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MULTI-TRACE METALS DETERMINATION OF PENINSULAR MALAYSIA STINGLESS BEE HONEYS FROM DIFFERENT REGIONS AND SEASONS USING INDUCTIVELY COUPLED PLASMA-OPTICAL EMISSION SPECTROMETRY AND CHEMOMETRIC TECHNIQUES

(Penentuan Logam Pelbagai Surih Madu Lebah Tanpa Sengatan dari Pelbagai Rantau dan Musim Berbeza Menggunakan Plasma Gandingan Teraruh-Spektrometri Pancaran Optik dan Teknik Kemometrik)

Syazwani Dzolin[1], Wan Aini Wan Ibrahim[1*], Naji Arafat Mahat[1,2*], Dzulkiflee Ismail[3] and Aidil Fahmi Shadan[4]

[1]Department of Chemistry,
Faculty of Science,
Universiti Teknologi Malaysia,
81310 UTM, Johor Bahru, Johor, Malaysia
[2]Centre for Sustainable Nanomaterials,
Ibnu Sina Institute for Scientific and Industrial Research,
Universiti Teknologi Malaysia,
81310 UTM, Johor Bahru, Johor, Malaysia
[3]School of Health Sciences,
Universiti Sains Malaysia,
16150 Kubang Kerian, Kelantan, Malaysia
[4]Department of Chemistry Malaysia,
Jalan Sultan, 46661 Petaling Jaya, Selangor, Malaysia

[*]Corresponding author: waini@utm.my, wawi4476@gmail.com

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Abstract

Demand for authenticating and distinguishing the provenance of bee honey among consumers is continuously rising. Considering vast variations in prices attributable to different geographical origins, suitable indication on labeling of honey products is important for consumer protection. Since concentrations of trace metals can be closely related to geographical origin, the use of spectroscopy and chemometric techniques for ascertaining the validity of such claim may prove relevant. In this study, multi-trace metals in stingless bee honey of *Heterotrigona itama* species, from different regions and seasons (rainy and dry) in Peninsular Malaysia (Kedah, Johor, Selangor and Pahang) were determined *via* inductively coupled plasma-optical emission spectrometry (ICP-OES). Principal component analysis (PCA) was then applied to recognize the distribution patterns. Subsequently, linear discriminant analysis (LDA) was applied to perform further classification. With the use of LDA, cross-validation was found as 95.8% and 89.6% during rainy and dry seasons, respectively. Combination of ICP-OES data with PCA and LDA techniques has provided accurate classification of the Malaysian stingless bee honey samples according to their respective origins. This study provided some elemental information on distribution of stingless bee honey samples at spatial and temporal levels, and could be used as a reference for their provenance establishment and authenticity.

Keywords: honey, stingless bee, provenance, authenticity, spectroscopy, chemometric

Abstrak

Permintaan untuk mengesahkan dan membezakan asal usul madu lebah di kalangan pengguna terus meningkat. Mengambil kira variasi besar harga byang disebabkan oleh asal geografi yang berbeza, petunjuk yang sesuai pada pelabelan produk madu adalah penting untuk perlindungan pengguna. Oleh kerana kepekatan logam surih berkait rapat dengan asal geografi, penggunaan teknik spektroskopi dan kemometrik untuk menentukan kesahihan tuntutan tersebut mungkin terbukti relevan. Dalam kajian ini, logam pelbagai surih dalam madu lebah tanpa sengatan dari spesies *Heterotrigona itama*, dari pelbagai rantau dan musim (hujan dan kering) di Semenanjung Malaysia (Kedah, Johor, Selangor dan Pahang) ditentukan melalui plasma gandingan teraruh-spektrometri pancaran optik (ICP-OES). Analisis komponen utama (PCA) kemudian digunakan untuk mengenal corak taburan. Seterusnya, analisis diskriminan linear (LDA) telah digunakan untuk melaksanakan klasifikasi lanjutan. Dengan penggunaan LDA, pengesahan silang masing-masing adalah 95.8% dan 89.6% pada musim hujan dan kering. Gabungan data ICP-OES dengan teknik PCA dan LDA telah memberikan klasifikasi tepat sampel madu lebah tanpa sengatan Malaysia mengikut asal usul masing-masing. Kajian ini memberikan beberapa maklumat unsur mengenai taburan sampel madu lebah tanpa sengatan pada tahap spasial dan temporal, dan dapat digunakan sebagai rujukan untuk asal usul dan keasliannya.

Kata kunci: madu, lebah tanpa sengatan, asal usul, ketulenan, spektroskopi, kemometrik

Introduction

Honey is the oldest natural sweetening agent with various nutritional and therapeutic values [1]. Being a natural product, composition of honey is diverse, ranging from sugars, flavor compounds, amino acids, enzymes, flavonoids, gluconic acid, lactone, nitrogenous compounds, proteins, vitamins (vitamin B6, thiamine, niacin, riboflavin and pantothenic acid) [2 - 5] phenols, pigments as well as multi-trace metals [2, 6, 7]. Modern researchers have reported several honey biological activities such as antioxidant activity [8], decreases factors of cardiovascular risk [9] and improve resistance against pathogenic organisms [10].

To date, consumer demands for genuine honey of high qualities are continuously increasing. Since the beginning of honey trade, negative perceptions concerning processed honey products (adulteration) are also growing. Honey adulteration is the major problem influencing the overall perception of honey in terms of quality, price and human health (potential genotoxic, cytotoxic and carcinogenic effects of hydroxymethylfurfural) [11]. According to [1], two major factors related to honey authenticity; (i) origin of the honey (geographical and botanical origins) and (ii) mode of honey production (harvesting and processing techniques). Since years ago, internationally, the Codex Alimentarius have developed the authenticity standard of honey by providing the essentials of identity and quality of honey requirements for human consumption. This standard can be applied to all types of honey produced by bees and it covers all types of honey conditions [12].

Recently in Malaysia, Department of Standards Malaysia have developed a standard for *Kelulut* (Stingless bee) honey (MS 2683:2017) in 2017 [13]. In this Malaysian standard, it covered the quality requirements, sampling methods, sample preparation, hygiene, packaging as well as labeling of stingless bee honey for direct human consumption. Besides being great pollinators in ecosystem, stingless bees able to modify floral nectars into distinctive aroma and flavor of honey [14] that is favorable by local consumers.

Honey produced by stingless bee species especially by Meliponinae is appreciated by consumers worldwide [14] including Malaysia. Meliponinae was reported to have distinct characteristics compared to other bee species such as absence of sting, short foraging distance and build hives in horizontal position to store nectar and pollen [15, 16]. Due to the insufficient knowledge of Malaysian stingless bee honey composition, there is a shortage of identity of its geographical origin especially related to the present of multi-trace metals signature. As reported by [2], multi-trace metals content is important as it much dependent on the soil and the environment of plants where the stingless bees collect nectar in the production of honey. In addition, anthropogenic activities such as agricultural practices and industrial can also influence the presence of multi-trace metals in honey [17]. Atanassova et al. [18] reported that during different vegetation season, honeys could exhibit different multi-trace metals concentrations even though it comes from same geographical region, same beehive and same locality.

The assessment of multi-trace metals concentrations can be useful in discriminating honey based on their geographical origin [19]. Few authors have begun to apply analytical approaches such as flame atomic absorption spectroscopy (FAAS) [20, 21], graphite furnace atomic absorption spectrometry (GFAAS) [22], inductively coupled plasma-mass spectrometry (ICP-MS) and inductively coupled plasma-atomic emission spectrometry (ICP-AES) [23] and inductively coupled plasma-optical emission spectrometry (ICP-OES) [24, 25] to determine the presence of multi-trace metals in honey samples.

In Malaysia, studies on multi-trace metals analysis have been performed by Moniruzzaman et al. [22] and Chua et al. in 2012 [23] on various type of Malaysian bee honey but none of the studies reported on the honey provenance establishment of stingless bee species. Shadan et al. [24] reported for the first time on the provenance establishment of stingless bee honey from five districts of Johor region (Segamat, Muar, Batu Pahat, Kota Tinggi and Johor Bahru). Their study focuses on the provenance establishment of the honeys using chemometric techniques namely principal component analysis (PCA) and linear discriminant analysis (LDA). The multi-trace metals profile detected the present of Ag, Al, As, B, Ba, Be, Cd, Co, Cr, Cu, Fe, Mg, Mn, Mo, Ni, Pb, Sb, Se, Sn and Zn. Despite its natural occurrence in most regions of Malaysia, there is still a shortage of studies on the geographical origin of stingless bee honey. The identity and quality standards of the honey are difficult to ascertain.

The incorporation of chemometric techniques into spectroscopy methods is an effective method for identifying minor and major compounds in honey that can serve as unique signatures for its botanical or geographical origin [26]. Therefore, the aims of our study were: (i) to determine eighteen multi-trace metals in stingless bee (*Heterotrigona itama*) honey samples collected from four regions of Peninsular Malaysia, namely Kedah, Johor, Selangor, and Pahang during two different seasons (rainy and dry); and (ii) to classify stingless bee honey samples by regions of each season based on their multi-trace metal concentrations using ICP-OES in combination with PCA and LDA techniques.

Materials and Methods

Instrument and apparatus

The analysis was performed using ICP-OES, Perkin Elmer (PE 4300 DV) model (Shelton, USA), located at the Department of Chemistry, Petaling Jaya, Selangor. The operating conditions for determining multi-trace metals in stingless bee honey samples were as follows: radio frequency power (0.7-1.5 kW; 1.2-1.3 kW for axial), Ar plasma gas flow (radial: 10.5-15 L/min; axial: 15 L/min), Ar auxiliary gas flow rate (1.5 L/min) and viewing height (5-12 mm).

Reagents

Analytical grade HNO_3 (65% v/v) was purchased from Merck KGaA, (Darmstadt, Germany). Ultra-pure water (resistivity, 18.2 MΩ cm^{-1}) was produced from a Milli-Q water purification system (Millipore, Bedford, MA, USA) and was used throughout the preparation of solutions. Multi-element standard solution was obtained from Agilent Technologies (Santa Clara, USA).

Sample collection, preparation and analysis

Honey samples from stingless bees (*Heterotrigona itama*) were harvested in November 2016 and February 2017, respectively, during rainy and less rainy seasons. At the beginning of this study, we decided to collect stingless bee honey samples throughout Peninsular Malaysia, but because of the limited number of beekeepers registered with the Malaysian Agricultural Research and Development Institute (MARDI), we are focusing only on the registered beekeepers from selected districts in this study. A total of thirty-two stingless bee honey samples were collected from the northern (Kedah: Padang Terap, Kulim, Yan and Baling), southern (Johor: Segamat, Johor Bahru, Pontian and Mersing), western (Selangor: Sabak Bernam, Klang, Kuala Selangor and Hulu Selangor) and eastern (Pahang: Jerantut, Rompin, Temerloh and Kuantan) regions of Peninsular Malaysia.

The honey samples were harvested directly from beekeepers during rainy and less rainy seasons, with the mean maximum temperature being 27.9℃ and 35.6℃, respectively. All the stingless bee honey samples were harvested without any treatment and kept in separate amber glass bottles at 12℃. To minimize the final acidity of honey samples after preparation, the acid mineralization method was performed, as suggested by Chua et al. [23] with a few modifications. Approximately 1 g of each honey sample was digested and mineralized using 65% concentrated HNO_3 (10 mL) by heating the mixture till near to dryness. The mixture was then made up to a volume of 50 mL with deionized water (DI), filtered and kept in a glass bottle at 12℃ prior to analysis. Using the same sample preparation procedure, a blank control sample that contained 10 mL of HNO_3 (65% v/v) was used in the preparation of calibration and quality control samples. The varying concentrations of working solutions and quality control samples were freshly prepared *via* serial dilution from a stock solution containing 100 mg/L of multi-elements in HNO_3.

Chemometric approach dataset

The data set consisted of thirty-two stingless bee honey samples separated by geographical origins and eighteen multi-trace metals. The samples which came from the sixteen districts of the four regions were divided as follows:

1. Northern region: Padang Terap (PT), Kulim (KM), Yan (YN) and Baling (BG)
2. Southern region: Segamat (ST), Johor Bahru (JB), Pontian (PN) and Mersing (MG)

3. Western region: Sabak Bernam (SB), Klang (KL), Kuala Selangor (KS) and Hulu Selangor (HS)
4. Eastern region: Jerantut (JT), Rompin (RN), Temerloh (TH) and Kuantan (KN)

Upon completion of data entry in Microsoft Excel® spreadsheet (Microsoft Corporation, Washington, USA) and IBM® SPSS statistics 20.0 software, the data were exported into Minitab® 17 software (Minitab Inc., State College, PA).

Principal component analysis (PCA) and linear discriminant analysis (LDA)

For studying the organization of the overall dataset, PCA was utilized following the steps proposed by Shlens [27] viz. standardization of dataset, calculation of covariance matrix, obtaining the principal components (PCs) and data translation into components. Being the supervised statistical method, LDA categorically clusters objects into user-defined groups/clusters by calculating linear discriminant function to create a model that would enable correct classification of objects within a given dataset. For validating the developed model for a given dataset, the "leave-one-out" technique cross-validation approach was utilized.

Results and Discussion

Analytical parameters and method accuracy

Thirty-two stingless bee honey samples were analyzed using ICP-OES to determine their multi-trace metal concentrations (3 replicates). The limits of detection (LOD) and limit of quantitation (LOQ) for ICP-OES were estimated from blank analysis (7 replicates). The LOD and LOQ were calculated as 3SDblank/slope and 10SDblank/slope, respectively. The proposed method with matrix matching calibration used in this study is shown in Table 1.

Table 1. Wavelength, R^2, regression equation, LODs, LOQs and % RSD for multi-trace metals determination in stingless bee honey using ICP-OES

Metals	Wavelength (nm)	Coefficient of Determination (R^2)	Equation	Limit of Detection (mg/L)	Limit of Quantitation (mg/L)	Relative Standard Deviation (%)
Ag	328.068	0.9999	y = 1.0445x + 0.004	0.048	0.159	3.6
Al	396.153	0.9992	y = 1.0255x +0.0045	0.069	0.230	5.3
As	193.696	0.9999	y = 0.9681x + 0.008	0.024	0.103	1.8
Ba	455.403	0.9998	y = 1.0301x - 0.0008	0.063	0.212	4.9
Be	313.107	0.9991	y = 1.0175x - 0.0033	0.056	0.186	4.3
Cd	226.502	0.9998	y = 1.0132x - 0.0024	0.043	0.145	3.4
Co	228.616	0.9998	y = 1.0294x - 0.003	0.048	0.159	3.7
Cr	267.716	0.9997	y = 1.03x - 0.0027	0.049	0.164	3.8
Cu	327.393	0.9999	y = 1.0369x - 0.0002	0.040	0.135	3.1
Mn	257.61	0.9993	y = 1.0398x - 0.0023	0.066	0.220	5.1
Ni	231.604	0.9999	y = 1.0247x - 0.0034	0.046	0.154	3.6
Pb	220.353	0.9999	y = 1.0019x + 0.0019	0.021	0.071	1.6
Se	196.026	0.9998	y = 0.98x + 0.0049	0.028	0.094	2.1
Tl	190.801	0.9999	y = 0.9923x + 0.0012	0.025	0.085	2.0
Th	283.73	0.9997	y = 1.015x + 0.0011	0.044	0.148	3.4
U	385.958	0.9983	y = 0.94x - 0.021	0.060	0.199	4.8
V	292.402	0.9999	y = 1.0184x - 0.0034	0.050	0.165	3.8
Zn	213.857	0.9997	y = 1.0072x + 0.0011	0.044	0.148	3.4

Note: Silver (Ag), aluminum (Al), arsenic (As), barium (Ba), beryllium (Be), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), lead (Pb), selenium (Se), thallium (Th), thorium (Th), uranium (U), vanadium (V) and zinc (Zn).

Multi-trace metals concentrations in stingless bee honey samples

The preliminary findings for multi-trace metals analysis presented in this study demonstrated the potential for provenance establishment of Malaysian stingless bee honey samples. Concurrently, a total of eighteen multi-trace metals were determined after digestion process using ICP-OES. For these metals, some trace metals cannot be detected in some types of honey samples and some exhibited low or high concentration based on certain areas and regions. It may be because of the differences in soil composition, floral type, floral density, nectar or pollen [28 - 30]. Study by Pohl [31] suggested another factor which may contribute to the diversified trace metals content such as beekeeping practices, environmental pollution and honey processing. In an industrial area, toxic trace metals have been reported to be presented high concentrations in honeys [32]. According to Fatima et al. [33], the composition of Malaysian stingless bee honey from Trigona species varies according to geographical or botanical origin, as well as fruit or flower season. Therefore, these trace metals may give useful information in order to validate the geographical origin (provenance) of stingless bee honey samples.

Table 2 shows the most abundant metals (Al and Zn) obtained in stingless bee honey from four regions of Peninsular Malaysia. Bees are exposed to Al from various sources of their foraging activities for nectar as this element is reported to be abundant in Earth's crust [34]. In this study, the highest concentration of Al in stingless bee honey samples of rainy and dry seasons were 0.666 and 0.477 mg/L, respectively. The Al values obtained in this study was slightly similar to the study by Chua et al. [23] as they reported the range of Al concentration was between 0.708 to 1.872 mg/L in various type of honey from Malaysia. Previous research by Altun et al. [35] on honey samples from South and East regions of Turkey using ICP-MS found the highest concentration was 0.96 mg/L. In addition, Di Bella et al. [36] reported the highest concentration of Al was 5.67 mg/L in all honeys from Sicily and Calabria, Italy whereas Czipa et al. [37] found 4.39 mg/L of Al in Hungary honeys. According to Oroian et al. [38], due to neurological and cancer effects, Al is not essential to be consumed in high dosage.

The recommended daily consumption of Zn was 7.5 mg/kg and 13.5 mg/kg for children and adults, respectively [39]. In this study, the lowest and the highest Zn concentrations were less than 1 mg/L in all honeys. Since the highest concentration of Zn in this study was 0.549 mg/L, it showed Zn level was within the acceptable limits. Honeys from Hatay, Turkey also showed lower Zn concentration (0.237 mg/L) [35]. Conversely, other studies obtained high Zn concentration values in Anatolia honey (1.1-12.7 mg/L) [21] and Eastern Solvakia honey (1.3 mg/L) [17]. Chua et al. [23] reported Zn concentration of forest Malaysian honeys between 2.353 to 18.112 mg/L which is higher compared to our study.

Table 3 shows the low concentrations metals present in stingless bee honey. The content of Cr and Ni in this study (<1 mg/L) attributed to the uncontaminated environment as the main sources of livelihood is agriculture and not heavily industrialized. The Cr concentrations of our honey samples are similar to the Cr concentrations of Turkey honey (0.0024-0.0379 mg/L) [21], Eastern Solvakia honey (0.0268-0.0433 mg/L) [17] and Hatay honey (0.1-0.54 mg/L) [40]. In contrast, various type of honey samples from Malaysia possessed high Cr concentration of the range 1.8-3.8 mg/L as discussed by Chua et al. [23]. In this study, the highest concentration of Ni during rainy and dry seasons was 2.939 and 0.0406 mg/L, respectively whereas Chua et al. [23] obtained 0.084 mg/L of Ni from Malaysian forest honey sample. The mean Ni concentration of Argentine honey was 0.03 mg/L [41].

Copper (Cu) is an important element in eukaryotes aerobic respiration [42] and vital in formation of haemoglobin as well as in production of melanin for skin, hair and eyes pigmentation [38]. The mean range concentration of Cu in this study (0.015-0.128 mg/L) was slightly similar to the mean range (0.046-0.236 mg/L) of various honey samples as reported by Chua et al. [23]. The maximum and minimum concentration values for Mn exhibited 0.031 and 0.795 mg/L, respectively. These results showed lower values compared to results from Chua et al. [23]. However, our study was higher than Irish honey samples (0.004 mg/L) [43].

Selenium (Se) is a nutritious element for humans and it plays an important role in reproduction process, DNA synthesis and protection against infection [35]. In this study, the highest Se concentration determined in stingless bee honeys was 0.023 mg/L which was lower than Se concentration of Malaysian Tualang honey produced by sting bees (17.202 mg/L) [23]. However, our result exhibited slightly higher compared to Argentine honeys [41] and honeys from North East of Romania [38].

Table 2. Most abundant trace metals in stingless bee honey samples from four regions of Peninsular Malaysia

Season	**Region**	**Al (mg/L)**	**Zn (mg/L)**
Rainy	**Pahang (East)**		
	Mean ± SD	0.561 ± 0.0965	0.3483 ± 0.1817
	Range (min-max)	0.425 - 0.666	0.127 - 0.549
	Kedah (North)		
	Mean ± SD	0.3778 ± 0.0592	0.0839 ± 0.019
	Range (min-max)	0.278 - 0.42	0.057 - 0.104
	Selangor (West)		
	Mean ± SD	0.3614 ± 0.0207	0.1322 ± 0.0267
	Range (min-max)	0.331 - 0.388	0.093 - 0.16
	Johor (South)		
	Mean ± SD	0.3792 ± 0.017	0.0858 ± 0.0444
	Range (min-max)	0.36 - 0.405	0.052 - 0.159
Dry	**Pahang (East)**		
	Mean ± SD	0.1763 ± 0.0748	0.0381 ± 0.0104
	Range (min-max)	0.119 - 0.316	0.029 - 0.057
	Kedah (North)		
	Mean ± SD	0.2806 ± 0.1468	0.1064 ± 0.0433
	Range (min-max)	0.1068 - 0.447	0.068 - 0.1808
	Selangor (West)		
	Mean ± SD	0.1225 ± 0.0457	0.0633 ± 0.0181
	Range (min-max)	0.091 - 0.203	0.0397 - 0.0885
	Johor (South)		
	Mean ± SD	0.2318 ± 0.1683	0.0575 ± 0.0035
	Range (min-max)	0.063 - 0.477	0.052 - 0.0615

SD = standard deviation (n = 12). One-way ANOVA with Tukey's HSD post hoc test with significant value of 0.05 was used for determining the mean differences in the concentrations of multi-trace metals in stingless bee honey samples among the different regions. *The mean difference is significant at the 0.05 level.

Table 4 shows heavy metals concentrations obtained from Malaysian stingless bee honey samples. Excess heavy metals such as As, Cd, Co and Pb cause an excessive production of reactive oxygen species (ROS) causing oxidative stress [44]. According to Bogdanov et al. [45], Cd and Pb are considered as bio-indicators for contamination of honey. Pollutants from industries might contaminate soil and air. Cd is an element that passed from soil to plants and nectar whereas Pb contamination correlates with air pollution [17]. The lowest and the highest concentrations of As, Cd, Co and Pb were <1 mg/L in all stingless bee honey samples. The honey samples of this study are harvested directly from beekeepers that are located mostly far from the main road and the factories area. Moreover, our data for As, Cd, and Pb concentrations were within the recommended limits as stated in Malaysia Food Act 1983 (Regulation 38) and Food and Agriculture Organization (FAO) and World Health Organization (WHO) [46].

Table 3. Low concentrations metals in stingless bee honey samples from four regions of Peninsular Malaysia

Season	Region	Ba (mg/L)	Cr (mg/L)	Cu (mg/L)	Mn (mg/L)	Ni (mg/L)	Se (mg/L)
Rainy	**Pahang (East)**						
	Mean ± SD	0.0926 ± 0.0073	0.0604 ± 0.0018	0.0360 ± 0.0083	0.064 ± 0.0037	2.1903 ± 0.5129	0.0196 ± 0.0028
	Range (min-max)	0.081 - 0.1	0.058 - 0.063	0.028 - 0.047	0.043 - 0.121	1.542 - 2.939	0.013 - 0.023
	Kedah (North)						
	Mean ± SD	0.0869 ± 0.0076	0.0592 ± 0.0017	0.03375 ± 0.0128	0.0614 ± 0.0254	0.0355 ± 0.0052	0.0176 ± 0.0042
	Range (min-max)	0.074 - 0.094	0.056 - 0.061	0.023 - 0.055	0.034 - 0.093	0.031 - 0.043	0.011 - 0.022
	Selangor (West)						
	Mean ± SD	0.0768 ± 0.0078	0.0589 ± 0.0024	0.0373 ± 0.0077	0.0563 ± 0.0269	0.1468 ± 0.0074	0.0203 ± 0.0027
	Range (min-max)	0.064 - 0.084	0.055 - 0.062	0.026 - 0.046	0.032 - 0.097	0.133 - 0.153	0.016 - 0.025
	Johor (South)						
	Mean ± SD	0.0703 ± 0.0109	0.0565 ± 0.0031	0.0522 ± 0.0447	0.0715 ± 0.0614	0.0348 ± 0.0069	0.0114 ± 0.0029
	Range (min-max)	0.053 - 0.082	0.053 - 0.062	0.024 - 0.128	0.031 - 0.175	0.029 - 0.046	0.005 - 0.016
Dry	**Pahang (East)**						
	Mean ± SD	0.08 ± 0.0203	0.0626 ± 0.003	0.0454 ± 0.0278	0.2428 ± 0.3086	0.0322 ± 0.0009	0.0036 ± 0.0014
	Range (min-max)	0.066 - 0.117	0.059 - 0.068	0.018 - 0.093	0.059 - 0.795	0.031 - 0.034	0.002 - 0.007
	Kedah (North)						
	Mean ± SD	0.065 ± 0.0106	0.0982 ± 0.0582	0.0258 ± 0.0022	0.086 ± 0.0446	0.0339 ± 0.0025	0.0143 ± 0.0028
	Range (min-max)	0.057 - 0.0843	0.056 - 0.2001	0.023 - 0.0292	0.046 - 0.1615	0.03 - 0.0372	0.01 - 0.0189
	Selangor (West)						
	Mean ± SD	0.0709 ± 0.0058	0.0653 ± 0.0033	0.0416 ± 0.0152	0.0553 ± 0.0185	0.035 ± 0.0039	0.0097 ± 0.0060
	Range (min-max)	0.0646 - 0.081	0.061 - 0.071	0.0226 - 0.058	0.034 - 0.0818	0.031 - 0.0406	0.003 - 0.0212
	Johor (South)						
	Mean ± SD	0.0712 ± 0.0095	0.0581 ± 0.0044	0.0239 ± 0.0086	0.0551 ± 0.0124	0.0314 ± 0.0017	0.0087 ± 0.0065
	Range (min-max)	0.058 - 0.0842	0.0505 - 0.061	0.015 - 0.037	0.037 - 0.0664	0.0293 - 0.034	0.001 - 0.0189
[a]Trigona Type 1	Lenggong, Perak			ND			
[a]Trigona Type 2	Lenggong, Perak			ND			
[a]Trigona Type 3	Serdang, Selangor			2.4 ± 0.06			
[a]Trigona Type 4	Serdang, Selangor			2.93 ± 0.36			

SD = standard deviation (n = 12); ND = not detected. One-way ANOVA with Tukey's HSD post hoc test with significant value of 0.05 was used for determining the mean differences in the concentrations of multi-trace metals in stingless bee honey samples among the different regions. *The mean difference is significant at the 0.05 level. [a]Results are expressed in mg/kg [22].

Table 4. Heavy metals in stingless bee honey samples from four regions of Peninsular Malaysia

Season	Region	As (mg/L)	Cd (mg/L)	Co (mg/L)	Pb (mg/L)
Rainy	**Pahang (East)**				
	Mean ± SD	0.0092 ± 0.0037	0.003 ± 0	0.0128 ± 0.0014	0.008 ± 0.0041
	Range (min-max)	0.004 - 0.014	0.003	0.011 - 0.014	0.004 - 0.014
	Kedah (North)				
	Mean ± SD	0.0068 ± 0.0021	0.004 ± 0	0.004 ± 0	0.0048 ± 0.0019
	Range (min-max)	0.004 - 0.01	0.004	0.004	0.002 - 0.008
	Selangor (West)				
	Mean ± SD	0.0019 ± 0.0033	0.004 ± 0	0.005 ± 0	0.0067 ± 0.0036
	Range (min-max)	0.002 - 0.009	0.004	0.005	0.003 - 0.011
	Johor (South)				
	Mean ± SD	0.0052 ± 0.0054	0.004 ± 0	0.0042 ± 0.0004	0.0075 ± 0.0061
	Range (min-max)	0.001 - 0.016	0.004	0.004 - 0.005	0.002 - 0.018
Dry	**Pahang (East)**				
	Mean ± SD	0.0123 ± 0.0019	0.0089 ± 0.008	0.004 ± 0	0.0012 ± 0.0011
	Range (min-max)	0.01 - 0.016	0.004 - 0.023	0.004	0.001 - 0.003
	Kedah (North)				
	Mean ± SD	0.0139 ± 0.0034	0.0268 ± 0.0371	0.0052 ± 0.0007	0.0043 ± 0.0015
	Range (min-max)	0.008 - 0.0185	0.004 - 0.0913	0.004 - 0.0059	0.001 - 0.006
	Selangor (West)				
	Mean ± SD	0.0144 ± 0.0028	0.0046 ± 0.0007	0.0049 ± 0.0009	0.0035 ± 0.002
	Range (min-max)	0.0096 - 0.018	0.004 - 0.0058	0.004 - 0.0059	0.002 - 0.0065
	Johor (South)				
	Mean ± SD	0.0150 ± 0.0059	0.0042 ± 0.0004	0.0045 ± 0.0007	0.0036 ± 0.0016
	Range (min-max)	0.006 - 0.0024	0.004 - 0.0049	0.004 - 0.0056	0.001 - 0.006
[a]Trigona Type 1	Lenggong, Perak	0.043 ± 0.002	1.03 ± 0.25	ND	ND
[a]Trigona Type 2	Lenggong, Perak	0.027 ± 0.001	0.78 ± 0.04	ND	0.691 ± 0.002
[a]Trigona Type 3	Serdang, Selangor	0.043 ± 0.001	0.51 ± 0.01	ND	0.043 ± 0.001
[a]Trigona Type 4	Serdang, Selangor	0.052 ± 0.002	0.33 ± 0.04	ND	0.052 ± 0.002
[b]Food Act 1983 (Regulation 38)	General limit for honey	1	1		2
[c]FAO and WHO	General limit for food		1		2

SD = standard deviation (n = 12); ND = not detected. One-way ANOVA with Tukey HSD post hoc test with significant value of 0.05 was used for determining the mean differences in the concentrations of multi-trace metals in stingless bee honey samples among the different regions. *The mean difference is significant at the 0.05 level. [a,b,c] Results are expressed in mg/kg [22, 46].

Trace metals concentrations of stingless bee honey samples from four regions of Peninsular Malaysia were shown in Table 5. In this study, the concentrations obtained for Ag and U were lower to those found by Chua et al. [23]. Additionally, Be and V metals have been determined in stingless bee honey samples but absent in various honey samples from Malaysia [23]. The presence of rare earth elements (REE), such as Ce, Dy, Er, La, Ho, Sm and Th are associated with the used of fertilizers to increase plants productivity [47]. From the results obtained, both rainy and dry seasons possessed low concentration of Th metal.

Table 5. Trace metals in stingless bee honey samples from four regions of Peninsular Malaysia

Season	Region	Ag (mg/L)	Be (mg/L)	Tl (mg/L)	Th (mg/L)	U (mg/L)	V (mg/L)
Rainy	**Pahang (East)**						
	Mean ± SD	0.0066 ± 0.0005	0.002 ± 0	ND	0.0132 ± 0.0018	ND	0.0034 ± 0.0005
	Range (min-max)	0.006 - 0.007	0.002	ND	0.011 - 0.016	ND	0.003 - 0.004
	Kedah (North)						
	Mean ± SD	0.0055 ± 0.0009	0.002 ± 0	ND	0.0118 ± 0.0015	ND	0.003 ± 0
	Range (min-max)	0.005 - 0.007	0.002	ND	0.009 - 0.014	ND	0.003
	Selangor (West)						
	Mean ± SD	0.0062 ± 0.0004	0.002 ± 0	ND	0.0105 ± 0.0012	0.0007 ± 0.0018	0.003 ± 0
	Range (min-max)	0.006 - 0.007	0.002	ND	0.009 - 0.012	0.002 - 0.006	0.003
	Johor (South)						
	Mean ± SD	0.005 ± 0	0.002 ± 0	ND	0.0098 ± 0.0011	ND	0.0033 ± 0.0005
	Range (min-max)	0.005	0.002	ND	0.008 - 0.011	ND	0.003 - 0.004
Dry	**Pahang (East)**						
	Mean ± SD	0.0052 ± 0.0004	0.002 ± 0	0.0002 ± 0.0004	0.0127 ± 0.0007	ND	0.003 ± 0
	Range (min-max)	0.005 - 0.006	0.002	0.001	0.011 - 0.014	ND	0.003
	Kedah (North)						
	Mean ± SD	0.0065 ± 0.0008	0.0037 ± 0.001	0.0002 ± 0.0004	0.0102 ± 0.0013	0.0103 ± 0.0066	0.0045 ± 0.0009
	Range (min-max)	0.005 - 0.0073	0.002 - 0.0043	0.0012	0.0083 - 0.013	0.0078 - 0.0173	0.003 - 0.0053
	Selangor (West)						
	Mean ± SD	0.006 ± 0.0011	0.0031 ± 0.0012	0.0002 ± 0.0004	0.0113 ± 0.0024	0.0064 ± 0.0107	0.0041 ± 0.0011
	Range (min-max)	0.005 - 0.0074	0.002 - 0.0043	0.0001 - 0.0013	0.0081 - 0.015	0.0002 - 0.0034	0.003 - 0.0053
	Johor (South)						
	Mean ± SD	0.0056 ± 0.001	0.0026 ± 0.001	ND	0.011 ± 0.0019	0.0042 ± 0.0076	0.0036 ± 0.001
	Range (min-max)	0.005 - 0.0073	0.002 -0.0043	ND	0.0082 - 0.013	0.0157 - 0.0175	0.003 - 0.0054

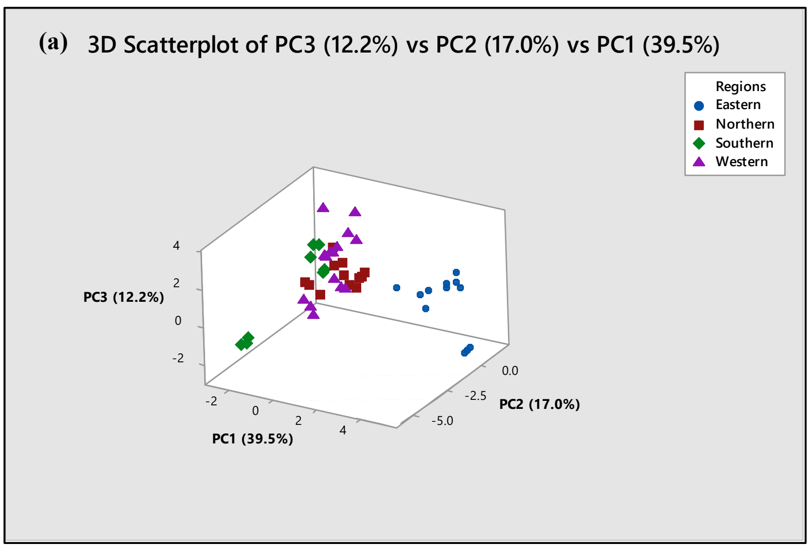
SD = standard deviation (n = 12); ND = not detected. One-way ANOVA with Tukey's HSD post hoc test with significant value of 0.05 was used for determining the mean differences in the concentrations of multi-trace metals in stingless bee honey samples among the different regions. * The mean difference is significant at the 0.05 level.

The data from multi-trace metal concentrations suggested that stingless bee honey samples from four regions of Peninsular Malaysia indicate the honey good quality. Different geographical regions are probably related to the variations in the multi-trace metal concentrations. Increases in anthropogenic activities such as agricultural practice, industries, traffic, coal mining and burning contribute to the accumulation of hazardous multi-trace metals in the ecosystem and become part of the regional environmental compartments [17, 24, 48, 49]. Atanassova et al. [18] reported that during different vegetation seasons, honey could exhibit different multi-trace metal concentrations even though it comes from the same geographical region, same beehive, and same locality.

Principal component analysis (PCA)

For better illustration, PCA was performed on eighteen multi-trace metals to study the overall organization of data for all stingless bee honey samples from the different geographical origins. In this study, it is possible to separate honey samples by their multi-trace metals distribution. The starting part of PCA calculations was the matrix of data with dimensions ($n \times p$), where n is the number of cases (rows) and p is the number of variables (columns). In the matrix, rows represented the honey samples geographical origins and the column represented the multi-trace metals in stingless bee honey samples. As a result of PCA analysis, thirteen and fourteen new variables were obtained, which were represented by eigenvalues for rainy and dry seasons, respectively.

The significance of a factor is explained by its eigenvalue. The factor with the highest eigenvalue is considered the most important. As a result, eigenvalues of 1 or greater are considered significant [50]. The results for the rainy season, as demonstrated in Figure 1(a), showed the first three principal components (PC1-3), with eigenvalues > 1, had 68.7% of the total variability among the thirteen variables in the original data, where in PC1, PC2 and PC3 contributed to 39.5%, 17.0% and 12.2%, respectively. Based on the eigenvalue criterion, PCs with eigenvalues of greater than 1 are considered important [51]. The first PC was principally correlated (loading > 0.30) with five trace metals (Al, Cd, Co, Ni and Zn) that were detected at low concentrations in stingless bee honey samples. While copper, manganese and lead metals were the dominant contents in PC2 and metal content of As, Th, U and V loaded highly in PC3.



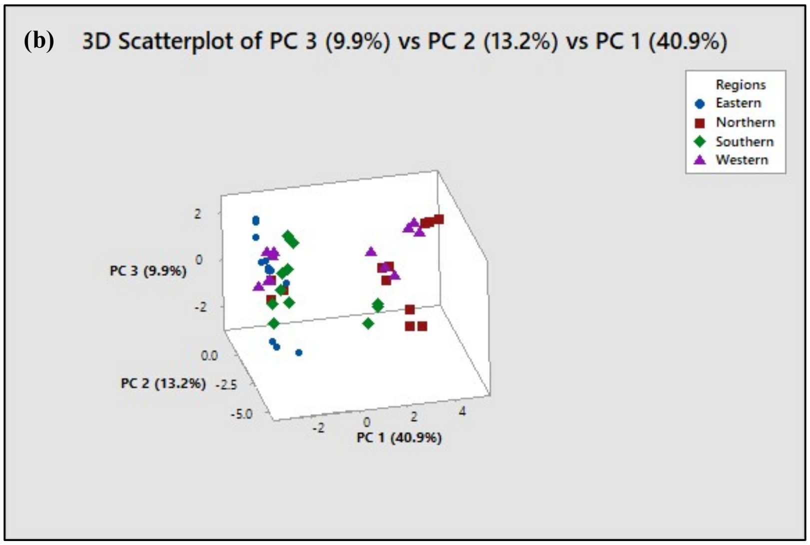


Figure 1. A three-dimensional PCA plot (PC1, PC2 and PC3) for the concentration of eighteen multi-trace metals during (a) rainy and (b) dry seasons

As shown in Figure 1(b), during the dry season, the first three principal components (PC1-3), with eigenvalues greater than one, had 64% of the total variability, where PC1, PC2 and PC3 accounted for 40.9%, 13.2% and 9.9%, respectively. The results showed that a three-factor model could explain 64% of the total variance. The first three PCs (loading plot > 0.3) showed that the Be, Co, Se, Th, U, and V metals have high weights in PC1; Ba, Cd, Mn, and Tl metals loaded high in PC2; and the dominating content in PC3 were Al, As, Cu, and Ni metals. The variability of these multi-trace metal concentrations could be a result of geographical variation. The distribution of stingless bee honey varies between rainy and dry seasons. During the rainy season, as shown in Figure 1(a), the northern, southern, and western regions are close to each other, indicating similarity in multi-trace metal compositions. The eastern region, on the other hand, is separated from the other three regions. As shown in Figure 1(b), the distribution pattern was different during the dry season. This study discovered a relationship between multi-trace metal compositions and the seasons.

As studied by Biluca et al. [52] and Pucholobek et al. [53], Mn metal exhibited the highest concentration in stingless bee honey produced in Brazil through PCA, indicating that geographical origin may influence the concentration of metals in honey. In this study too, Mn is evidently present in high concentrations in the stingless bee honey samples in Peninsular Malaysia during both the rainy and dry seasons. The fact that specific studies focusing on the distributions of Mn across the different locations in Peninsular Malaysia remain unreported, suitable attempt to elucidate such a matter proves necessary.

Linear discriminant analysis (LDA)

LDA simplifies the observation and interpretation of information on graphical representations [51]. The classification results obtained using LDA technique is shown in Figure 2(a) and (b). An accuracy of 95.8% (rainy season) and 89.6% (dry season) success rate in honey samples regions classifications were achieved by the cross-validation (leave-one-out) LDA technique, in order to evaluate the performance of developed classification models. A neat and no neat separation between regions can be observed during rainy and dry seasons, respectively. Although no neat separation was obtained during dry season, however, all the stingless bee honey sample was confirmed to be harvested from the beekeepers of selected districts of four regions.

A study by Nomikos and MacGregor [54], explained that, with highly correlated variables, only few PCs are needed to explain the significant in the data set. Additionally, by using cross-validation technique, the number of PCs to retain can also be determined. The power of PCA technique can be observed by plotting the scores of PCs, in which the objects will exhibit measurement similarities to form clusters and moreover, an isolated object (outlier) can be seen clearly. As shown in Figure 1(a) and (b), an exploratory PCA was run on the entire data set to get a sense of the data distribution for each region.

During both rainy and less rainy seasons, overlapping regions for stingless bee honey were observed, with a minor clustering for their geographical origins. Therefore, in this study, by considering three PCs, rainy and less rainy seasons represented 68.7% and 64.0% of the variations, respectively. Further classification by the LDA technique exhibited an improvement in percent of classification to 95.8% (rainy) and 89.6% (less rainy). The selection techniques allowed a reduction from eighteen trace metal concentrations to only ten. The presence of Al, As, Cd, Co, Cu, Mn, Ni, Th, U, and V in both rainy and less rainy seasons appears to provide suitable chemical signatures of the Malaysian stingless bee hone's geographical origins. This percentage can be considered satisfactory as the number of stingless bee honey samples was small. A previous study by Shadan et al. [24] reported 70.2% of variations using the first three PCs and improved to 96.2% using the LDA technique. Their study was similar to our study, but they only examined five districts in the southern region (Johor) of Peninsular Malaysia during the less rainy season in February to July 2016. The composition of stingless bee honey varies according to geographical or botanical origin, as well as fruit or flower season [33].

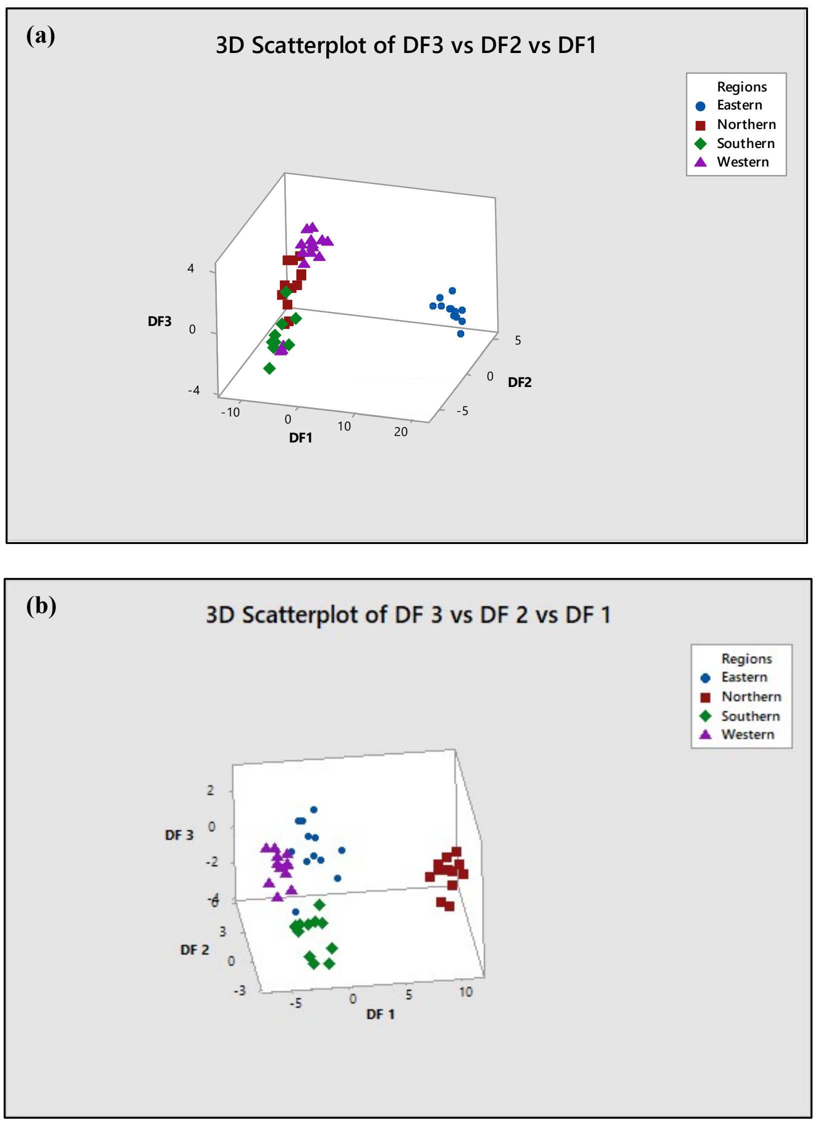


Figure 2. A three-dimensional LDA plot (DF1, DF2 and DF3) after removal of outliers and (b) dry season

Conclusion

This study filled some information gaps on multi-trace metals in *Heterotrigona itama* stingless bee honey from four regions of Peninsular Malaysia, namely Kedah, Johor, Selangor, and Pahang. For the first time, discussion on eighteen multi-trace metals (Ag, Al, As, Ba, Be, Cd, Co, Cr, Cu, Mn, Ni, Pb, Se, Th, Tl, U, V and Zn) concentrations of stingless bee honey sample collected from Padang Terap, Kulim, Yan and Baling (Kedah), Pontian and Mersing (Johor), Sabak Bernam, Klang, Kuala Selangor and Hulu Selangor (Selangor) as well as Jerantut, Rompin, Temerloh and Kuantan (Pahang) were made. Cross-validation results were excellent during the rainy and less rainy seasons, with 95.8% and 89.6%, respectively. It could be attributed to regional differences in the composition of multi-trace metals. This study supported the possibility of assigning a relationship between multi-trace metal compositions and their geographical origins, following the integration of such data with chemometric techniques like PCA and LDA. Further research should concentrate on bioavailability studies to estimate the degree of intake that is suitable for the human digestive system as metals show varied absorption interactions.

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Conflict of Interest Statement

The authors declare no conflict of interest regarding the publication of this paper.

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