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MULTIVARIATE ANALYSIS OF THE ATR-FTIR OF HONEY SAMPLES

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Abstract

This study explores the chemical composition and authenticity of various honey types (wildflower, clover, pure, and organic unfiltered honey) utilizing Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy coupled with multivariate statistical analyses. The ATR-FTIR spectra revealed key vibrational bands that indicate the presence of hydroxyl groups, aliphatic hydrocarbons, and carbonyl functionalities, highlighting significant moisture content and sugar composition common to all samples. Principal component analysis (PCA) demonstrated that the spectral data variances primarily reflected differences in floral sources, with wildflower and clover honeys clustering closely due to their analogous chemical profiles. Conversely, pure honey distinguished itself with a more standardized chemical constitution, while organic unfiltered honey exhibited unique characteristics linked to its less processed nature. Hierarchical cluster analysis (HCA) reinforced these findings by visually representing the relationships among honey types, confirming that floral sources and processing methods critically influence chemical diversity and authenticity. This research underscores the potential of PCA and HCA as effective tools for evaluating honey quality, supporting industry efforts to ensure product integrity and consumer confidence.

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Introduction

Honey, a natural substance synthesized by bees from plant nectar, has been consumed by humans for centuries due to its sweet taste and health benefits, including antimicrobial, antioxidant, anti-inflammatory, and anticancer properties.[1] Its complex composition, consisting of over 200 compounds, is dominated by carbohydrates but also includes water, amino acids, organic acids, minerals, and enzymes.[2] The composition of honey is highly variable and influenced by factors such as geographical and botanical origins, bee species, harvesting methods, and storage conditions.[3] These variations contribute to the categorization of honey as either unifloral, derived predominantly from one flower type, or multifloral, made from nectar collected from diverse floral sources.[4]

The increasing commercial demand for honey has led to diversification into various flavors and types, such as clover, wildflower, pure, and organic unfiltered honey. However, the high value of honey has also made it a target for adulteration through methods such as the addition of glucose, fructose, or high-fructose corn syrup, or by feeding bees low-quality sugars.[5] Such adulteration compromises consumer trust and underscores the need for reliable authentication and quality control methods.

Among the analytical techniques available for honey quality assessment, attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy has emerged as a powerful and versatile tool.[6-8] ATR-FTIR is a non-destructive method that requires minimal sample preparation and provides rapid, reproducible results. It works by measuring the absorbance of infrared light, which interacts with molecular vibrations in the sample, producing a spectrum that reflects the chemical composition of the honey. This method is particularly well-suited for analyzing honey due to its ability to detect subtle compositional differences and identify adulterants without extensive chemical preprocessing.

ATR-FTIR spectroscopy offers significant advantages over traditional methods like melissopalynology, which requires skilled expertise aside from being a destructive and time-consuming method,[6] and chromatographic techniques,[9] which are time-consuming and costly. When combined with chemometric techniques such as principal component analysis (PCA), hierarchical clustering analysis (HCA), and partial least squares (PLS) regression, ATR-FTIR enables comprehensive data analysis, allowing for the differentiation of honey based on botanical and geographical origins and the detection of adulteration.[6,10] While PCA has been extensively applied in honey studies, the inclusion of hierarchical cluster analysis (HCA) to evaluate spectral similarities and identify hierarchical relationships among honey types is a novel aspect of this work. This approach provides a robust framework for distinguishing honey types based on their spectral data, offering invaluable insights for producers, regulators, and consumers concerned with honey authenticity and quality.

This study analyzed the chemical composition of four honey variants produced by Urban Meadows: clover, wildflower, pure, and organic unfiltered. Using ATR-FTIR spectroscopy paired with multivariate statistical methods, specifically principal component analysis (PCA) and hierarchical clustering analysis (HCA), the research aimed to identify compositional differences among the variants. The combination of these techniques not only enhances our understanding of the chemical diversity among honey samples but also validates the efficiency of ATR-FTIR spectroscopy in routine honey quality control and authentication.

Materials and Methods

Honey Samples

Four commercially available honey variants from Urban Meadows were selected for analysis: clover, wildflower, pure, and organic unfiltered (Figure 1). Each variant represents a distinct type of honey, differentiated by processing methods, floral sourc-

es, or labeling claims. The samples were stored in sealed containers at room temperature prior to analysis to maintain their original composition.

ATR-FTIR Analysis

The honey samples were analyzed using attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. A small amount (25 uL) of each honey sample was applied directly to the ATR crystal surface to ensure uniform contact. After each measurement, the ATR surface was thoroughly cleaned with methanol to prevent cross-contamination between samples. Each honey variant was analyzed five times, with a fresh sample scooped from the container for each run to account for potential heterogeneity.

Multivariate Statistical Analysis

The spectral data obtained from ATR-FTIR analysis were subjected to multivariate statistical analysis to evaluate compositional differences among the honey variants. Principal component analysis (PCA) was used to identify patterns and reduce dimensionality in the data, allowing for visualization of sample clustering. Hierarchical clustering analysis (HCA) was performed to assess the similarity between samples and classify them into distinct groups. These statistical methods provided a comprehensive understanding of the chemical variability across the honey samples. PCA and HCA were performed based on the methods used by Grabato et al (2022).[11]

Results and Discussion

ATR-FTIR

Figure 2 shows the ATR-FTIR spectra of the four honey samples, with their corresponding peaks listed in Table 1 along with their associated functional groups. The analysis of ATR-FTIR spectra for various honey samples- including wildflower, clover, pure, and organic unfiltered honey - revealed distinct vibrational characteristics that provide insight into their chemical composition. The data from the wavenumbers (Table 1) exhibited several key vibrational bands associated with the functional groups present in these honey types.

Figure 1. Honey samples used in the study.

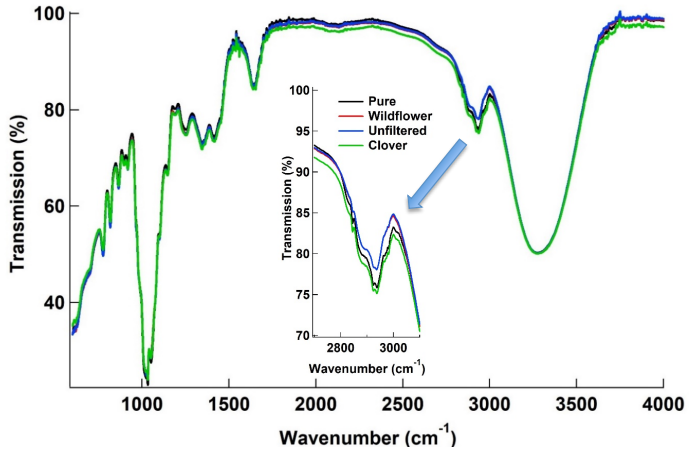

Figure 2. ATR-FTIR spectra of different honey samples.

The O-H stretching vibrations were observed between 3271 to 3291 cm^{-1} range, indicating the presence of hydroxyl groups that are characteristic of sugars and phenolic compounds in honey. This broad peak suggests a significant moisture content across all honey samples, which is vital for the texture and preservation of honey.[12,13] The consistency in the wavenumber reflects a similar hydrophilic character across these samples, reaffirming their natural origins. In the C-H stretching region, peaks located around 2935 cm^{-1} were notable for their differences. These peaks are characteristic of aliphatic hydrocarbons found in the sugar components of honey. The consistent presence of this band across all samples indicates that the primary constituents - glucose and fructose remain relatively uniform, aligning with findings from Sahlen et al (2019).[14] Although the region is usually ignored for discrimination since the C-C and C-H groups is common among all of organic compounds, subtle differences in its intensity and shape suggest variations in composition and processing. Both the wildflower and unfiltered samples appear slightly broader (2946 to 2999 cm^{-1}) and less intense compared to the other two samples. This suggests a slightly different distribution of sugar molecules or the presence of additional organic compounds such as pollen, wax, or other minor components, which could contribute to spectral broadening. The pure honey on the other hand, has a less broader peak and a lower intensity as that of the clover honey that shows a slightly more intense peak**,** indicating a more defined hydrocarbon structure. This may be related to a higher proportion of certain carbohydrates or a more refined composition due to filtration.

The C=O stretching, indicated by a peak at 1653 cm^{-1}, may suggest the presence of carbonyl groups, which can be linked to aldehyde in glucose and ketones in fructose that influence the flavor and aroma profile of honey.[11] The region 1540-1175 cm^{-1} is the results of deformations of O-H, C-O, C-H and C-C in the carbohydrate structure.[15] C-H bending vibrations were reflected in the peaks around 1415 cm^{-1}. These bending modes represent CH_2 and/or CH_3 groups, confirming the presence of aliphatic structures in

Table 1. ATR-FTIR peaks (cm^{-1}) observed on the four honey samples.

Wildflower	*Clover*	*Pure*	*Unfiltered*	*Vibrational Bands*
3278	3291	3271	3278	O-H stretching (hydroxyl group)
2937	2937	2937	2936	C-H stretching (aliphathic CH_2/CH_3)
1653	1654	1654	1654	C=O stretching (carbonyl groups)
1417	1417	1415	1416	C-H bending (CH_2 groups)
1350	1346	1346	1343	C-O stretching (carbohydrates)
1253	1254	1253	1252	C-O-C stretching (carbohydrates)
1032	1033	1033	1032	C-O stretching (primary alcohols)
917	918	918	918	C-H out-of-plane bending
865	865	865	865	ring vibrations (carbohydrates)
817	817	817	816	C-H out-of-plane bending (aromatic or sugars)
776	776	776	776	C-H out-of-plane bending (sugars)

honey. Slight variations in the peak positions may indicate subtle structural differences attributable to floral sources. Further investigation into the C-O stretching or deformation vibrations yielded results in the 1350 cm^{-1} range. This region highlights the presence of ether or alcohol functionalities, which are indicative of the carbohydrate structures that dominate honey's chemical profile.[14] In the lower wavenumber regions, peaks were maintained at 1250 cm^{-1}, corresponding to C-O stretching alongside O-H bending vibrations. This consistency demonstrates the complexity of the carbohydrate structures present in honey.[14]

The vibrations in the regions 1175-940 cm^{-1} are due to the C–OH group as well as the stretches C–C and C–O. while the region between 940 and 700 cm^{-1} is due to C–H bending and ring vibrations (mainly from carbohydrates).[15] The peaks at 1030 cm^{-1} further confirmed the presence of C-O stretching, reflecting a stable composition of sugar-related compounds across all honey samples. Additionally, peaks at 920 cm^{-1} showcased out-of-plane bending vibrations of C-H bonds, suggesting the possible orientation of aromatic compounds within the honey matrix. The C-H deformation peaks at 865 cm^{-1} were consistent across samples, reinforcing the notion that similar aliphatic structures are prevalent in various types of honey. Finally, peaks at 776 cm^{-1} signify C-H wagging vibrations, suggesting that, despite the differences in floral sources, the natural structural arrangement of honey remains consistent across the various samples analyzed.

The spectral data obtained from the ATR-FTIR analysis illustrate the utility and effectiveness of this technique in differentiating honey types based on their chemical signatures. By establishing the presence of unique functional groups and structural features, one can infer the authenticity and quality of honey products effectively.

Multivariate Analysis

Continuing from the analysis of the IR spectra, PCA and HCA were employed to further investigate the relationships among the different honey samples. The differentiation of pure honey from other samples based on chemical profiles has been explored in various studies. Principal Component Analysis (PCA) is a common method used to distinguish honey varieties by analyzing their chemical compositions. For instance, a study on Czech acacia and linden honey utilized PCA to assess volatile compound profiles and physico-chemical parameters, aiding in honey characterization and quality assessment.[16]

The PCA plot (Figure 3) of the FTIR-ATR region of 600-4000 cm^{-1} visually represents how the spectral data clusters, with PC1 accounting for 73.2% of the total variance and PC2 accounting for 23.7%. This delineation suggests that together, these two principal components explain a significant portion of the information contained within the spectral data. This high cumulative variance indicates that the majority of the compositional information is effectively represented by these two components. The PCA plot reveals distinct clustering patterns, with pure and clover honey forming a closely related group on one side, while wildflower and unfiltered honey clustered on the opposite side. These patterns align with the spectral observations, where pure and clover honey exhibited similar carbohydrate profiles, while wildflower and unfiltered honey showed more variation due to their multifloral origins and retained organic components. The clear separation of pure honey from the others further underscores its refined and standardized composition. The pure honey, positioned at a negative value on the PC1 axis, indicates a distinctly different chemical profile. This separation can imply that pure honey, possibly being more refined or standardized, lacks some of the complex floral attributes present in the more diverse samples. Such differentiation holds implications for honey quality assessments, as it positions pure honey as a potential benchmark for evaluating other types.

Contributions of specific spectral features to PC1 and PC2 were analyzed to better understand the observed clustering at different regions. Table 2 shows the quantitative loadings for PC1 and PC2 at the other different regions. The 2700-3100 cm^{-1} region gave the highest percentage which confirmed the difference observed in the 2953 cm^{-1} range. This is also in agreement with one study suggesting the region of 2400–4000 cm^{-1} of the FTIR spectra as the most significant for discrimination of thyme (monofloral) versus the multifloral honey.[17]

Further validation comes from the HCA dendrogram (Figure 4) of the full region (600-4000 cm^{-1}), which complements our findings from the PCA by illustrating the hierarchical relationships among the samples. The dendrogram distinctly shows how

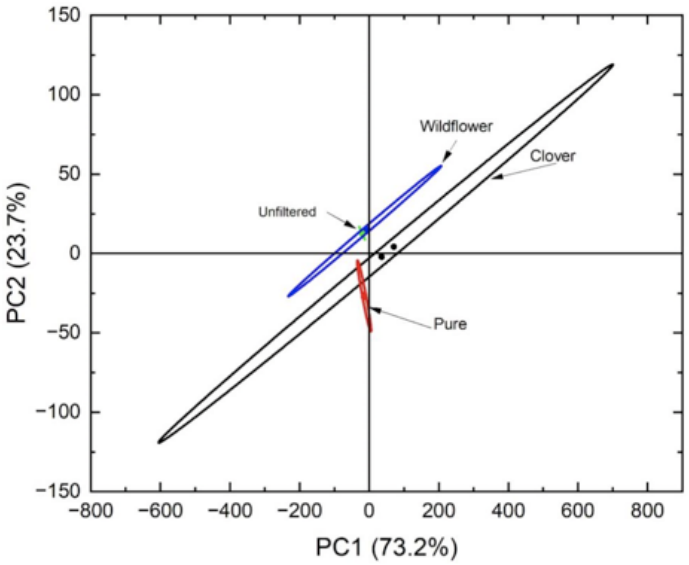

Figure 3. PCA plot of the different honey samples.

Table 2. PCA quantitative loadings at different regions.

Region	Sum (PC1 +P2) (%)
600-4000 cm^{-1}	96.9
2500- 4000 cm^{-1}	98.4
2700-3100 cm^{-1}	99.7
1000-1700 cm^{-1}	91.2
600-1700 cm^{-1}	85.8

closely related certain honeys are based on spectral similarities. If wildflower and clover honeys appear in the same cluster, it reinforces their spectral similarities observed in PCA, leading to an understanding that their chemical compositions are intertwined. The unfiltered honey's position in the dendrogram suggests that it shares attributes with both wildflower and clover while containing unique features likely due to its less refined nature. Conversely, if the pure honey is distinctly separated in the dendrogram, this reinforces the concept of its unique composition, further supporting the PCA conclusions about its role as a reference point within honey analysis.

The combination of PCA and HCA not only enhances our understanding of the chemical diversity among honey samples but also validates the efficiency of ATR-FTIR spectroscopy in these assessments. PCA has demonstrated sensitivity and specificity exceeding 87% for distinguishing botanical origins[18] and when paired with HCA successfully differentiated honey from various bee species, revealing closer compositional similarities between honey from *A. mellifera* and *A. dorsata* compared to *A. cerana* and *Trigona* sp.[11] The clear distinctions derived from these analyses provide a robust framework for distinguishing honey types based on their spectral data, offering invaluable insights for producers, regulators, and consumers concerned with honey authenticity and quality. Moving forward, continuous advancements in multivariate analysis techniques will support the broader application of these methodologies in the honey industry, aiding in quality control and the prevention of adulteration.

Conclusion

The ATR-FTIR spectroscopy analysis of the four honey samples demonstrated distinct chemical profiles, supporting the accuracy of their distinct labeling as different products. The variations in functional group presence and the limited overlap in principal components among the samples indicate that the differences are not superficial but reflect genuine compositional differences. This finding suggests that the honey flavors are not heavily diluted or artificially flavored to appear unique but instead possess unique chemical characteristics consistent with their advertised origins and names. These results reinforce the integrity of labeling practices for the brand, Urban Meadows and highlight the utility of IR spectroscopy in verifying the authenticity of food products.

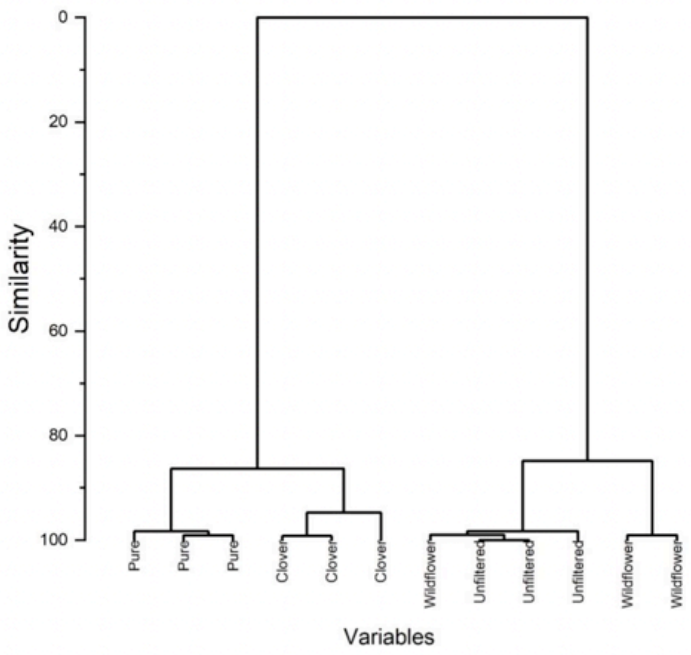

Figure 4. HCA dendrogram of different honey samples.

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