Differential Analysis of Organic and Non-Organic Honey for Pesticides and Pollutants by LC Time-of-Flight Mass Spectrometry

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Introduction

In recent years, organic certified foods have grown in popularity due to their perceived potential to contain, for example, less pollutants and pesticides. Food must meet certain criteria established by an organic certification body in order to be certified organic. Certified organic honey, for example, must meet criteria, including but not limited to, the source of the nectar, the honey bees foraging area, bee management and the honey extraction processes. For honey, in particular, this requires considerable resource as it is hard to restrict bees' travel, making it possible for them to forage on non-organic pollen. For many beekeepers it is too difficult to adhere to these strict organic guidelines thus questioning the purpose of purchasing organic honey.

Research Goal

To screen different types of honey (some certified organic) for pesticides and pollutants using identical separation and mass spectrometry conditions. Using differential analysis, the data sets will be reduced and similarities between the samples will be visualized. A judgment will be made in order to determine if the extra cost associated with organic honey justifies its additional purchase price over non-certified honey.

Experimental

Sample Description

Six different types of honey (given in Table 1), from a mix of different regions and some certified organic (by different certifying bodies) were purchased. The honey samples were each treated using an identical QuEChERS extraction protocol.

Sample Designation	Comments
Honey 1	Orange Blossom-USA
Honey 2	Clover Honey-USA
Organic Honey 3	Raw, Mexico certified organic by Agency 1
Organic Honey 4	Wild Collected Raw, India certified organic by Agency 1
Organic Honey 5	Agave Nectar-Mexico, Certified organic by Agency 2
Organic Honey 6	Raw-Agave Nectar, Certified organic by Agency 3

Table 1: List of honey samples analyzed

Experimental

QuEChERS Extraction Protocol

Five grams of honey was weighed into a 50 mL centrifuge tube. Ten mL of water was added to the tube and it was vortexed for 30 seconds. Fifteen mL of acetonitrile containing 1% acetic acid was added to the tube and it was vortex for 30 seconds. An AOAC extraction salt packet (containing sodium acetate and magnesium sulfate, Agilent PN 5982-5755) was added to the tube and it was shaken vigorously for 1 minutes. The tube was then centrifuged at 5000g for 5 minutes. Eight mL of the extract was transferred to a d-SPE tube (contains C18, magnesium sulfate, Agilent PN 5982-4956). A d-SPE tube was chosen without PSA because we are screening for unknowns with possibly carboxylic acid functionalities. The tube was vortexed for 1 min and then centrifuged at 5000g for 5 mins. Five mL of the extract was transferred to a 15 mL conical tube and evaporated to dryness with nitrogen. The sample was reconstituted in 100 µL of 40% acetonitrile/60% water.

Instrumentation and Method Conditions

An Agilent 1290 Infinity UHPLC was coupled to an Agilent 6230 Time-of-Flight mass spectrometer. The UHPLC consisted of a binary pump with integrated degasser (G4220A), a thermostatted autosampler (G4226A), and a thermostatted column compartment (G1316C). A Zorbax Eclipse Plus C18 (2.1x100mm, 1.8 µm, part number 959758-902) column (thermostatted at 45°C) was used for all analysis. The mobile phase was water (A) and acetonitrile (B) containing 0.1% formic acid (FA), run using the gradient conditions in Table 2 at 0.3 mL/min flow rate. The mass spectrometer was run with the source settings also given in Table 2. The injection volume was set to 1 µL and each sample was injected 6 times. The data was processed using Masshunter Qualitative software (version B.05) and Mass Profiler Professional (version 12).

LC Gradient Conditions		MS Source Settings	
Time (min)	%B	Source	+ESI
0.0	5	Gas Temperature	325°C
2.0	5	Gas Flow	10 L/min
23.0	95	Nebulizer	50 psig
25.0	95	Capillary Voltage	4000 V
25.1	5	Fragmentor	175 V

Table 2: LC-MS Method conditions

Results and Discussion

Initially, the mass accuracy and the retention time precision of each replicate injection (n=6) was examined and deemed satisfactory. Also, the blanks between each set of 6 replicate injections were found to be clean thus eliminating the question of carry over between samples. Initial comparison of the samples was done by examining the BPC for each sample (as shown in Figure 1). Organic honey 3 shows the most complex and intense BPC overall, while organic honey 5 and 6 show the least complex and intense BPC. This reveals the complexity of the samples and although some common patterns and differences can be seen overall, there still remains difficulty in a more specific sample comparison.

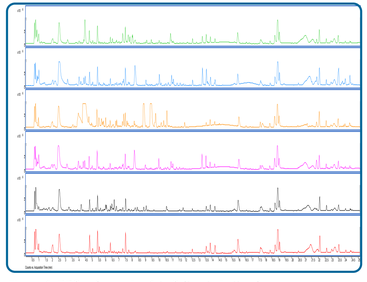

Figure 1: BPCs of samples 1 to 6 (top to bottom)

Next, the sample data was processed using an algorithm (find by molecular feature) capable of reducing the data set into unique compounds found throughout the chromatographic run. The compound lists were then furthered refined by filtering the results against a pesticide database (Agilent PN:G6854AA) to screen for potential pesticides and their relative amounts in each type of honey. The database matching compares the exact mass, isotope abundance and isotope spacing (weighted 100, 60 and 50, respectively) of the experimental to the theoretical formula and computes a score. Compounds with scores less than 70.00 were discarded.

The results indicate that each sample contains a variety of pesticides/pollutants at different relative amounts. It is important to note, however, that this result is somewhat anticipated. After honey is produced and it is subsequently tested for food safety purposes, it is conceivable that the honey does contain pollutants and/or pesticides but that each of these substances it is tested for is below a pre-established cut-off for the approved analytical method. Since these samples were prepared and concentrated by evaporation, the absolute levels found in the sample are not indicative of the amount found by the original testing protocol that the honey passed in order to be sold. The relative amounts though however are indicative of the quality of each sample relative to the other.

Since the database matching was based solely on MS only data (exact mass, isotope abundance, and isotope spacing), the possibility of false positives is large. The number of false positives can be significantly reduced by also matching a found compound's retention time to the retention time of a standard.

A standard containing approximately 200 common pesticides was also injected 6 times using the same instrument conditions used to acquire the sample data.

Compound	Sample 1	Sample 2	Organic Honey 3	Organic Honey 4	Organic Honey 5	Organic Honey 6
4-Nonylphenol					83973	19087
ADBI		7506			8838	
Aldimorph	65847		98196	78113	33415	39316
Carbendazim						24271
Carbofuran-3-OH-7-phenol					174122	17870
Carbofuranphenol-3-keto	53645	47695	64687	58857	56740	61059
Dodemorph	2082999	1203038	2318342	2043600	2107973	2632697
Embelin	8272	11548	11875	10172	10437	15098
Phenylacrylic acid	20020	22195	64790	18092	14978	21057
Pirimicarb		13872				
Pyrimidifen	18085		13902	13768	12627	14313
Tri-n-butyl phosphate	6711	6414	7227			

Table 3: List of compounds and their average abundance (N=6) found in each sample using exact mass and retention time matching

Results and Discussion

The pesticide standard was processed into compounds and searched against the pesticide database. If a pesticide was found in at least 4 out of the 6 runs, a retention time was associated with the compound and it was added to an abbreviated pesticide database. The compound lists for each sample were then filtered by both retention time and the MS data in order to generate an abbreviated list of pesticides for each sample which is reported in Table 3.

Table 3 is useful in illustrating several common and unique pesticides among the various honey samples and their relative amounts. For example, carbofuranphenol-3-keto, dodemorph, embelin, and phenylacrylic acid appear at consistent levels in all samples while aldimorph and pyrimidifen appear consistently except for in sample 2. Meanwhile, 4-nonylphenol, ADBI, carbendazim, carbofuran-3-OH-7-phenol, pirimicarb and tri-n-butyl phosphate appear in only one to two of the samples. This type of comparison, although useful for a low chance of false positives is however limited because it only illustrates a subset of the sample that was well matched to the specific pesticide database (approximately 200 entries).

In order to compare the overall data set for each sample to all the other samples, differential analysis was performed and a PCA (principal component analysis) plot was generated. A PCA plot reduces the complexity of the data and correlates the similarities in the samples using a visual format. To generate the PCA plot, the list of compounds for each replicate of each sample is exported to mass profiler professional for analysis. The PCA plot in Figure 2 was generated from a compound list (limited to the largest 3000 compounds) that was unfiltered. This PCA plot illustrates that honey 1, honey 2 and organic honey 4 cluster together and overall are fairly similar.

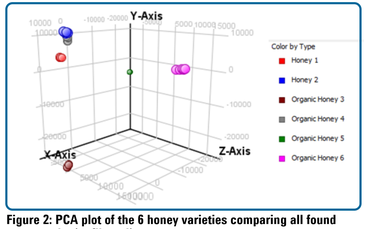

Figure 2: PCA plot of the 6 honey varieties comparing all found compounds (unfiltered)

Another PCA plot was generated from these compounds lists filtered by the pesticide database (compounds with scores below 70.00 were discarded) as shown in Figure 3. This plot shows that honey 1, honey 2, and organic honey 4 show similarities in their pesticides. Organic honey 5 and 6 are also very similar.

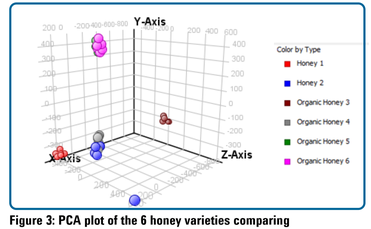

Figure 3: PCA plot of the 6 honey varieties comparing compounds matched to a pesticide database

In order to search for a broader range of pollutants (such as from environmental or packaging) in the samples the compound lists were filtered against a vet-drug database (Agilent) and compounds with a score below 70.00 were again discarded. A PCA plot generated from this set of compound lists is shown in Figure 4. In this case, close to 3 pairs of clusters are formed between honey 1 and organic honey 3, honey 2 and organic honey 3 and organic honey 5 and organic honey 6.

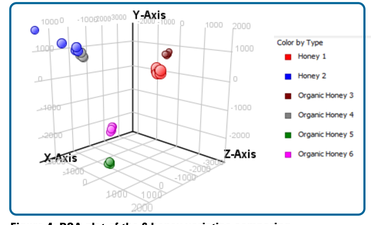

Figure 4: PCA plot of the 6 honey varieties comparing compounds matched to a vet-drug database

Conclusions

A list of common and unique pesticides found in each sample was generated. This list however is too specific to provide an overall evaluation of the sample so differential analysis was used. Differential analysis demonstrated that overall organic honey 4 was very similar to the two non-certified organic honey samples. Based on pesticides, the same conclusion can be drawn. Based on pollutants, organic honey 3 and honey 1 were similar as was organic honey 4 and honey 2. This indicates that one should consider the organic certifying agency and its criteria before deciding to spend extra money on organic as some of the organic honey clustered with the non-certified honey.