for relative analyte comparisons between samples.

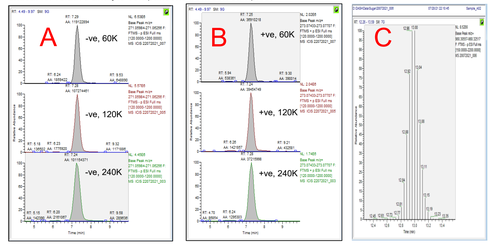


Figure 6: Response of Naringenin in a honey sample, displayed in positive (A) and negative (B) polarities during Pos/Neg switching on the Exploris Orbitrap 240 MS. Resolution of 60K, 120K, and 240K show virtually identical response. For quantitation, plenty of scans are acquired across chromatographic peaks. Maltododecaose is shown as an example in (C), with > 15 scans/peak.

CONCLUSIONS

- A fit-for-purpose methodology using both reverse phase and HILIC approaches was shown to effectively screen honey samples for native compounds and metabolites. Over 200 analytes in honey samples following stringent mass accuracy (< 5 ppm) criteria were detected.
- The methodology combined with Compound Discoverer software allowed clear differentiation of the six honey samples through principal component analysis. The PCA analysis helped to identify adulteration of some samples due to externally added sugars. This complete workflow not only provided information regarding adulteration but also offers the chemical name of the adulterant.
- The Exploris 240 Orbitrap provides excellent response for analytes at 60, 120, and 240K resolution settings. In addition, quantitation ability with high number of scans/peak is maintained when performing polarity switching experiments, improving laboratory productivity.

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