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Original Research Article

Amino acid determination by HPLC combined with multivariate approach for geographical classification of Malaysian Edible Bird's Nest

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ABSTRACT

Edible Bird's Nest (EBN) is mainly used as a functional food where its quality is affected by many factors including geographical region. This study aims to differentiate the EBN from West Malaysia (WM) and East Malaysia (EM) based on amino acid profiles by high-performance liquid chromatography (HPLC) combined with multivariate approach. A total of 33 authentic EBN samples were collected from WM ($n = 23$) and EM ($n = 10$) for classification. The data obtained was used to identify the reliable potential markers between WM and EM via serial multivariate analysis including hierarchical clustering analysis (HCA), principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). EBN samples from WM and EM were clearly distinguished by the developed OPLS-DA model with high prediction ability (Q^2) of 62.7 %. The model's robustness was validated and blind test samples were 100 % properly allocated to their respective groups. Glycine, cysteine, tryptophan and aspartic acid were proposed as potential markers to classify the EBN from WM and EM. Overall, the predictive model shows high accuracy for EBN classification.

1. Introduction

Edible Bird's Nest (EBN) or "yànwō" in Mandarin with the meaning of bird nest, is the secretion of *Aerodramus* swiftlets for building their nest during breeding seasons. It is a delicacy and has been enjoyed by humans since ancient times owing to its nutritious benefits. EBN is a luxurious food with the curative effect being documented in the classical book Compendium of Materia Medica (Ben Cao Gang Mu) in Tang Dynasty (618-907 A.D.) of the Chinese history for the human wellness (Dai et al., 2020). To date, Chinese physicians stated that EBN can be used as traditional Chinese medicine to nourish lung, maintain a youthful and radiant complexion (Babji et al., 2018). Modern pharmacological research further indicated that EBN contained various biological activities such as antioxidant (Quek et al., 2018a), neuroprotective (Ismaeil et al., 2021), anti-aging (Hwang et al., 2020), bone strengthening (Hou et al., 2021) and immune promotion (Teh and Ma, 2018). Due to its health benefit, EBN has been developed as an additive for health supplementary and has caught more attention from pharmaceutical, nutraceutical and cosmeceutical industry. Furthermore, EBN has increasingly being cherished by consumers which resulted in market expansion to worldwide, especially among the Chinese community in Singapore, China, Taiwan, Japan, Korea, the North and Middle East America (Jamalluddin et al., 2019). The prominent source of EBN can be harvested in Indonesia and Thailand, while Malaysia represents the third main supplier of EBN in the world (Dai et al., 2020). In Malaysia, the EBN production has been well developed due to favourable geographical and climatic conditions that provide suitable habitat for swiftlet farming (Rahman et al., 2018).

Swiftlet farming is unique in the agricultural sector of Malaysia. The Malaysia government identifies EBN as a high-value animal by-products with good agribusiness prospects in the National Key Economic Areas (NKEA) (Fatin et al., 2019). Swiftlet farming also considered as a

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lucrative business where the investors can earn 2000 USD per kilogram on the international market (Connolly, 2017). According to the Federation of Malaysia Bird's Nest Merchants Association, there have been around 63,000 registered swiftlet farms in Malaysia in 2020 with the contribution of 20 % in the total EBN production of the world (Kamaruddin et al., 2019). These swiftlet farms are located in Malaysian Borneo (known as East Malaysia) and Peninsular Malaysia (known as West Malaysia) being the largest contributor of EBN. In the market, the EBN products are graded based on its colour, productions environment and geographical origin, consequently the price (Jamalluddin et al., 2019). EBN from different cultivation places could vary in composition and market price. Thus, searching potential methods to differentiate the original cultivation place of EBN has becoming an important topic.

High quality EBN is a relatively high-value commodity. Fraudulent activities by irresponsible manufacturers such as mislabelling of its brand and geographical origin can be encountered (Jamalluddin et al., 2019). Extensive chemical studies on the EBN content have been done by food technologists to prevent the falsification. Study on the composition of EBN such as protein content, carbohydrate, minerals, ash and fats (triglyceride) have been well developed (Hun et al., 2020; Quek et al., 2018a). The evaluation of EBN physicochemical characteristics is highly recommended because the properties of EBN change depending on geographical resources. The major constituent of EBN is protein (62–63 %) that plays a key role in the EBN products (Lee et al., 2021). For this reason, food scientists have started to pay attention to exploring the amino acid contents in EBN. It was reported that amino acid content could be useful to identify EBN from two swiftlet species (*A. maximus* and *A. fuciphagus*) (Quek et al., 2018b). However, the classification of EBN by amino acids is still ambiguous to evaluate the geographical sources of EBN.

Among the techniques employed for amino acid determination in EBN, liquid chromatography and gas chromatography have been frequently reported for geographical classification (Azmi et al., 2021; Seow et al., 2016). Amino acids are the building block of protein and these compounds can be a useful indicator for the classification of EBN (Quek et al., 2018a). There are 18 types of amino acids (9 essential and 9 non-essential amino acids) detected in EBN (Azmi et al., 2021). The quantification and qualification of the amino acids are more accurate compared with protein. This could be due to the heat-sensitive characteristic of proteins that are easily denatured at high temperatures, causing inconsistency in the result obtained (Azmi et al., 2021). With this consideration, the analysis of amino acids could be an alternative method in EBN classification. For example, amino acids analysis was successfully used in the identification and classification of EBN samples based on species origin, production origin, and geographical origin (Quek et al., 2018a). Besides, chromatographic techniques using phytochemical composition and microscopic analysis to observe the morphology of the samples are also have been exploited previously to distinguish samples from different geographical origin (Mocan et al., 2018; Mollica et al., 2018). However, these procedures are time consuming and expensive as it involves lots of procedures and well trained personnel who are specialized in those particular methods. Since EBN contains multiple chemical components with varying physicochemical characteristics, the investigation is usually integrated with chemometric (multivariate analysis) approaches to investigate the possibility of any distinctive patterns based on the geographical origin of EBN (Guo et al., 2018). Nevertheless, the study on the determination of the geographical origin of EBN in East and West Malaysia is limited. Moreover, with the abundant resource of EBN in East and West Malaysia, it is worthy to explore the combination of amino acids profiling followed by multivariate analysis to identify suitable indicators for determining the EBN geographical origin in the effort of protecting consumers against EBN frauds. Previously, chemometrics model has been successfully applied in authentication study. They employed linear discriminate analysis (LDA) and partial least squares discriminant analysis (PLS-DA) to simulate adulterations of saffron (Morozzi et al., 2019) and grappa spirit (Arduini et al., 2021). Both LDA and PLS-DA models have shown an excellent classification to discriminate the artificial adulteration mixture with pure and pure test samples. These approaches have been proved to be more convenient as it allows a fast, simple and inexpensive method. These findings proved that the application of chemometrics models is time and cost effective compare to conventional method.

This study aims to propose and explore the reliable unique indicators between the EBN from East and West Malaysia by the liquid chromatography based on their amino acids profiling. To aid in data visualization, chemometrics model through multivariate analysis such as principal component analysis (PCA), hierarchical cluster analysis (HCA), and orthogonal partial least-squares discriminant analysis (OPLS-DA) were used for sample classification and marker identification. The findings are envisioned to reveal the distributions of important amino acids and most importantly, to protect the consumers from the fraudulence activity related to EBN quality.

2. Materials and method

2.1. Chemical and reagents

Water (Milli-Q ultrahigh-purity water), AccQ-Fluor derivation buffer, AccQ-Fluor reagent kit, AccQ-Tag Ultra Eluent A and AccQ-Tag Ultra Eluent B were purchased from Waters Corp (USA). Lithium hydroxide monohydrate ($LiOH{\bullet}H_2O$), nitrogen gas (N_2), sodium acetate, formic acid, hydrogen peroxide, hydrochloric acid (HCl) were supplied by Merck (Germany). Methanol (HPLC grade), hydrobromic acid, alpha aminobutyric acid (AABA) were obtained from Sigma-Aldrich (USA). Standard for tryptophan, cysteic acid, methionine and Pierce standard H with a purity of 98 % were procured from Thermo Fisher Scientific (USA).

2.2. Collection of Edible Bird's Nest (EBN)

A total of 33 EBN samples (house nest) studied in this work were collected from 11 states in West Malaysia (WM) (Perak (WM1), Selangor (WM2), Kelantan (WM3), Pahang (WM4), Johor (WM5), Kedah (WM6), Melaka (WM7), Negeri Sembilan (WM8), Pulau Pinang (WM9), Perlis (WM10), and Terengganu (WM11)) and 2 states in East Malaysia (EM) (Sarawak (EM1) and Sabah (EM2)) to ensure the geographical location variations are covered (Table 1). Fig. 1a shows the sampling sites in WM and EM. All EBN samples were obtained directly from the licensed swiftlets premises in WM and EM from 1st January to 31st December 2016. The collected EBN samples were further authenticated by the Department of Veterinary Services Malaysia via Radio Frequency Identification. The genuine EBN were labelled with a voucher specimen number as shown in Table 1. These EBN samples were yellowish in colour and about 9 cm in length with a shape resembling a half-bowl (Fig. 1b).

2.3. Preparation of Edible Bird's Nest (EBN)

The EBN samples were undergone the cleaning process according to the Good Manufacturing Practice (GMP) of industrial cleaning method [Raw-Unclean and Raw-Clean Edible Bird's Nest (MS 2333:2010)] as described by Lee et al. (2018). Briefly, the EBN samples were sprayed with the reverse osmosis water on the surface individually for swelling its strains. The feathers, eggshells, dirt, and other impurities were manually removed using tweezers. After that, the EBN samples were further dried in a forced-air convection oven (UM 100, Mememert, Germany) at 40 °C for 5 h until the moisture content was less than 15 % to control the growth of the pathogens and preventing spoilage. Then, the dried EBN samples were pulverized into a fine powder using a mechanical grinder (DFY-1000C, NewSwan, China) followed by passing through a sieve (1 mm). The ground EBN samples were labelled based on

Table 1
Locations of Edible Bird's Nest (EBN) collected in West Malaysia and East Malaysia.

Regions	Province	Part	Label[#]	Voucher specimens
West Malaysia	WM1	West	WM1a	800016
		West	WM1b	800019
		West	WM1c	860029
		West	WM1d	800011
		West	WM1e	850098
	WM2	West	WM2a	700690
	WM3	West	WM3a	302910
		West	WM3b	302910
		West	WM3c	302910
	WM4	West	WM4a	430001
		West	WM4b	430003
		West	WM4c	430005
		West	WM4d	430004
	WM5	West	WM5a	110003
		West	WM5b	140052
	WM6	West	WM6a	270074
		West	WM6b	270080
	WM7	West	WM7a	410008
		West	WM7b	410012
	WM8	West	WM8a	550006
	WM9	West	WM9a	750008
	WM10	West	WM10a	900048
	WM11	West	WM11a	400330
East Malaysia	EM1	East	EM1a	600570
		East	EM1b	600580
		East	EM1c	600620
		East	EM1d	600700
		East	EM1e	601520
	EM2	East	EM2a	500010
		East	EM2b	100070
		East	EM2c	100150
		East	EM2d	100160
		East	EM2e	300140

[#] The small letter represents the individual samples.

their collection site and stored in separate sterile containers at room temperature prior to analysis.

2.4. Extraction of amino acids

The procedure used for the amino acid extraction prior to free amino acid content analysis was performed by three different hydrolysis methods, namely acid hydrolysis, alkaline hydrolysis and performic acid oxidation. Acid hydrolysis was performed according to the methods of Hun et al. (2020) to measure all the amino acids content except tryptophan, cysteine and methionine. Alkaline hydrolysis was carried out the method adapted from Cour et al. (2019) to determine the tryptophan content. Sulphur containing amino acids (cysteine and methionine) were determined through performic acid oxidation adapted from Thera et al. (2018). The details of the three extraction procedures were briefly described in the following sub-sections.

2.4.1. Acid hydrolysis

0.1 g of ground EBN sample was weighed and transferred into a 50 mL hydrolysis tube along with 15 mL of 6 N HCl. The use of HCl was performed in a properly functioning chemical fume hood. The tube was tightly sealed and heated at 110 °C ± 1 °C in an electric oven (UF30, Mememert, Germany) for 24 h. After the heating process, the mixture was cooled and transferred into a 100 mL volumetric flask with a 400 µL internal standard of 50 µmole/mL alpha amino butyric acid (AABA). The mixture (AABA and sample hydrolysate) was topped up with 100 mL of Milli-Q water. Then, it was filtered using Whatman No.1 filter paper followed by a 0.2 µm cellulose acetate syringe filter (Waters Corporation, USA). After that, 10 µL of the mixture was pipetted and added with 70 µL of AccQ-Fluor derivation buffer and 20 µL of AccQ-Fluor reagent kit for the derivatization of amino acids. Then, it was vortexed for 10 s and transferred into a high-performance liquid chromatography (HPLC) autosampler vial with 4.6 mm micro-volume insert. The vial was heated on the heating block (Dry Block Heater 1, IKA, Germany) at 55 °C for 10 min. Then, the mixture was filtered again using the Whatman No.1 filter paper followed by the 0.2 µm cellulose acetate syringe filter. About 2 µL of the derivatized sample was then injected into the HPLC system for amino acid analysis.

2.4.2. Alkaline hydrolysis

0.1 g of ground EBN sample was placed in a hydrolysis tube and 15 mL of 4.3 N $LiOH{\bullet}H_2O$ was added. The tube was flushed with N_2 gas to provide oxygen-free conditions, sealed and placed in an electric oven at 120 °C for 16 h. After cooling, the pH of the sample was adjusted to 4.5 using a diluted HCl solution (0.1 M). Then, the sample was made up to 100 mL with distilled water in a volumetric flask. 50 µL of the sample was pipetted out and topped up to 10 mL with mobile phase solution, containing 86.7 % of 8.5 mM sodium acetate (pH 4), and 13.3 % of methanol. Then, the mixture was filtered using a Whatman No.1 filter paper followed by 0.2 µm cellulose acetate syringe filter and 2 µL was injected into the HPLC system for amino acid analysis.

2.4.3. Performic acid oxidation

The performic acid was prepared immediately prior to use by mixing formic acid and hydrogen peroxide in a ratio of 9:1 (v/v) and then incubated at room temperature for 30 min. An amount of 0.1 g of ground EBN sample was mixed with 2 mL of performic acid and then the mixture was kept at 0 °C for 16 h. After that, 0.4 mL of hydrobromic acid was added and stored at 4 °C for 30 min in a chiller. The sample was then slowly rotary-evaporated under a vacuum (200 mbar) to remove the performic acid. The sample was further hydrolysed using 15 mL of 6 N HCl at 110 °C for 24 h with a sealed hydrolysis tube. The following step was similar to Section 2.4.1 until the sample injected into HPLC.

2.4.4. Standard amino acid preparation

The standard mixture of amino acids solution (Pierce Standard H) containing the mixture of 2.5 mM of the hydrolysed amino acids (aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine) and 1.25 mM of cysteine was prepared. Generally, 1.5 mL of the standard mixture was mixed with 5 mL of 50 µmole/mL AABA and the subsequent process was similar to Section 2.4.1. The tryptophan standard was prepared by dissolving 50 mg of tryptophan with 50 mL of 0.1 N HCl in a volumetric flask. The subsequent process was similar to Section 2.4.2. After that, another standard solution containing 10 mL of 25 µmole/mL cysteic acid and 10 mL of 25 µmole/mL methionine was prepared by mixing 100 mL of 0.1 N HCl and 5 mL of 50 µmole/mL AABA which labelled as Solution A. About 1.5 mL Solution A was immediately mixed with 1.5 mL of amino acid standard Pierce Standard H and labelled as Solution B. Subsequently, 160 µL of Solution B was diluted with 840 µL of distilled water. Similar preparation was performed as in Section 2.4.3 until ready for injection into the HPLC system.

2.5. Amino acids analysis using HPLC

After the extraction process, all the amino acid contents were detected and quantified based on Hun et al. (2020) while tryptophan content was measured according to Lee et al. (2016). Briefly, amino acids were analyzed using a Waters 2695 series HPLC system (Waters, USA) equipped with a FLR-2475 fluorescence detector (Waters, USA). Separation of amino acid was carried out on an AccQ-Tag column (3.9 mm x 150 mm) at 36 °C with an elution rate of 1.0 mL/min (Eluent A: 200 mL AccQ-Tag Ultra Eluent A mixed with 2 L of Mili-Q waters; Eluent B: AccQ-Tag Ultra Eluent B) according to the gradient described in Table 2. The fluorescence detector was set at excitation wavelength ($E\lambda_x$) 340 nm and emission wavelength ($E\lambda_m$) 455 nm. Moreover, the separation of tryptophan was carried out by Waters Nova-Pack C_{18}

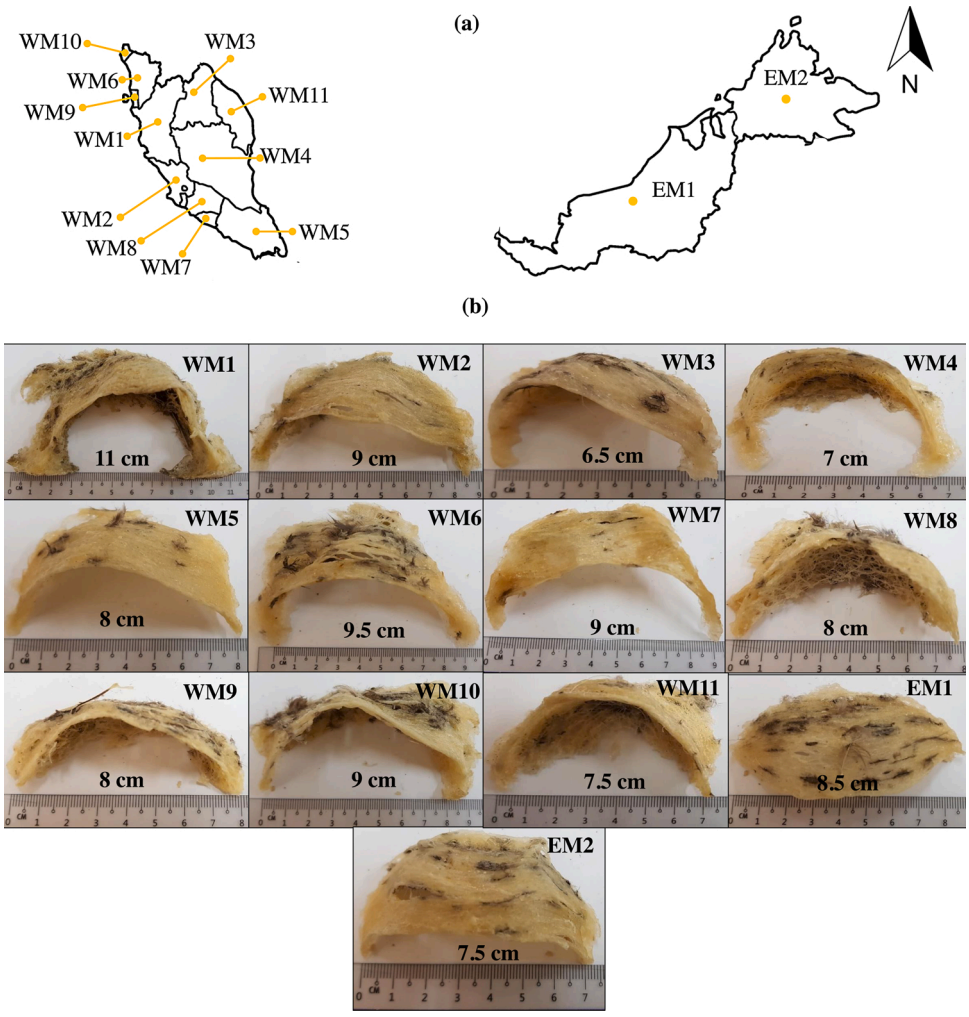

Fig. 1. Sampling sites in West Malaysia (WM1-11) and East Malaysia (EM1-2) (a); morphological characteristics of Edible Bird's Nest (EBN) (b).

Table 2
Mobile phase gradient for amino acid (except tryptophan) analysis.

Time (min)	Elution rate (mL/min)	Eluent A (%)	Eluent B (%)
0	1.0	100.0	0.0
0.8	1.0	98.0	2.0
15	1.0	90.0	10.0
19	1.0	87.0	13.0
32	1.0	65.0	35.0
33	1.0	65.0	35.0
34	1.0	0.0	100.0
37	1.0	0.0	100.0
38	1.0	100.0	0.0
42	1.0	100.0	0.0

column (3.9 mm x 150 mm) with a flow rate of 1.0 mL/min at ambient room temperature. The mobile phase used was a mixture of 0.0085 M sodium acetate (pH 4) and methanol (86.1: 13.3, v/v). The fluorescence detector was set at excitation wavelength ($E\lambda_x$) of 285 nm and emission wavelength ($E\lambda_m$) of 345 nm. The data were recorded and evaluated via the Millennium Chromatography Manager Software (Waters Corporation, USA). The standard of amino acids was injected in the middle of each run of sample to calibrate the amino acid quantification.

2.6. Quantification of amino acids

Eqs. 1 and 2 were used to calculate the amino acid content from acid hydrolysate sample (acid hydrolysis and performic acid oxidation) while Eq. 3 was used to determine the tryptophan content from the alkaline hydrolysate sample. The amino acid content was expressed in weight per weight percentage (w/w %).

$$\text{Response factor (Rf)} = \frac{\text{Weight of standard AA}/\text{Area of standard AA}}{\text{Weight of IS}/\text{Area of IS}} \quad (1)$$

$$\text{Amino acid (\%)} = \frac{\text{Rf} \times \text{Area of AA in sample}}{\text{Area of IS in sample}} \times \frac{\text{IS weight}}{\text{Sample weight}} \times 100\% \quad (2)$$

$$\text{Trp (\%)} = \frac{\text{Area of Trp in sample}}{\text{Area of Trp standard}} \times \text{Weight of Trp standard} \times 100\% \quad (3)$$

Where, AA = Amino acid; IS = Internal Standard (AABA); Trp = Tryptophan.

2.7. Multivariate analysis

The data set resulting from HPLC was subsequently exported into SIMCA software Version 13.0 (Umetrics, Umea, Sweden) for multivariate pattern recognition analysis. Two multivariate analyses namely unsupervised principal component analysis (PCA) and supervised orthogonal partial least-squares discriminant analysis (OPLS-DA) was used. Prior to the development of the chemometric models, unit variance scaling was employed on the OPLS-DA datasets. The 18 types of amino acids data as the X-matrix while the analysed regions (EM and

WM) were used as the Y-matrix to investigate the differences among the EBN. Briefly, the PCA was run to obtain a general overview of the variance of the EBN amino acids profile. PCA summarized the original dataset by reducing the dimension of the large dataset. Subsequently, OPLS-DA was carried out to investigate the differences among the EBN amino acid of the analysed regions (EM and WM). The combination of unsupervised and supervised information could ensure whether these data are appropriate to identify a geographical classification of the analysed regions.

The model strength performance was assessed using R^2 (goodness of fit) and Q^2 (goodness of prediction). The model was considered stable and robust when the values of R^2 and Q^2 greater than 0.5, and values closer to 1.0 indicated the excellent model. The robustness and significance of the created model were evaluated by Cross-Validation Analysis of Coefficient Variance (CV-ANOVA) at a 95 % confidence level ($p < 0.05$).

2.7.1. Model validation

Internal and external validations were used as a figure of merit that was assessed by the R^2 and Q^2 value to ensure the accuracy of the model. Seven-fold internal cross-validation was first employed to validate the OPLS-DA model, where the data matrix was split into 7 ($k = 7$) separated groups. The OPLS-DA model was randomly built from the $k = 7-1$ group, known as the 'training set', while the remaining group was predicted as 'test set' and used to measure the generalization error. Following this, the model was then validated by a sample blind test as an external validation to ensure the accuracy of the model. Briefly, 3 samples from each cluster (analysed regions: EM and WM) were randomly selected from the original data and treated as 'unknown sample' (test set) to project into the predictive model. The remaining samples (training set) were used to build the predictive model (Wang et al., 2021).

2.7.2. Selection of potential marker

The identified marker (amino acids) was sorted and summarized according to their importance by their variable importance in the projection (VIP) values. The identified marker with VIP greater than values 1.0 that led to group separation was chosen as a potential marker to discriminate the amino acids of EBN from analysed regions. The most significant marker generated by the SIMCA software and their differences in abundance were depicted with an S-plot.

2.8. Statistical analysis

All experiments were carried out in triplicate unless indicated. An independent samples *t*-test was performed to compare the amino acids content between the WM and EM. Hierarchical clustering analysis (HCA) was applied to demonstrate the contributions of factors in grouping EBN samples based on the analysed regions. The HCA was displayed in a dendrogram via the SIMCA software. A heat map of the correlations among the 33 EBN samples was performed by GraphPad Prism® version 8 (GraphPad Software Inc., San Diego, CA, USA).

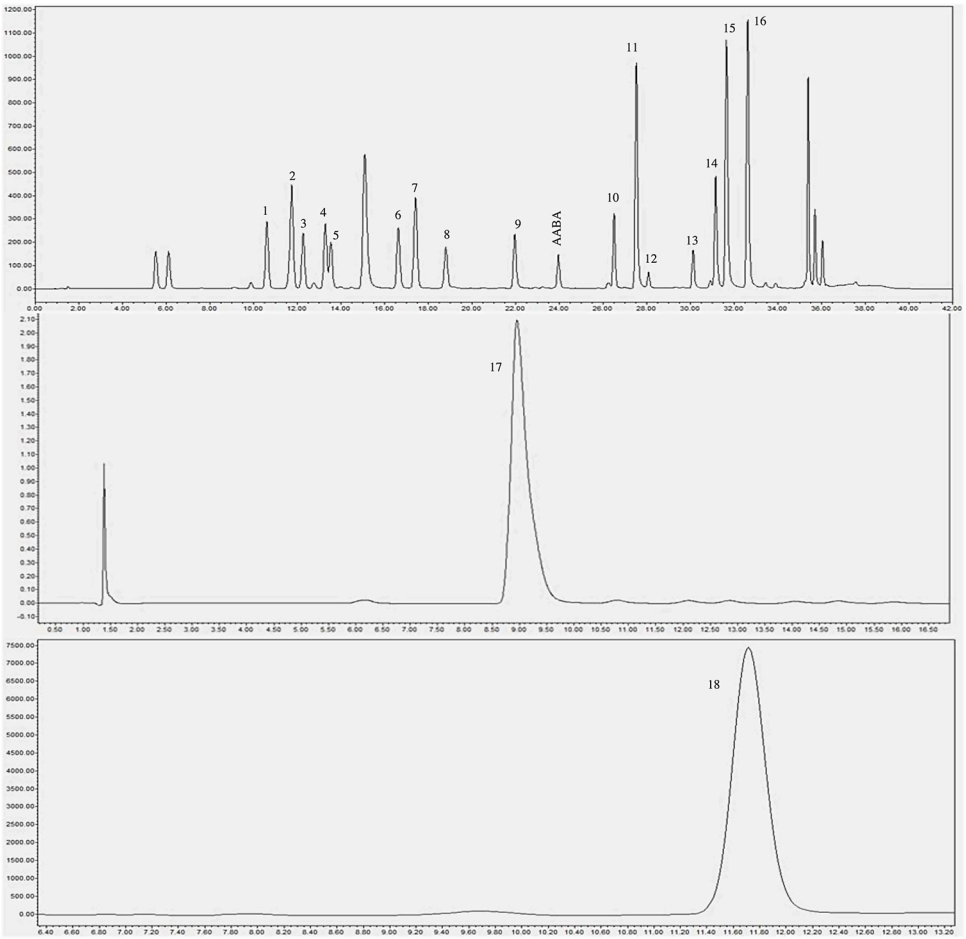

Fig. 2. Typical HPLC chromatogram of amino acid from EBN extracts with the internal standard (AABA). Amino acid profiles: (1) aspartic acid, (2) serine, (3) glutamic acid, (4) glycine, (5) histidine, (6) arginine, (7) threonine, (8) alanine, (9) proline, (10), tyrosine, (11) valine, (12) methionine, (13) lysine, (14) isoleucine, (15) leucine, (16) phenylalanine, (17) cysteine and (18) tryptophan.

3. Result and discussion

3.1. Amino acid composition of EBN

Due to the complex biochemical compositions of EBN resulting from different geographical regions, it is necessary to identify an appropriate EBN extraction method to determine the amino acid compositions of EBN. In this study, three different hydrolysis methods were used. The amino acid content of all EBN collected from different locations were quantified using the HPLC technique. The HPLC parameters set allowed to separate and quantify 18 types of amino acids with good resolution (Fig. 2). The intention was to extract as many amino acids constitute as possible, to obtain the most comprehensive information for EBN sample assessment. It was reported by Marcone (2005) that only 15 amino acids were found in EBN via HCl hydrolysis where the HCl destroyed the tryptophan, cysteine and methionine, resulting only 15 types of amino acids being identified by HPLC. In order to identify the 3 remaining amino acid (i.e. cysteine, methionine and tryptophan), performic acid oxidation and alkaline hydrolysis were required (Hun et al., 2020). Therefore, it was revealed that the application of acid hydrolysis, performic acid oxidation and alkaline hydrolysis allowed the detection of 18 amino acids in the EBN samples. The result is consistent with Gan et al. (2020), as 18 amino acids were also identified in EBN samples.

The amino acids of the EBN from 33 different locations are tabulated in Table S1 and vary from 0.20 to 4.41 %. In total, 18 amino acids were detected and quantified, constituted mainly of 9 essential amino acids and 9 non-essential amino acids. Among them, three essential amino acids (phenylalanine, threonine and leucine) and three non-essential amino acids (serine, aspartic acid, and proline) were the highest constitutes in EBN. This finding is consistent with other research works (Ali et al., 2019; Azmi et al., 2021; Gan et al., 2020), hence strengthen the reliability of the results of the amino acid detected in this study. However, the difference in amino acid compositions between these research works could be due to the EBN's sources obtained from different origins and the cleanliness processed of EBN from impurities (feathers and eggshell). Table 3 shows that EBN has high content of total non-essential amino acids (22.88 ± 2.2 % from WM, 21.84 ± 1.80 % from EM) than total essential amino acids (17.30 ± 1.68 % from WM, 16.79 ± 1.27 % from EM). Human cells able to synthesise the non-essential amino acids for tissue growth and immune functions while essential amino acids need to be acquired from food (Wei et al., 2020). Since human cells unable to produce essential amino acids, it is important to supply the human body with sufficient essential amino acids for regulating supply of important building blocks (amino acids). According to Table 3, the total of essential amino acids found in the EBN samples (17.30 ± 1.68 % from WM 16.79 ± 1.27 % from EM) were similar in other protein-rich foods like commercial chicken egg (19.12 %) and higher than milk drink (1.38 %) (Ali et al., 2019; Kakimov et al., 2017). This reveals that EBN is a good source of essential amino acids. It is believed that essential amino acids in EBN can offer sufficient building blocks for the protein synthesis that will benefit human health in tissue repair and nutrient absorption. In fact, the essential amino acids content presented in EBN was well above the daily requirement for adults as recommended dosage by the World Health Organization/Food and Agriculture Organization of the United Nations/United Nations University (WHO/FAO/UNU) (Iacone et al., 2018; Seow et al., 2016; WHO/FAO/UNU, 2007). Thus, EBN can be a healthy food alternative that should be incorporated into the human diet due to their excellent sources of essential amino acid profile and potential health benefits.

An independent samples *t*-test was applied to the dataset for comparing the amino acid content of EBN samples in West Malaysia (WM) and East Malaysia (EM). No significant difference ($p > 0.05$) was observed in total amino acids obtained in the WM (40.18 ± 3.88 %) and EM (38.63 ± 3.07 %) regions as shown in Table 3. These results suggest that the EBN contained a homogeneous total amino acid content in WM and EM, indicating EBN is mainly built by glycoproteins components.

Table 3
Amino acid contents of EBN from West and East Malaysia characterized by HPLC.

Amino acid	Amino acid content (w/w %)		
	West Malaysia	East Malaysia	WHO/FAO/UNU (2007 report)[a]
Essential			
Phenylalanine, Phe	3.17 ± 0.34 *	2.91 ± 0.21*	0.0025
Threonine, Thr	3.02 ± 0.25	2.87 ± 0.19	0.0015
Leucine, Leu	2.92 ± 0.22	2.92 ± 0.17	0.0039
Valine, Val	2.77 ± 0.21	2.76 ± 0.17	0.0026
Histidine, His	1.86 ± 0.17 *	1.70 ± 0.14 *	0.0010
Lysine, Lys	1.56 ± 0.14	1.60 ± 0.10	0.0030
Isoleucine, Ile	1.40 ± 0.10	1.41 ± 0.08	0.0020
Methionine, Met	0.38 ± 0.03	0.37 ± 0.03	0.0015
Tryptophan, Trp	0.22 ± 0.22 *	0.25 ± 0.18 *	0.0004
Total essential amino acid	17.30 ± 1.68	16.79 ± 1.27	0.0184
Non-essential			
Serine, Ser	3.67 ± 0.31	3.53 ± 0.23	
Aspartic acid, Asp	3.29 ± 0.34 *	3.59 ± 0.31*	
Proline, Pro	3.07 ± 0.24	2.87 ± 0.17	
Tyrosine, Try	3.01 ± 0.31 *	2.78 ± 0.27 *	
Arginine, Arg	2.99 ± 0.24	2.82 ± 0.20	
Glutamic acid, Glu	2.75 ± 0.25	2.91 ± 0.15	
Glycine, Gly	1.53 ± 0.27 **	0.93 ± 0.30 **	
Cysteine, Cys	1.44 ± 0.15 **	1.25 ± 0.11 **	
Alanine, Ala	1.13 ± 0.09	1.16 ± 0.06	
Total non-essential amino acid	22.88 ± 2.20	21.84 ± 1.80	
Total amino acid	40.18 ± 3.88	38.63 ± 3.07	

Values are mean ± standard deviation. Asterisk (*) show statistical significance. Independent samples *t*-test: * $p < 0.05$; ** $p < 0.01$.

[a] Essential amino acid requirements of adults suggested by World Health Organization/Food and Agriculture Organization of the United Nations/United Nations University (WHO/FAO/UNU).

Since the amino acids are mainly originated from the protein, thus the similar content observed could be due to the glycoproteins in EBN (Dai et al., 2020; Quek et al., 2018a). However, the composition analysis of EBN amino acids showed that different habitats possess qualitative similarities among these amino acids constitutes with considerable variations in quantitative levels of individual amino acid constitutes. In terms of the individual amino acids, it was found that phenylalanine, histidine, tryptophan, aspartic acid, tyrosine, glycine, and cysteine were significantly different between the WM and EM (Table 3). The variations in the individual amino acid contents could be due to genetic, environmental variation, or both (Guo-Lian et al., 2018). A heat map was generated to visualize the amino acid content in WM and EM EBN (Fig. 3). The heat map of the correlations among the 18 amino acids of the EBN samples collected from the various locations in WM and EM produced an overview of the amino acid compositions, hence indicating the potential amino acids (glycine and cysteine) to differentiate the EBN samples. When compared with other amino acids, both glycine and cysteine showed a distinctive colour, suggesting the differences of amino acid composition between WM and EM. Thus, both glycine and cysteine could be the potential geographical markers to classify EBN samples between WM and EM.

3.2. Hierarchical clustering analysis of EBN samples based on amino acid profiles

Hierarchical clustering analysis (HCA) was conducted to

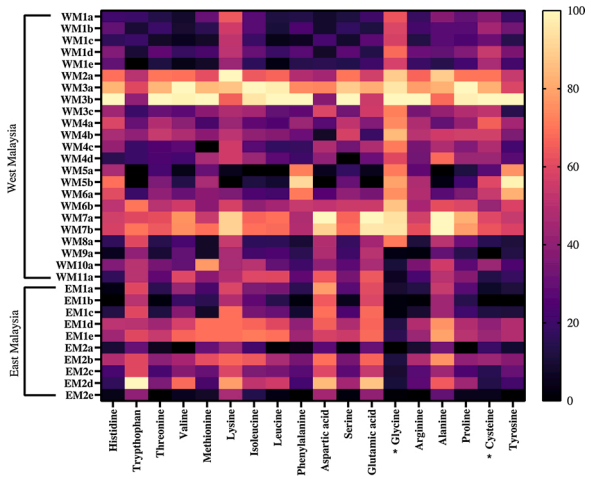

Fig. 3. Heatmap of EBN samples from different geographical regions (WM and EM) based on amino acids data. Asterisk (*) show the statistical significance ($p < 0.01$).

characterize the role of amino acids in classifying the geographical regions of EBN. Amino acids quantification enabled the determination of the similarity between the geographical regions, resulting in the formation of two clusters. A dendrogram generated from the statistical analysis showed that the WM and EM were grouped in Cluster I and Cluster II, respectively (Fig. 4a). It was found that there were three outliers originated from the WM grouped in the Cluster I. These outliers were distant from the WM group (Cluster II) and caused the unclear differentiation between the classes. In this case, the three outliers were omitted from further data analysis. Thus, the finding obtained was corroborated with Granato et al. (2018) where the perfect segregation can be obtained when the dataset are analyzed after removal the outliers. As such, the HCA results in Fig. 4b were based on the data without the three outliers. As shown in Fig. 4b, no samples were assigned to the wrong group where an ideal result was obtained. In particular, the observed outliers were eliminated so that the dataset obtained in the HCA can be prior to classification approach using multivariate analysis.

3.3. Classification of EBN from WM and EM using multivariate analysis

3.3.1. Principal component analysis

EBN is a kind of natural animal-based product with a complex matrix, it is difficult to conclude by analyzing the data obtained from HCA. PCA was applied to investigate for similarity between samples based on their amino acid composition and geographical regions. PCA is an unsupervised chemometric analytical approach for reducing dataset dimensionality by identifying the most essential information for preliminary classification. It aims to give a first view of the data structure. Score plots (Fig. 5a) shows the overview of the group clustering based on the amino acid contents of the EBN samples from WM and EM in order to determine outlier, study possible trend and pattern. The first principal component (PC1) shows 73.9 % of variance while the second principal component (PC2) shows 16.7 % of variance. The sum of these two PCs account for 90.6 % of the original information of the data obtained. The values are considered appropriate when the total variance are more than 70 % (Seow et al., 2016). Moreover, the robustness of the PCA models can be explained by the goodness of the fit (R^2) and the predictive ability (Q^2), with 94.3 % and 78.6 % respectively. The results revealed these two components were well-fitted into the dataset.

For better visual illustrations and understanding, the score plots in the model were coloured. Green dots represent EBN from WM while red dots represent EBN from EM (Fig. 5a). The EBN samples from the EM formed a tight group which means good similarity between the samples. Unlike the EBN samples from WM that were different within their groups. The result could be due to most of the amino acid contents are more similar within the EM as compared to WM. Apart from that, some of the EBN samples in WM were overlapped with EM group. The result of this study indicates that the PC1 and PC2 were still unable to classify the two clusters. The reasons could be due to Malaysia has a different type of topography for each state and different location of swiftlet habitat consist of different insect species where mainly come from the forest, rice field, grassland and plantation (Yan et al., 2020). For example, the WM6 area has the most paddy fields in Malaysia, while at WM2 area is more concentrated with industrial activities. Previous investigations of the environmental factors regulating the insects' community from both forest and developed areas (Hashim et al., 2017; Hudin et al., 2019). Since swiftlet is an insectivorous animal in the ecosystem, insects play significant roles in food chains by providing energy for them. Thus, different vegetation area may contribute to a different population of insect and lead to a different composition of micro and macro bioactive compound in EBN (Lukman and Wibawan, 2018). Consequently, it might be contributed to the significant differences in amino acid composition in EBN. Based on the findings above, the data were further processed and analyzed by the supervised orthogonal partial least-squares discriminant analysis (OPLS-DA) approach.

3.3.2. Orthogonal partial least-squares discriminant analysis

The PCA score plot provides a good starting point for sample clustering, then application of a supervised OPLS-DA method was able to differentiate amino acid content of EBN from WM and EM. The OPLS-DA model was performed based on 18 types of amino acid content from WM and EM. Total 17 samples from WM and 7 samples from EM were randomly selected from the original data to construct a predictive OPLS-DA model. The remaining 3 samples from each group was used for testing set (blind test) to project into the predictive OPLS-DA model to validate the accuracy. Seven-fold cross-validation used as internal

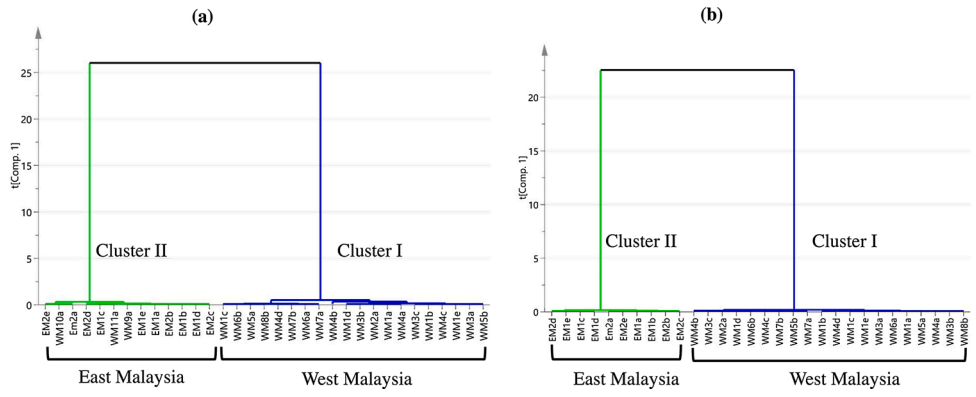

Fig. 4. Dendrogram of the cluster analysis of EBN samples (a) included with outliers samples (b) excluded with outliers samples.

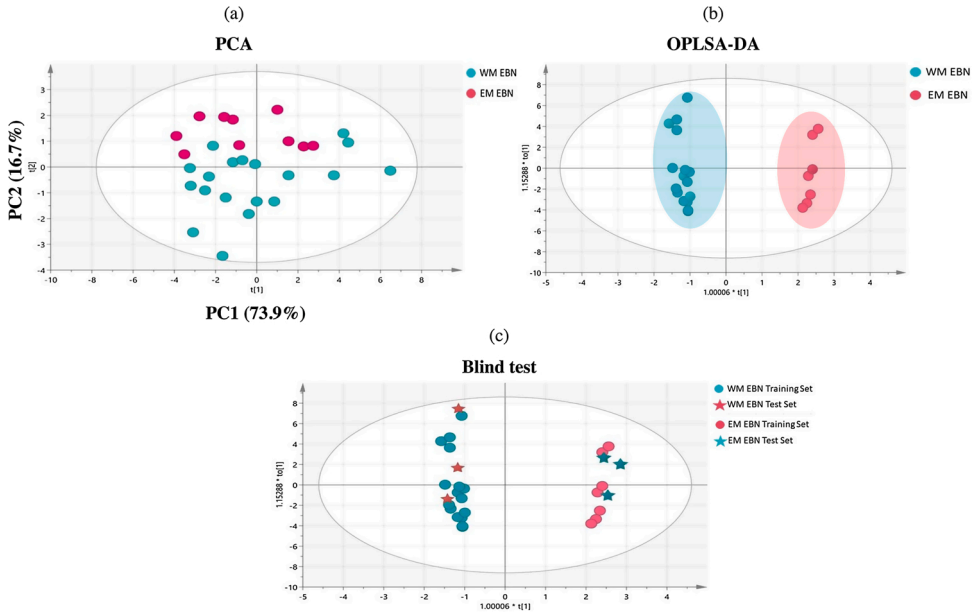

Fig. 5. Multivariate analysis conducted on the data of EBN extracts from WM and EM. Score plot for (a) PCA and (b) OPLS-DA models; (c) Blind test.

validating method to validate the prediction ability of the model. As shown in Fig. 5b, the OPLS-DA score plot showed a high degree of discrimination among the group. The robustness of the OPLS-DA model was supported by high R^2 (0.72) and Q^2 (0.63) values obtained, revealing great predictability and goodness of fit. Besides, the OPLS-DA score plot also revealed the R^2Y value of 0.99, deduced WM and EM had a good separation on its amino acid contents. Moreover, CV-ANOVA further confirmed the validation of the model and reported with *p*-value of 6.62×10^{-20}. The result indicates that the separation of EBN into WM and EM group was significant. The OPLS-DA score plot also showed the score scatters, indicating that sample classification is highly sensitive to classify EBN from WM and EM. Repetitive dots of different groups mapped on the OPLS-DA score plot showed a strong separation, suggesting that the WM and EM could be distinguished based on the content of the amino acids observed. Overall, the established OPLS-DA model was robust and it was strong enough for the further application to classify the EBN from WM and EM. This provides a credible base for the identification of EBN from WM and EM.

3.4. Model validation

United States Pharmacopoeia suggested that the chemometric model (OPLS-DA) has to be validated with an external set of samples that have been treated as 'unknown' to certify and evaluate the robustness of the developed predictive model. Their classification should be predicted to certify sample clustering is real. In this study, the predictive model robustness was assessed using external validation of the 3 samples from WM and EM group respectively. These samples were treated as an 'unknown' sample to validate the sample classification in the exact group and not over-fitting to the predictive model. This validation method which also known as "blind test" served as a figure of merit to ensure the accuracy of the model. The model classified all 6 samples correctly and the 3 EBN samples from WM and EM were grouped into their respective groups (Fig. 5c). It is interesting to find that the blind test sample classification accuracy was 100 %, and the 'unknown' EBN samples fall into their exact respectively group. A close similarity between the predicted ('unknown' EBN samples) and experimental values (EBN samples from WM and EM) were within 5 % which verified the validity of the OPLS-DA model.

3.5. Identification of potent amino acid markers

Potential amino acids of interest could be selected from the *S*-plot generated from validated OPLS-DA. In this case, the *S*-plot analysis was performed to select the critical variable (potential marker) to distinguishing the EBN from WM and EM. When the contribution of the variables to the discrimination is higher, the spots will be located at the ends of the plot. In the comparison between WM and EM, the higher amino acids in WM were showed in the lower left quadrant of the *S*-plot while the upper right quadrant showed the higher amino acids in EM (Fig. 6a). As a result, two amino acids content (glycine and cysteine) were higher in WM, while three other amino acids content (tryptophan, aspartic acid, and glutamic acid) were higher in EM. In addition, in order to determine the potential marker, the result of *S*-plot result was cooperated with variables importance in projection (VIP) values and its value should be greater than 1. VIP suggested the weightage of each variable within the model to differentiate between the two classes on the basis of their differences (Azmi et al., 2021). Fig. 6b shows that glycine, cysteine, tryptophan, and aspartic acid all have VIP values greater than 1. Thus, the key amino acids were selected based on two criterions: less than -0.6 and more than 0.4 in *S*-plot with the VIP value above cut-off (>1) are selected as potential markers. Therefore, the glycine, cysteine, tryptophan and aspartic acid were considered as the potential markers suggested by the model which could discriminate the EBN from WM and EM.

4. Conclusion

In this study, HPLC combined with multivariate analysis were applied to distinguish the amino acids profile between the EBN obtained from WM and EM for further classification. The HPLC method successfully quantified 18 amino acids content in EBN from WM and EM. Results showed that EBN are rich in amino acids and contain all the essential amino acids. Although most of the EBN samples showed similar total amino acids content in WM and EM, some significance differences in individual amino acids were found. Additionally, the use of OPLS-DA improved the recognition and prediction ability, allowing EBN classification according to its geographical regions (WM and EM). The optimum result in classification was obtained using OPLS-DA which offered the excellent separation of the "unknown samples" into their geographical region with the highest accuracy. Glycine, cysteine,

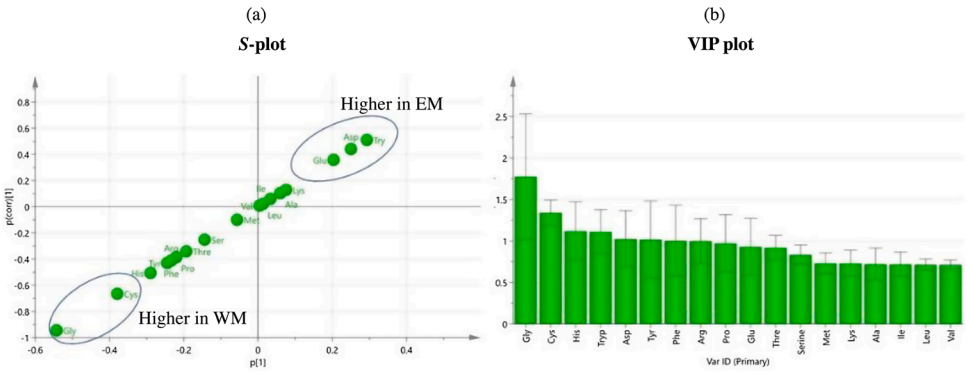

Fig. 6. Construction and assessment of potential markers based on OPLS-DA model for WM and EM. (a) *S*-plot of OPLS-DA model for WM and EM; Glutamic acid, tryptophan and aspartic acid content are higher in EM; Glycine and cysteine content are higher in WM. (b) VIP plot of OPLS-DA model. Abbreviation: Ala, alanine; Arg, arginine; Asp, aspartic acid; Cys, cysteine; Gly, glycine; Glu, glutamic acid; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

tryptophan and aspartic acid were the potential markers proposed by the OPLS-DA to classify the EBN from WM and EM. Overall, this study revealed the exceptional promise of OPLS-DA to classify EBN samples based on their amino acid contents. In the future, additional research with a larger number of samples in each class is needed to verify and establish the identification database of EBN in Malaysia.

Data availability

No data was used for the research described in the article.
Data will be made available on request.

CRediT authorship contribution statement

Ting Hun Lee: Conceptualization, Supervision. **Chia Hau Lee:** Formal analysis, Investigation, Writing - original draft. **Nurul Alia Azmi:** Methodology, Investigation, Writing - original draft. **Rock Keey Liew:** Writing - review & editing. **Norfadilah Hamdan:** Resources. **Syie Luing Wong:** Writing - review & editing. **Pei Ying Ong:** Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that there were no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2022.104399.

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