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Profiling of Australian Stingless Bee Honey Using Multivariate Data Analysis of High-Performance Thin-Layer Chromatography Fingerprints

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Abstract: The complex chemical composition of honey presents significant challenges for its analysis with variations influenced by factors such as botanical source, geographical location, bee species, harvest time, and storage conditions. This study aimed to employ high-performance thin-layer chromatography (HPTLC) fingerprinting, coupled with multivariate data analysis, to characterise the chemical profiles of Australian stingless bee honey samples from two distinct bee species, *Tetragonula carbonaria* and *Tetragonula hockingsi*. Using a mobile phase composed of toluene:ethyl acetate:formic acid (6:5:1) and two derivatisation reagents, vanillin–sulfuric acid and natural product reagent/PEG, HPTLC fingerprints were developed to reveal characteristic patterns within the samples. Multivariate data analysis was employed to explore the similarities in the fingerprints and identify underlying patterns. The results demonstrated that the chemical profiles were more closely related to harvest time rather than bee species, as samples collected within the same month clustered together. The quality of the clustering results was assessed using silhouette scores. The study highlights the value of combining HPTLC fingerprinting with multivariate data analysis to produce valuable data that can aid in blending strategies and the creation of reference standards for future quality control analyses.

Keywords: high-performance thin-layer chromatography (HPTLC); stingless bee honey; Australia; multivariate data analysis; fingerprint; chemometrics; quality control

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1. Introduction

Honey is a complex natural product, composed of a wide variety of compounds, that has been used in traditional medicine for thousands of years due to its range of biological activities, including antibacterial and antioxidant properties [1,2]. Among the various types of honey, Australian stingless bee honey, also known as sugarbag honey, holds a unique place with a rich history of medicinal use by the country's First Nations Peoples [3,4]. Despite this, research on the bioactive properties and chemical profile of Australian stingless bee honey remains limited particularly when compared to the much more extensively studied honeys produced by European honeybees (*Apis mellifera*) [5]. This

is a shortcoming since stingless bee products, attributed to the rich vegetation in tropical and subtropical regions where stingless bees are found, are often considered superior sources of biologically active compounds, such as phenolic constituents [6,7].

These compounds have been associated with the health-promoting properties of stingless bee honey, driving its increasing potential for use in the food, pharmaceutical, and cosmetic industries over the past two decades [7,8]. In addition to its unique properties, the collection of stingless bee honey appears to have some distinct advantages over honey derived from European honey bees. Stingless bees, as their common name suggests, do not sting and do not tend to abandon their nests (in contrast to European honey bees that can swarm). Due to their small size, they are also excellent pollinators for crops which have small flowers (e.g., macadamia) that are difficult to access by the much larger European honey bees and, possibly most importantly, they do not tend to be susceptible to common bee diseases that threaten European honey bee colonies around the world and that require regular pesticide treatments of their hives [6,9]. Despite these benefits, the production of stingless bee honey is still limited. This is not only because stingless bees produce significantly less honey compared to European honey bees [10] but also due to the absence of quality control standards and a lack of comprehensive research, both of which hinder its broader commercialisation [7,11].

Stingless bee honey is typically sold at significantly higher prices than honey derived from European honey bees, reflecting the limited supply but also growing demand for the product. Expanding stingless bee breeding could not only increase honey production but also foster honey industry in tropical regions, offering ecological, social, and economic benefits [7,11]. However, achieving these goals also requires the establishment of general quality standards for stingless bee honey to ensure product authenticity and to prevent fraudulent practices such as the sale of adulterated or lower-quality honey.

The current lack of standardisation of stingless bee honey presents a major challenge [7,8]. Establishing these standards is complicated by the fact that honey is a complex natural product with its composition varying widely due to factors such as bee species, floral sources, climate conditions or harvest time [12].

Given this complexity, non-targeted analytical approaches, such as generating chemical "fingerprints", are powerful tools for extracting detailed information from natural products like honey [13,14]. These approaches are, for example, powerful tools for ensuring food authenticity [15,16]. In addition, fingerprints also offer a representation of the distribution of chemical constituents in natural products. This is particularly valuable for honey, considering that its chemical composition can vary significantly due to factors such as botanical origin, bee species, geographical origin, seasonality, and storage conditions [17,18].

Chromatographic and spectroscopic techniques are frequently employed to generate fingerprints with chromatographic methods being especially useful for separating complex food mixtures and providing reliable qualitative and quantitative data [19,20]. While techniques such as gas chromatography and liquid chromatography coupled with mass spectrometry (GC/LC-MS) are well established, they often require extensive instrumentation and lengthy analysis times, making them less ideal for routine quality control [21,22]. High-performance thin-layer chromatography (HPTLC), by contrast, offers distinct advantages, including faster analysis time and lower costs due to the ability to simultaneously analyse a large number of samples on a single plate with minimal reagent and solvent input [22,23]. Additionally, HPTLC's advanced image recording systems and capacity to scan samples at multiple wavelengths make it particularly suitable for both qualitative and quantitative food analysis [15,23].

The large amount of data generated by HPTLC analysis requires the use of advanced data analysis techniques such as chemometrics to fully interpret obtained results. Various chemometric methods, including principal component analysis (PCA), hierarchical cluster analysis (HCA), k-nearest neighbours (k-NN), artificial neural network (ANN), Uniform Manifold Approximation and Projection (UMAP), and partial least squares-discriminant analysis (PLS-DA), are commonly employed to evaluate chromatographic fingerprints and reveal patterns in complex data sets [22].

To date, chemometrics analysis has been mainly used to confirm the geographic origin and authenticity of *Apis mellifera* honey [21,24–26]. However, to the best of our knowledge, this is the first paper that reports on HPTLC-generated fingerprints of Australian stingless bee honey and its profiling based on the chemometric analysis of these fingerprints. The combination of the richness of HPTLC-derived fingerprints with multivariate data analysis can provide valuable insights into honey samples with similar chemical profiles, facilitating the creation of blends for quality control. Analysing blends, rather than individual samples, offers several advantages, including reduced variability across samples, improved cost-effectiveness and the ability to generate reference samples in the absence of an absolute standard [27].

To demonstrate the usefulness of this approach and to generate valuable data that might support quality control efforts in the future, the aim of this study was to analyse HPTLC-derived fingerprints of 32 Australian stingless bee honey samples. Applying multivariate data analysis, the samples were profiled to support blending strategies for the preparation of reference standards for future quality control.

2. Materials and Methods

2.1. Chemicals and Reagents

The chemicals and reagents used in this study and their suppliers are as follows: vanillin (Sigma Aldrich, St. Louis, MO, USA); 2-aminoethyl diphenylborinate (Chem Supply, Port Adelaide, SA, Australia); toluene (APS Chemicals, Sydney, NSW, Australia); 4,5,7-trihydroxyflavanone (Alfa Aesar, Lancashire, UK); ethyl acetate, formic acid and sulfuric acid 98% (Ajax Finechem, Wollongong, NSW, Australia); polyethylene glycol 400 (PharmAust, Welshpool, WA, Australia); methanol (Merck KGaA, Darmstadt, Germany); ethanol (ChemSupply, Gillman, SA, Australia); dichloromethane (Merck KGaA, Darmstadt, Germany); and HPTLC calibration mix (UHM) (Sigma Aldrich, p/n 91816).

Silica gel 60 F254 HPTLC glass plates (20 cm × 10 cm) were obtained from Merck KGaA (Darmstadt, Germany).

2.2. Stingless Bee Honey Samples

For this study, thirty-two stingless bee honey samples from two of the most cultivated bee species, *Tetragonula carbonaria* (n = 26) and *Tetragonula hockingsi* (n = 6), were sourced directly from a local beekeeper in Burpengary East, Queensland, Australia (Table 1). Samples were collected in May (n = 12), September (n = 10), and November (n = 10) 2022. All samples were stored at 4 °C until further analysis.

Table 1. Stingless bee honey samples.

Bee Species	Sample	Harvest Date
Tetragonula carbonaria	TC-01 TC-02 TC-03 TC-04	May 2022

Table 1. *Cont.*

Bee Species	Sample	Harvest Date
	TC-05	
	TC-06	
	TC-07	
	TC-08	
	TC-09	
	TC-10	
Tetragonula hockingsi	TH-01	May 2022
	TH-02	
Tetragonula carbonaria	TC-13	September 2022
	TC-14	
	TC-15	
	TC-16	
	TC-17	
	TC-18	
	TC-19	
	TC-20	
Tetragonula hockingsi	TH-05	September 2022
	TH-06	
Tetragonula carbonaria	TC-21	November 2022
	TC-22	
	TC-23	
	TC-24	
	TC-25	
	TC-26	
	TC-27	
	TC-28	
Tetragonula hockingsi	TH-07	November 2022
	TH-08	

2.3. HPTLC Fingerprinting

High-performance thin-layer chromatography (HPTLC) was used to obtain fingerprints of organic extracts of the honey samples. For the organic extraction, approximately 1 g of each honey was dissolved in 2 mL of deionised water. The aqueous solution was then extracted three times with 5 mL of dichloromethane. The combined organic extracts were evaporated at 35 °C and stored at 4 °C until further analysis. Before high-performance thin-layer chromatography (HPTLC) analysis, the organic extracts were reconstituted with 100 µL of dichloromethane. A methanolic solution of 4,5,7-trihydroxyflavanone (0.5 mg/mL) and the HPTLC calibration mix (UHM) were used as reference standards.

The mobile phase was composed of a mixture of toluene, ethyl acetate, and formic acid at a ratio of 6:5:1 (*v*/*v*/*v*) [28]. Vanillin spraying reagent (VSA) was prepared by dissolving 1 g of vanillin in 100 mL of 96% ethanol, which was followed by the gradual addition of 2 mL of 98% sulfuric acid. Natural product spraying reagent (NP) was prepared by dissolving 1 g of 2-aminoethyl diphenylborinate in 100 mL of methanol. Polyethylene glycol (PEG) solution was prepared by dissolving 5 g of PEG 400 in 100 mL of 96% ethanol.

The reference standard (4 µL) and the organic extracts of the honey samples (5 µL) were applied as 8 mm bands at 8 mm from the lower edge of the HPTLC plate (glass plates 20 × 10 cm, silica gel 60 F254) at a rate of 150 nLs^{-1} using a semi-automated HPTLC application device (Linomat 5, CAMAG, Muttenz, Switzerland). The chromatographic separation was performed in a saturated and activated (33% relative humidity) automated development chamber (ADC2, CAMAG, Muttenz, Switzerland). Samples were developed to a distance of 70 mm at ambient temperature. The obtained chromatographic results

were documented using an HPTLC imaging device (TLC Visualiser 2, CAMAG, Muttenz, Switzerland) under 254 nm, 366 nm, and white light.

Following initial documentation of the chromatographic results, each plate was derivatised using either the vanillin/sulfuric acid (VSA) reagent or natural product/PEG (NP-PEG) reagent. For the NP-PEG derivatisation, plates were first sprayed with 3 mL of 1% NP reagent using a green nozzle at level 3 and then dried for 5 min at 40 °C using a TLC Plate Heater III (CAMAG, Muttenz, Switzerland). The plates were then sprayed again, this time with 5% PEG reagent using a blue nozzle at level 2, dried for 5 min at 40 °C, and the resulting image was captured at 366 nm. To derivatise using the VSA reagent, plates were sprayed with 3 mL of 1% vanillin sulfuric acid reagent using a yellow nozzle at level 3, then heated for 3 min at 115 °C using the TLC Plate Heater III, and after cooling for 2 min, the plates were visualised at 366 nm and white light. The chromatographic images were digitally processed and analysed using a specialised HPTLC software (visionCATS v3.1, CAMAG, Muttenz, Switzerland).

2.4. Multivariate Data Analysis

2.4.1. Data Pre-Processing

The initial output from the HPTLC samples consisted of RGB values and absorbance units (AU) for each sample at the respective wavelengths. The retardation factor (R_F) values were standardised based on the HPTLC calibration mix track, which was obtained after development at 366 nm. The R_F values across all samples were constrained to a range between 0.100 and 0.805, covering most of the detected bands.

The raw data were stored in CSV format, containing the RGB and AU values for each sample. These CSV files were processed and converted into a single HDF5 file containing the data for all the samples analysed for efficient storage and subsequent analysis. The HDF5 format was chosen for its ability to store large datasets while allowing data to be quickly read using the free available software Python™ (v. 3.12), facilitating efficient data handling and analysis.

2.4.2. Multivariate Analysis Approach

Multivariate data analysis was conducted to discern patterns and relationships within the dataset. Initially, a simpler approach was applied by performing K-means clustering using R and R Studio (v. 2024.04.1-748). This was followed by applying a more sophisticated method, Uniform Manifold Approximation and Projection (UMAP) [29] for dimension reduction and clustering of the high-dimensional HPTLC data. Finally, the Plotly (v.32.0) tool was used to create 3D plots to enhance visualisation. The silhouette score was employed as a metric to assess the quality of the clusters created.

3. Results and Discussion

3.1. HPTLC Fingerprints

The separation and analysis of honey constituents presents significant challenges due to its complex chemical composition, which can vary widely based on factors such as botanical source, geographical location, bee species, harvest time, and storage conditions [30,31]. Consequently, the complete composition of honey remains largely unexplored, particularly so in the case of stingless bee honeys. Fingerprinting methods capture the richness of chemical constituents and therefore can serve as the basis for a comprehensive characterisation of stingless bee honey samples.

Two commonly used derivatisation reagents were selected for the HPTLC analysis. The NP/PEG system is primarily employed for detecting flavonoids, while vanillin–sulfuric

acid (VSA) is mainly used to identify a wide range of lipophilic compounds including terpenoids, sterols and alkaloids [32].

While the resulting chromatograms provide two-dimensional data (Rf vs. intensity), the HPTLC generated fingerprint images offer greater detail, as the bands vary in colour in each chosen visualisation condition prior to and also after derivatisation, which can be recorded numerically in the form of RGB values. When displayed side by side, the HPTLC fingerprints allow for the visual assessment of both unique characteristics and shared features.

Figure 1 shows exemplary HPTLC images obtained at 254 nm (Figure 1a) and 366 nm (Figure 1b) after development with the mobile phase, and at 366 nm (Figure 1c) and white light (Figure 1d) after derivatisation with VSA, as well as at 366 nm (Figure 1e) after derivatisation with NP/PEG. Visual examination of the HPTLC fingerprints revealed both similarities and differences in chemical composition among the honey samples. Several bands were observed between Rf 0.085 and 0.805, indicating the presence of a range of (most likely phenolic) compounds. Table 2 highlights some of the main bands found. Images of the fingerprints of all samples can be found in the Supplementary Material (Figure S1).

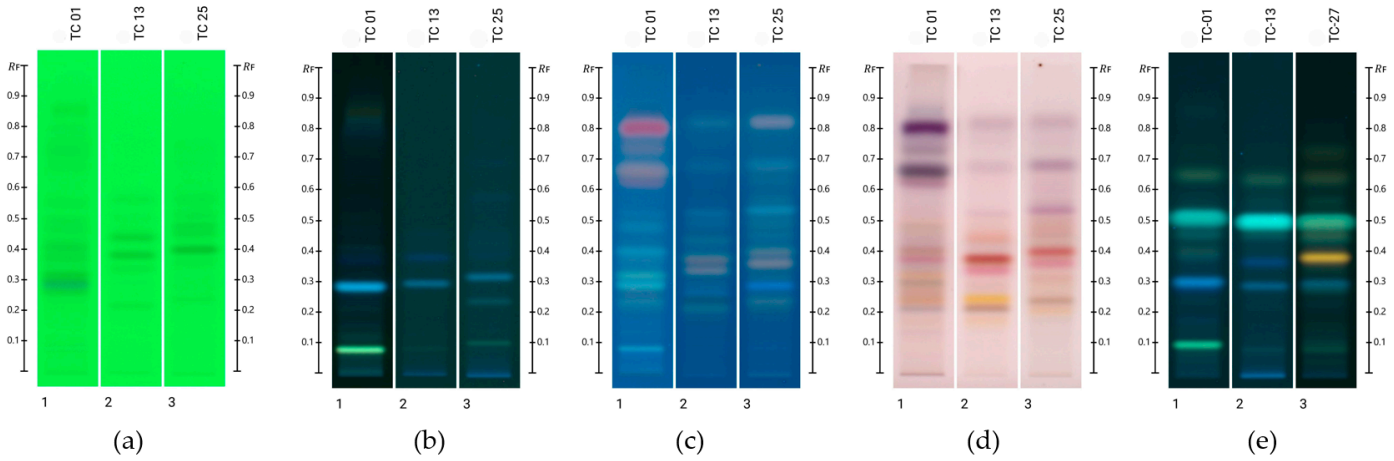

Figure 1. Images of HPTLC fingerprints taken at (**a**) 254 nm after development; (**b**) 366 nm after development; (**c**) 366 nm after derivatisation with VSA; (**d**) white light after derivatisation with VSA; (**e**) 366 nm after derivatisation with NP/PEG. Track 1—sample TC-01; track 2—sample TC-13; track 3—sample TC-25.

Table 2. Main bands found in Australian stingless bee honey samples.

Rf	Colour	Visualisation	Spray Reagent
0.259		254 nm	NA (after development)
0.290		254 nm	NA (after development)
0.389		254 nm	NA (after development)
0.443		254 nm	NA (after development)
0.720		254 nm	NA (after development)
0.104		366 nm	NA (after development)
0.292		366 nm	NA (after development)
0.213		White light	VSA
0.251		White light	VSA
0.341		White light	VSA

Table 2. *Cont.*

Rf	Colour	Visualisation	Spray Reagent
0.384		White light	VSA
0.677		White light	VSA
0.082		366 nm	VSA
0.292		366 nm	VSA
0.328		366 nm	VSA
0.341		366 nm	VSA
0.407		366 nm	VSA
0.664		366 nm	VSA
0.100		366 nm	NP-PEG
0.302		366 nm	NP-PEG
0.371		366 nm	NP-PEG
0.384		366 nm	NP-PEG
0.394		366 nm	NP-PEG
0.501		366 nm	NP-PEG
0.651		366 nm	NP-PEG

3.2. Multivariate Data Analysis

As previously mentioned, honey composition can vary widely, challenging its quality control. Many quality assurance methods for natural products, including honey, rely on the qualitative and/or quantitative analysis of specific marker compounds [33]. However, standardising these marker compounds presents challenges, especially when the bioactivity and sensory qualities of a natural product cannot be attributed to one or a few compounds [34] but are instead the result of a complex interaction among various constituents [35] or when key constituents have not yet been chemically identified [36]. Therefore, the use of profile chromatograms, which capture the typical phytochemical composition of a natural product for comparison purposes, has become a common approach to support the quality control of natural products and food items [36–38]. However, the determination of what constitutes a suitable reference standard for comparative purposes remains a challenge. Since the chromatographic profile of a single sample might not accurately represent the typical chemical composition, pooling multiple samples to obtain a chromatographic profile which is more representative of common features see across samples may provide a more accurate evaluation [39].

In this approach, multiple samples that are considered representative of the natural product extract are combined to create a pooled reference sample. While individual samples in the blend may vary, the overall profile from this pooled reference sample and its associated chromatogram reflects the extract's typical phytochemical characteristics. Pooling minimises the impact of uncommon constituents while amplifying those constituents that are common across samples, supporting the effective quality control of complex natural products. However, a challenge remains in selecting appropriate samples for inclusion in this pooled reference sample [27]. In this study, multivariate data analysis of HPTLC-generated fingerprints was therefore explored for its suitability to assist sample pooling using Australian native bee honey as an example for a complex natural product.

To identify which honey samples to pool, multivariate data analysis was performed on the R_F values, RGB data, and absorbance units, enabling the clustering of samples based on their HPTLC-generated fingerprints. Cluster analysis, a key technique for classifying data, was employed to identify groups within the dataset based on similarities within

clusters and dissimilarities between them [40]. K-means clustering was initially used because it is one the most commonly used and simplest unsupervised learning algorithms for cluster analysis. The algorithm starts by setting a target number of clusters (k), which represents the number of centroids to be determined [40]. This point-based method begins by randomly placing cluster centres and then iteratively adjusts them to minimise the clustering error [41].

The initial hypothesis was that the bee species involved in honey production would significantly influence the HPTLC fingerprints with samples from the same species clustering together. However, as shown in Figure 2, when the number of clusters was set to two, species did not appear to be a defining factor, as samples from different species were grouped together. Nevertheless, it was observed that cluster 1 consisted entirely of samples harvested in September and November, while cluster 2 contained only samples harvested in May.

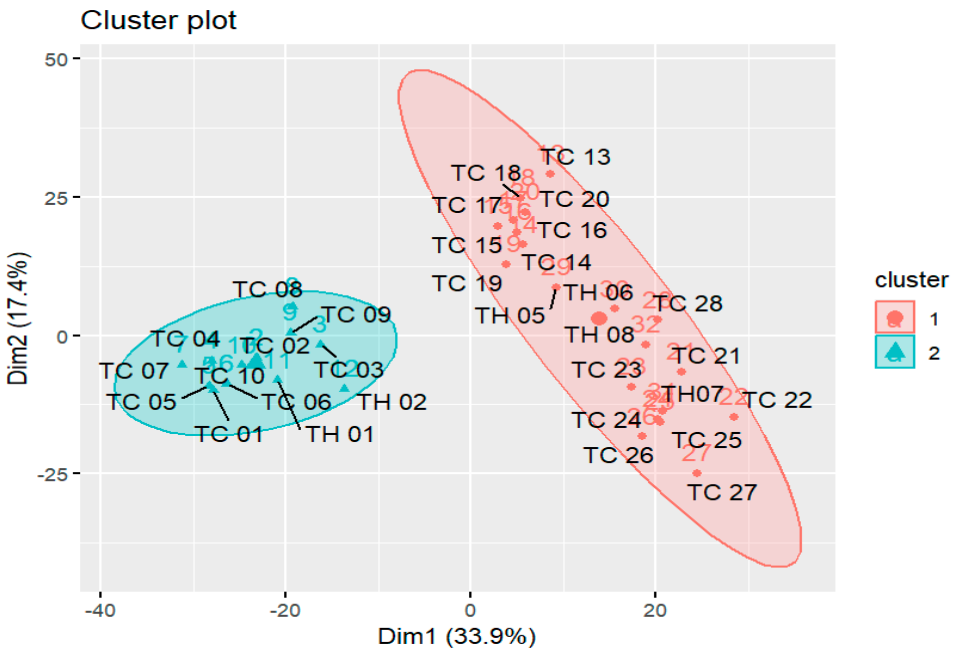

Figure 2. K-means clustering plot (k = 2).

The K-means analysis was then performed again with the number of clusters set to three. In this case, three distinct clusters emerged which grouped samples mostly according to their harvest month; thus, it could be concluded that the chemical profiles of the honey samples were found to be more strongly influenced by the time of harvest rather than by bee species (Figure 3). This finding is consistent with previous studies: research on honey from Croatia demonstrated that the beekeeping season significantly influences its elemental composition [42]. Similarly, a study on stingless bee honey from Malaysia (*H. itama* and *T. binghami*) revealed that harvest time had a notable impact on its physicochemical properties, antioxidant activity, and antimicrobial effects [43]. Additionally, a study on Italian honeys reported that seasonality plays a major role in the diversity of pollen collected by honeybees, which directly leads to variations in honey composition [44].

Uniform Manifold Approximation and Projection (UMAP) [29] was also used for dimension reduction and clustering of the high-dimensional HPTLC data generated with both spraying systems. UMAP was chosen for its ability to preserve both local and global data structures while reducing dimensions, making it a powerful tool for visualising complex relationships in datasets.

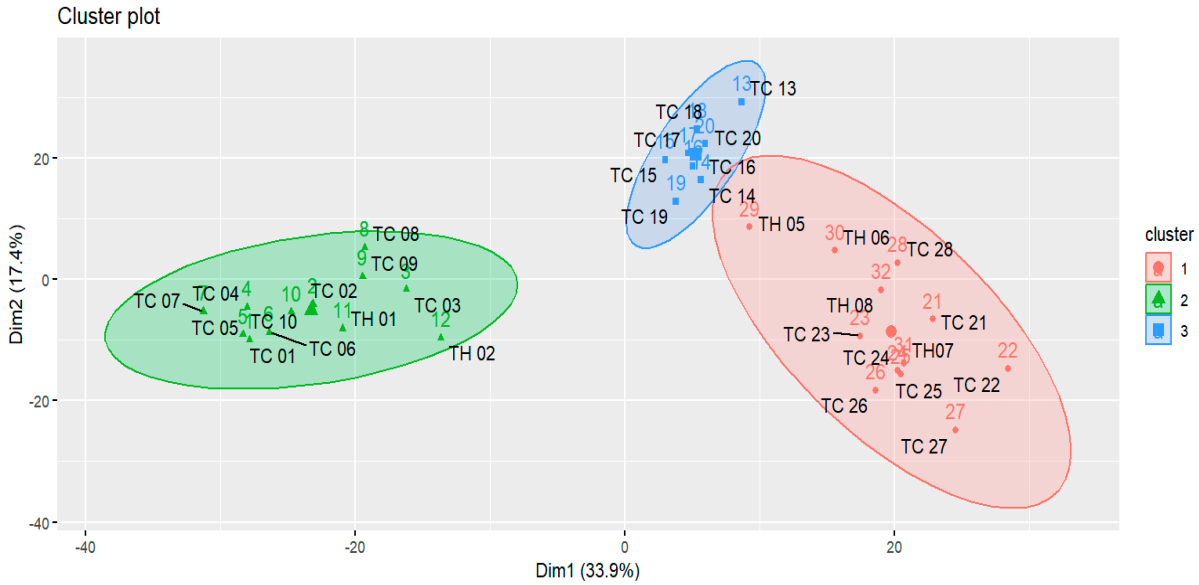

Figure 3. K-means clustering plot (k = 3).

UMAP's ability to maintain both the local and global topological structure of the data allowed for the clear identification of clusters, revealing natural groupings within the honey samples. This provided a robust visualisation of how different honey samples were related or distinct from one another. UMAP was particularly effective in distinguishing subtle differences in chemical composition that may not have been as discernible using other methods.

Compared to basic K-means analysis, this approach allowed for a more comprehensive exploration of the relationships between samples by enabling rotation and zooming to examine the data from different perspectives. The interactive nature of the 3D plots, along with adjustable visual parameters such as colour scales and point size, facilitated a detailed and dynamic interpretation of the UMAP clustering results.

Figure 4 shows the UMAPs generated from the fingerprints sprayed with NP-PEG (a) and VSA (b). The dynamic 3D plots are included in the Supplementary Material Link S1.

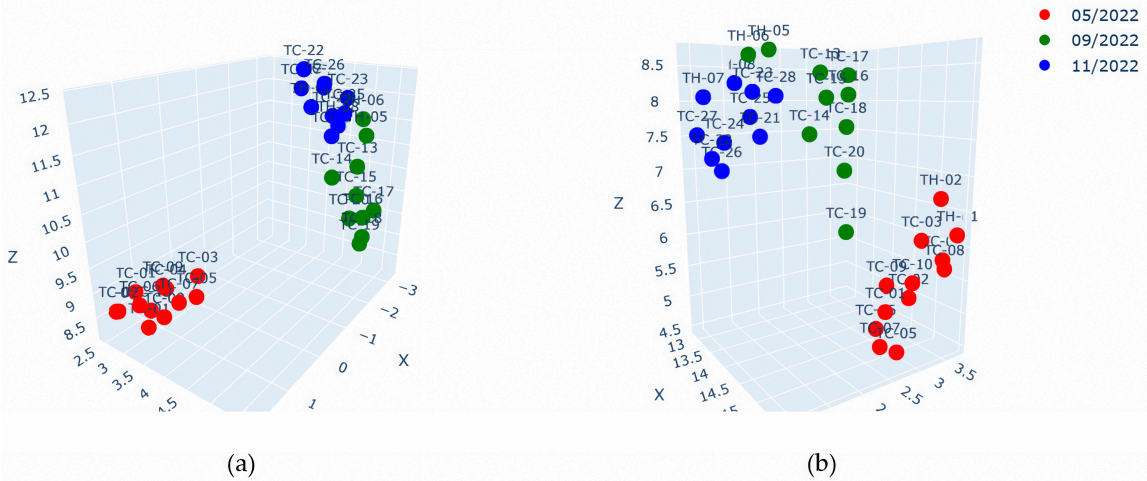

Figure 4. UMAPs of HPTLC fingerprints sprayed with (**a**) NP-PEG and (**b**) VSA. Honey samples harvested in May (red), September (green) and November (blue).

The 3D plots revealed the same consistent pattern that had already been revealed in the K-means analysis with most samples from the same harvest month clustering together.

Notably, the clusters formed by fingerprints sprayed with NP-PEG were tighter, likely due to the points sharing more common features, indicating a stronger relationship among them.

A silhouette score was employed as a validation metric to assess the quality of the clustering results. The silhouette score is a metric used to evaluate the quality of a clustering model by measuring how similar an object is to its own cluster compared to other clusters. It ranges from -1 to 1, where values closer to 1 indicate well-defined clusters, values around 0 suggest overlapping clusters and negative values suggest poor clustering, where points may have been assigned to incorrect clusters [45].

This study applied K-means clustering with the number of clusters set to two and three. For two clusters, the silhouette score was 0.48, indicating well-separated clusters. For three clusters, the silhouette score slightly decreased to 0.44, but this was still indicating good clustering. The lower score might be attributed to the proximity of the harvest dates, particularly in September and November, when it is possible that the same flora present in September remained available to the bees in November. The UMAP scores, NP-PEG = 0.44, VSA = 0.49, show the harvest time-based clusters are well-defined clusters, which aligns with the findings from the K-means clustering and further underscores the influence of harvest time over bee species. The scores show that the clusters have strong validity.

Therefore, the multivariate data analysis indicates that to create an accurate reference standard for stingless bee honey samples from the Burpengary East region, it is essential to pool samples collected in the same month. This combined sample will effectively capture the key characteristics and natural variations of the product at different times throughout the year, acknowledging the changing composition of this complex natural product. Such blends can then be utilised for quality control purposes or for further in-depth analysis to identify compounds of interest that can serve either as marker compounds or that contribute significantly to the honey's bioactivity.

Additionally, this approach has potentially practical implications for the honey industry, particularly in detecting honey adulteration and preventing fraud. Since honey is subject to natural variations due to factors such as geographical origin and seasonality, assessing its authenticity can be challenging. The proposed method addresses these variations by creating pooled reference samples that more accurately reflect the composition of honey from a specific area and season. As a result, evaluating profile chromatograms from these pooled reference samples ensures reliable authentication. While this method was applied to stingless bee honey from Burpengary East in Queensland, Australia, it is also applicable for preparing pooled reference samples for other types of honey.

4. Conclusions

The significant variability in honey composition highlights the complexities involved in developing effective quality assurance methods for natural products. Quality control using chromatographic fingerprints often relies on pooled samples to represent the typical chemical profile of a specific sample. However, determining which samples have sufficiently similar fingerprints to be grouped together as a pooled sample remains a challenge. This study employed multivariate data analysis to interpret the HPTLC fingerprints of stingless bee honey samples from the Burpengary East region of Australia. Contrary to initial assumptions, the findings revealed that the time of harvest has a far greater impact on the chromatographic profile than the bee species involved in the production of the honeys. These results provide valuable insights for future research into the quality control of stingless bee honey samples from this region and might also serve as the basis for future more in-depth studies aiming to identify key constituents that can serve as marker compounds or contribute significantly to the honey's bioactivity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/chemosensors13020030/s1, Figure S1: Images of HPTLC fingerprints taken at (a) 254 nm after development; (b) 366 nm after development; (c) 366 nm after derivatisation with VSA; (d) white light after derivatisation with VSA; (e) 366 nm after derivatisation with NP/PEG; Link S1: Dynamic 3D plots (UMAPs) of HPTLC fingerprints sprayed with (a) VSA and (b) NP-PEG.

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