CHEMOMETRIC ANALYSIS OF AMINO ACIDS AND MINERALS COMPOSITION FOR TRACEABILITY OF EDIBLE BIRD'S NEST

by

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LIST OF ABBREVIATIONS

Ag	Silver
Al	Aluminium
ALA	Alanine
As	Arsenic
ASN	Asparagine
ASP	Aspartic acid
B	Boron
Ba	Barium
Be	Beryllium
Bi	Bismuth
Ca	Calcium
C-C	Cystine
Cd	Cadmium
Co	Cobalt
Cr	Chromium
Cu	Copper
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
EBN	Edible bird’s nest
EGF	Epidermal growth factor
Fe	Iron
Ga	Gallium
GC-MS	Gas chromatography-mass spectrometry
GLN	Glutamine
GLU	Glutamic acid

GLY	Glycine
HIS	Histidine
HLY	Hydroxylysine
HNO_3	Nitric acid
H_2O_2	Hydrogen peroxide
HYP	4-hydroxyproline
ICP-OES	Inductively coupled plasma-optical emission spectrometry
ILE	Isoleucine
In	Indium
IS	Internal standard
K	Potassium
LEU	Leucine
LOD	Limit of detection
LOQ	Limit of quantitation
LYS	Lysine
MET	Methionine
Mg	Magnesium
Mn	Manganese
Mo	Molybdenum
Na	Sodium
NANA	N-acetylneuraminic acid
Ni	Nickel
NO_2	Nitrite
NO_3	Nitrate
OPLS-DA	Orthogonal partial least square-discriminant analysis
Pb	Lead
PC	Principal component

PCA	Principal component analysis
PCR	Polymerase chain reaction
PHE	Phenylalanine
PRO	Proline
R2	Coefficient of determination
Sb	Antimony
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Se	Selenium
Si	Silicone
SIMCA	Soft independent modeling of class analogue
Sr	Strontium
THR	Threonine
Ti	Titanium
Tl	Thallium
TRP	Tryptophan
TYR	Tyrosine
V	Vanadium
VAL	Valine
Zn	Zinc

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ANALISIS KIMOMETRIK KOMPOSISI ASID AMINO DAN MINERAL UNTUK PENGESANAN SARANG BURUNG

ABSTRAK

Sarang burung ialah makanan tonik berharga tinggi yang digemari oleh komuniti Cina. Pengguna telah terpedaya untuk membeli sarang burung rumah pada harga premium sarang burung gua. Dalam kajian ini, satu kaedah yang boleh dipercayai dan tepat telah dicadangkan untuk pembezaan sarang burung rumah dan gua. Kalsium (Ca), natrium (Na), tyrosine (TYR) dan asid glutamik (GLU) telah dicadangkan sebagai pembolehubah discriminasi yang menjanjikan untuk pembezaan sarang burung rumah dan gua. Pendekatan yang sama diaplikasikan untuk pengesanan asal sarang burung rumah dari kawasan yang berlainan tetapi pemisahan antara kelompok yang terbentuk adalah tidak ketara. Justeru, profil asid amino dan mineral didapati bukan penunjuk yang sesuai untuk pengesanan asal sarang burung. Profil asid amino dan mineral yang ditentukan dengan gas kromatografi spektometri jisim (GC-MS) and induktif ditambah plasma spektometri emisi optik (ICP-OES), masing-masing telah dianalisa dengan analisis korelasi Pearson, analisis komponen utama (PCA) dan analisis perbezaan ortogon separa kuasa dua terkecil (OPLS-DA). Corak korelasi yang berbeza dan signifikan kelihatan antara pasangan asid amino dan pasangan mineral dalam setiap kumpulan sarang burung. PCA telah digunakan untuk mengkaji kemungkinan pengelompokan, di mana sarang burung rumah dan gua dapat dipisahkan oleh dua komponen utama (PC), iaitu PC1 dan PC3 yang mana menjelaskan 43.6% and 12.6% daripada jumlah variasi set data, masing-masing.

Model yang dibina oleh OPLS-DA didapati merupakan satu alat yang menjanjikan dengan kebolehan ramalan yang tinggi sebanyak 89.5%. Keteguhan model ini telah dikenalpasti dengan penetapan sampel test buta kepada kelompok masing-masing dengan tepat.

CHEMOMETRIC ANALYSIS OF AMINO ACIDS AND MINERALS COMPOSITION FOR TRACEABILITY OF EDIBLE BIRD'S NEST

ABSTRACT

Edible bird's nest (EBN) is a high-priced tonic food favored by the Chinese community. Consumers have been deceived into buying house-farmed EBN at premium price of cave-harvested EBN. In the present study, a reliable and accurate method was proposed to differentiate EBN of house and cave origin. Calcium (Ca), sodium (Na), tyrosine (TYR) and glutamic acid (GLU) were proposed as promising discriminating variables for differentiating between house and cave EBN samples. Similar approach was applied for provenance traceability of house EBN from different regions but the clusters formed were not distinctly separated. Thus, amino acids and minerals profiles have been found not able to serve as good indicators for provenance traceability of EBN. The amino acids and minerals profile determined by gas chromatography-mass spectrometry (GC-MS) and inductively coupled plasma-optical emission spectrometry (ICP-OES), respectively were analyzed using Pearson correlation analysis, principal component analysis (PCA) and orthogonal partial least square-discriminant analysis (OPLS-DA). There were significant different correlation patterns seen between different amino acids pair and minerals pair within each EBN group. PCA was applied to study possible clustering, wherein house and cave EBN were separated by two principal components (PC), PC1 and PC3 which explains 43.6% and 12.6% of the total variability in data set, respectively. The model constructed by OPLS-DA was found to be a promising tool with high predictive ability of 89.5%. Robustness of the model was validated and blind test samples were correctly assigned to their respective cluster.

CHAPTER 1

INTRODUCTION

1.1 Background

Edible bird's nest (EBN) is highly consumed by the Chinese community, because they uphold the belief handed down based on anecdotal evidences that EBN is beneficial to relief respiratory ailments and enhance body energy. The work by Kong *et al*. (1987), who suggested the presence of epidermal growth factor (EGF)-like substance in EBN, has drawn the attention of consumers as well as researchers. Since then, extensive research activities have been conducted to confirm the presence of EGF-like substance in EBN and its potential use in medical field and cosmetic industry for cell proliferative effect. This idea was substantiated by positive results reported in studies using human adipose-derived stem cells (Roh *et al*., 2012), corneal keratocytes (Zainal Abidin *et al*., 2011) and Caco-2 cells (Aswir & Wan Nazaimoon, 2010). Apart from that, EBN extract has been found effective in curing erectile dysfunction (Ma *et al*., 2012), improving bone strength and dermal thickness (Matsukawa *et al*., 2011) and inhibiting influenza virus infection (Guo *et al*., 2006).

EBN has been the sought after as lavish tonic food since Tang Dynasty (Lim, 2006). Generally, EBN is built by gelatinous strand of nest cement secreted by swiftlets, namely White nest swiftlet (*Aerodramus fuchipagus*) and Black nest swiftlet (*Aerodramus maximus*) during breeding seasons (Koon & Cranbrook, 2002). These swiftlets are found in the South-East Asia region and inherently inhabit in the caves (Chantler & Driessen, 1999). Comparatively, EBN produced by the White nest swiftlet is of higher economic value as it is entirely made of pure salivary nest cement with only traces of impurities. On the other hand, though the nest of Black nest swiftlet is full with feathers and requires tedious cleaning process, it is still heavily harvested as the exploitation is worthwhile due to the fact that the nest is of high price.

With the increasing demand of EBN, the price of this product is skyrocketing as the stock available in the market could not fulfill the growing needs. A recent survey reported by Manan & Othman (2012) revealed that the raw pre-processed EBN was sold at RM 3000/kg to RM 4500/kg in the market in year 2010 to 2011. The market price of EBN is always doubled after the laborious and time consuming cleaning process (Lim, 2006). Therefore, many investors are lured by the lucrative revenue and ventured into EBN house-farming. Efforts have been done by the house farmers to ensure that only the pure breed of White nest swiftlet, which could produce EBN of high commercial value, would inhabit and breed in the farm (Lim, 2006). Unfortunately, EBN harvested from the house farm is much lower priced in the market than those harvested from the cave.

Driven by the unscrupulous desire, unethical EBN manufacturers tend to adulterate cave EBN with lower price house EBN, some even make intentional false claims by selling house nest as cave nest. Besides, adulteration of EBN with addition or substitution with less expensive materials such as egg white, *Tremella* fungus, gelatin, karaya gum, fried porcine skin, starch, soybean and red seaweed (Ma & Liu, 2012b; Marcone, 2005), is commonplace.

Authentication methods at molecular level using Taqman-based real time PCR (Guo *et al*., 2014), combination of DNA based PCR and protein based two dimensional gel electrophoresis methods (Wu *et al*., 2010), DNA sequencing-based method (Lin *et al*., 2009) and SDS-PAGE electrophoresis (Marcone, 2005) have been proposed. However, these techniques are rather tedious, time-consuming and costly.

The aim of this study was to distinguish EBN samples harvested from the cave and the house farm based on amino acids and minerals profile analyzed using gas chromatography-mass spectrometry (GC-MS) and inductively coupled plasma-optical emission spectroscopy (ICP-OES), respectively. Correlation of amino acid and mineral pairs within each group of sample was analyzed using Pearson correlation analysis and unsupervised principal component analysis (PCA) and supervised orthogonal partial least square-discriminant analysis (OPLS-DA) were employed to investigate the relationship between amino acids and elemental concentration and the type of EBN samples studied. Construction of classification model for determination of unknown samples was also carried out.

1.2 Problem statement

There is no protocol for differentiation and traceability to the origin of edible bird's nest, consumers could be duped into buying such counterfeit products.

1.3 Objectives

The general objective of the present study was to develop a protocol for the differentiation and provenancing of edible bird's nest. Two specific objectives of the study are listed as follows:

a. To propose the use of amino acids and minerals profile as discriminating variables for authentication of house nests and cave nests.
b. To discern the bird's nest of different geographical origins based on amino acids and minerals profile.

1.4 Hypothesis

Nutritional composition of cave and house edible bird's nests might be different due to significant different habitat macro- (insect species available, geographical locations, etc.) and micro- (supporting materials, air quality, etc.) environmental factors.

CHAPTER 2

LITERATURE REVIEW

2.1 Edible bird's nest (EBN)

Bird's nest is generally made to serve as a shelter for breeding. Different from other birds that construct nests using grass, twig, sticks, muds and etc., swiftlet is known to be unique in its nest building behavior in a way that it produces edible bird's nest (EBN) using saliva. The edible nest swiflets from the *Collocaliini* tribe under *Apodidae* family, could be further classified into two main divisions: non-echolocating Glossy swiftlets, genus *Collocalia* (Gray, 1840) and echolocating swiftlets, genus *Aerodramus* (Oberholser, 1906). White-bellied swiftlet (*Collocalia esculenta cyanoptila*) and Kinabalu swiftlet (*Collocalia linchi dodgei*) are two common species found that fall under genus *Collocalia*. Two species under *Aerodramus* which are heavily exploited are White-nest swiftlet (*Aerodramus fuciphagus*) and Black-nest swiftlet (*Aerodramus maximus*). *Aerodramnus fuciphagus* could be subdivided into *Aerodramus fuciphagus vestitus, Aerodramus fuciphagus amechamus, Aerodramus fuciphagus perplexus* and *Aerodramus fuciphagus fuciphagus*, while two common Black-nest swiftlet seen are *Aerodramus maximus lowi* and *Aerodramus maximus tichelmani* (Koon & Cranbrook, 2002). However, among the 24 species of swiftlets identified in the world, only three species (i.e. *Aerodramus fuciphagus, Aerodramus maximus* and *Collocalia esculenta*) that produce edible nests are found in Malaysia. According to Lim (2006), little is known about the distribution of subspecies of *Aerodramus fuciphagus* in Peninsular

Malaysia and it was suggested that species inhabit in coastal areas and inland may be different.

Disputes over the taxonomic affinity and classification of swiftlets' species had not been resolved for years until the taxonomic conventions, which proposed the use of molecular approach for classification, which till now is widely accepted and followed (Stimpson, 2013; Thomassen *et al*., 2005, 2003; Lee *et al*., 1996). The classification originally started with a single genus, i.e. *Collocalia* (Gray, 1840) and was later subdivided by Brooke (1970) into three genera: *Collocalia*, *Aerodramus* and *Hydrochous* by taking into account the echolocating ability. It is interesting to note that the original classification with one genus was reused by Salomonsen (1983) and Chantler & Driessens (1995). Sibley & Monroe (1990) then reclassified the swiftlets into two genera which are *Collocalia* (including *Aerodramus*) and *Hydrochous* and again, classification proposed by Brooke was used by Del Hoyo *et al.* (1999). The several attempts of reshuffling the swiftlets into different number of genera were actually based on outer morphological characters of the nests but apparently the reliability was not significant (Thomassen *et al*. 2003). The echolocating ability was once thought as one of the useful characteristics to separate the *Aerodramus* from the *Collocalia*. The discovery of pygmy swiftlet (*Collocalia troglodytes*) with the ability to echolocate has subverted the postulation and the echolocating ability was suggested to be a synapomorphy of both genera which could have been lost in most *Collocalia* during evolution (Price, 2004).

The lack of exposure and knowledge about EBN had induced people in the old days to generate and create stories which were repleted with myths, legends and strange beliefs regarding the origin and composition of the nests. The earliest known record described swiftlets as birds fed on certain mollusc with two very strong and white fine tendons, which were believed to contain tonifying, strengthening and antitubercular properties. It was believed that the tendons were indigestible by swiftlets and hence being spitted out together with saliva for nest building (Koon & Cranbrook, 2002; Sallet, 1930). A postulation made by Bontius (1658) was that swiftlets built their nests with a foam of sea water and Ray (1678) had a different opinion and suggested the nest building materials were actually whales' sperm or fishes. Another surmise proposed by de Rhodes (1653) was that the birds sucked the scented timber tree and mixed it with sea froth as materials for nest construction. An idea which accurately postulated by Rumpf was that EBN was built using saliva secreted by the swiftlets. However, this suggestion was not accepted by Wood who strongly believed the nest materials are actually seaweeds (Koon & Cranbrook, 2002).

Interestingly, EBN produced by different species of edible nest swiftlets carry different economic values. The nest build by White-nest swiftlet is inevitably the most sought after nest with the highest quality, which attracts immense commercial interest. The half-cup shaped nest adheres to the rocky surface of cave wall, composed of almost entirely of pure salivary nest cement, with only traces of impurities such as plumage and faeces. It is formed by strands of nest cement that gradually frame the shape, which is self-supporting and attach firmly to the supporting surface. Unlike White-nest swiftlets, Glossy swiftlets and Black-nest swiftlets' nests are not solely constructed by nest cement but with their feather and impurities incorporated. The edible portion of nest for Black-nest swiftlets and Glossy swiftlets is 10-15% and 1-2%, respectively. These nests require laborious and tedious cleaning process and thus considered as nest of inferior quality. Raw unprocessed nests of Black-nest swiftlets are sold at the market price of one fourth or one fifth lower than White-nest swiftlets'. Yet, driven by the lucrative profits, the edible part of these nests could be extracted in order to cater to the high demands (Koon & Cranbrook, 2002; Sankaran, 1998; Lau & Melville, 1994).

Edible nest swiftlets are cave dweller with a lifespan of around 15-25 years (Manan & Othman, 2012) and they are predominantly found in limestone caves (Ma & Liu, 2012b). Flying paths of edible nest swiftlets are discovered to be confined to India sub continental, Hainan island in the South of China (Lim, 2006) and South-east Asia regions, including Cambodia, Indonesia, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam (Koon & Cranbrook, 2002). They are non-migratory (Manan & Othman, 2012) and exhibit colonial behavior which is likely to flock with conspecifics (Sankaran, 1998). More than one species of swiftlets could be inhabiting within the same cave but different species are probably seen building nests in their own associated groups (Koon & Cranbrook, 2002). According to Sankaran (1998), caves in the Andaman islands could be occupied exclusively by swiftlets, or bats, or both. It is worth noted that reduction of swiftlets population could be due to ecological problem where their nesting space is tenanted by other cave dwellers especially bats.

2.2 Physical appearance of swiftlets

Very often, sparrows (*Passeride*) and swallows (*Hirundinidae*) that could be prevalently seen on electric lines have been mistaken as the edible nest birds. Albeit their size and appearance resemble to swiftlets, swiftlets still possess distinctive characteristics that make them distinguishable from others. Swiftlets' legs are weak and short that they couldn't even walk or perch. Nevertheless, they normally cling with the aid of their sharp and re-curved claws, on the rim of nest at night. Not only that, swiftlets are known to have more rapid flight strokes in addition to possessing acute eyesight. The privilege of having the ability to fly at greater manouverability and velocity facilitates the foraging activity with their short bill and wide gape. Swiftlets are aerial insectivores fed on airborne insects and they capture the flying insects and water droplets in the air with their mouth open while flying (Koon & Cranbrook, 2002). The swiftlets are feeding their young with pellets of compressed insects which are diverse arthropods with weight ranging from 0.01-0.69 g (Lourie & Tompkins, 2000; Medway, 1962b). Study on swiftlets' diet using food boluses has discovered the swiftlets' preferences where food boluses of black-nest swiftlets made up of more large-bodied hymenoptera and less diptera; more coleoptera in boluses of Glossy swiftlets while white-nest and Mossy-nest swiftlets' diets demonstrated no significant difference but with the white-nest swiftlet's prey size being significantly smaller. The diversity of insects found in white-nest swiftlets' diet also suggested that this species is possibly well-adapted to the environments with different preys as compared to the black-nest swiftlets with more specialized diet, that make them the suitable candidate and target of swiftlet farming industry (Lourie & Tompkins, 2000).

Swiftlets initiate their foraging activity in early morning and return to the roosting place when the sunlight fades. Owing to the limitation of their morphological feature, they have no chance to perch but to spend the day entirely on their wings (Koon & Cranbrook, 2002). Flying with mouthful of whole day's catch, swiftlets navigate their way home via echolocation, a simple yet effective way (Medway, 1959; Novick, 1959). They utter the echolocating call (a succession of clicks) at a range of frequencies for human hearing, in a dissimilar pattern according to species. Black nest swiftlets emit a single click while white nest swiftlets utter double clicks, with a silent interval of merely a couple of milliseconds. Thomassen *et al.* (2004) has discovered that a number of echolocating swiftlet species emit both single and double clicks but the use of single clicks occasionally remains unknown. Unlike bats that echolocate to detect surrounding prey by the returning echoes, swiftlets' echolocation call is comparatively simpler and less sensitive, which is mainly aimed at detection of obstacles in dimly lighted areas and for orientation in the total darkness of caves. Emission of echolocation directs swiftlets to return to their roost as dusk approaches, with the super memory conferred to trace their own nest among thousands of others. Swiftlets only start clicking when they are approaching the cave entrance where the light is not sufficient for them to see. In addition, they may increase the rate of clicking when they are approaching obstacles, wall, or their own nests, for a clearer picture of the soundscape (Koon & Cranbrook, 2002).

2.3 Nest building and breeding

Instead of selecting cavities in trees or man-made structures such as buildings as breeding sites, swiftlets normally build their nests on the rock surfaces in cave. Nest building is usually accomplished by a pair of swiftlets during the breeding seasons: August-November, December-March and April-July (Manan & Othman, 2012). Both parent swiftlets are responsible in constructing the nest using the salivary secretions from sublingual salivary glands beneath the tongue. This is evidenced by a recent research which discovered numerous minor salivary glands in the lingual apparatus of White-nest swiftlets that could provide copious amount of saliva for nest building (Shah & Aziz, 2014). Interestingly, the glands are only activated during nesting and breeding periods which will expand to achieve 160 mg in weight from 2.5 mg (inactive state), for maximum secretory activity (Medway, 1962a). According to Kang *et al*. (1991), production of saliva and egg formation requires body energy reserves. Thus, female swiftlet that has greatly consumed energy for both processes is less actively participating in nest building as compared to male swiftlet (Ramji *et al.,* 2013). Salivary nest cement which is freshly produced is sticky and soft but it binds firmly and strongly to the rock wall as supporting surface when it slowly dries and hardens due to the air exposure. It is made up of irregular thin strands of salivary materials that gradually expand layer by layer daily to form the desired size of half-cup shaped nest, which could support the weight of eggs (Lim, 1999) and accommodate the swiftlets nestlings at the later stage (Koon & Cranbrook, 2002). White-nest swiftlets normally take 30 days to complete a nest wholly made up of saliva (Medway, 1969) while 35-125 days are required for a nest constructed using both saliva and feathers by Black-nest swiftlets (Koon & Cranbrook, 2002). Approximately 7-10 days are needed before they lay the first egg in the shallowed bowl-shaped cavity. A new nest will be rebuilt on the same site instantly which requires shorter period of time if the nest is harvested at this stage. However, a delay of 10-14 days is expected if the nest is removed together with the eggs or nestlings inside (Manan & Othman, 2012, Koon & Cranbrook, 2002). Nguyen Quang (1994) has found that white-nest swiftlets build their nests in dry season and start breeding in the first rainy season when the aerial insects are in abundance.

Edible nest swiftlets pairs are sedentary and they are special for their faithfulness to their nest sites (Sankaran, 1998). Both parent swiftlets are involved in incubation of the eggs but the assiduity of either gender remains unknown due to their indistinguishable appearance. Unlike White-nest swiftlets that produce two eggs per clutch, Black-nest swiftlets lay only a single egg per clutch. Under the multi-brooded reproductive strategy, swiftlets try to optimize the production of clutches and raise the young birds during the favorable breeding periods (Koon & Cranbrook, 2002). Given that a conducive and safe environment is provided during breeding seasons, swiftlets will attempt to achieve greater annual breeding success by laying eggs. It might be rare but it is not uncommon for some pairs of swiftlets to produce a fourth clutch (Phach & Voisin, 1998).

2.4 Cave-harvested edible bird's nest (EBN)

Edible nest swiftlets are cave dwelling animals that were discovered in the Andaman and Nicobar islands of India, Szechuan and Hainan island of China, Palawan island of Philippines, Cambodia, Vietnam' coasts and islands, Myanmar, Thailand, Peninsular Malaysia and Borneo, Singapore and the Indonesian archipelago such as Java, Sumatra and the Lesser Sunda islands (Manan & Othman, 2012; Lim, 2006; Koon & Cranbrook, 2002). Other than the White-nest swiftlets which produce premium quality of edible bird's nests, other edible nest swiftlets i.e. Glossy swiftlets and Black nest swiftlets are also found to reside in the caves (Koon & Cranbrook, 2002; Sankaran, 1998). The caves are not exclusively for only one species, White-nest swiftlets and Black-nest swiftlets are normally building their nests in the total darkness area of caves in their own colonies. Nesting sites of Glossy swiftlets are rather unique as they would colonize the caves' mouths and entrance passages. This is due to their limitation of being non-echolocating swiftlets which are unable to navigate in the dark (Koon & Cranbrook, 2002). A study on the nest site preference of white-nest swiftlets has found that this species prone to select the smooth and concave surface with supporter as their nesting site. These characteristics could serve as contributing factors for the development and enhancement of the swiftlet farms' wall structure (Viruhpintu *et al.* 2002).

EBN as a valuable commodity in maritime trade could be traced back to Tang (AD 618-907) or Sung (960-1279) dynasties, as evidenced by the discovery of iron harvesting tools among the ceramics of the above-mentioned dynasties in Niah Cave, Sarawak which suggests Chinese merchants have possibly stepped into Borneo those times. Another saying was that EBN was introduced to China by Admiral Cheng Ho, the well-known eunuch of Ming dynasty for his voyages to the South Seas. The belief was supported by his seven magnificent voyages which covered major EBN regions. However, there are no written sources as references to support the views and the first Chinese literature mentioned about EBN is *Yin Shih Hsu Chih* written by Chia Ming. Initially, the ownerships of the caves were claimed by the indigenous people who discovered them. Personal or shared proprietary rights are applied to caves owned by personal or family, and communal caves, respectively. Nowadays, caves in certain places have been appropriated by the government and are tendered to competitive private contractors. Nevertheless, driven by distinctive social and environmental factors, various harvesting routines have been practiced at different areas. Despite of some owners who are aware of the importance of sustainability of edible nest swiftlets and EBN, there are still people allured by the lucrative monetary returns who caused over-exploitation of EBN. Therefore, rules and regulations are now set to govern the harvest and trade of EBN. They will only harvest the nests with considerable size in every May and November (set by Bird's nest Association), or sometimes only once a year (Koon & Cranbrook, 2002). It is also suggested to leave the EBN for at least 85-90 days and only start harvesting after the offspring leave the nests (Manan & Othman, 2012). The cave owners normally don't harvest the nests by themselves but sub-contracted or sub-leasing to others to hire skilled collectors for the painstaking and risky nest harvesting. They will gain the revenue in a passive way by sharing certain percentages from the profit. In order to safeguard the caves, temporary shelter, tents and guardhouses are set up to prevent invasions into caves.

Raw pre-processed white nests and black nests freshly harvested from the caves are sent to cleaning houses or processing centres before they are ready for sale. The nests are soaked in water to soften them to ease the removal of feathers and plumages manually using tweezers. White nests with traces of tiny plumages picked will be placed on mold to restore their original half-cup shape form for drying. Likewise, clean water is used for soaking black nests but with minute amount of cooking oil added to separate the large feather through floatation method. The subsequent steps in the process are rather more tedious and laborious. Since it is the nature of Black-nest swiftlets' building behavior to incorporate feather in nests, to remove them from the loosened laminae is apparently challenging and time-consuming. During the first treatment in the processing stages, the basal parts of the nests with edible materials are sorted out. Later, the edible portions of proper structures with lesser feathers are collected. Finally, chips are used for rearrangements and molding of the cleaned strands into different shapes which are then dried, packaged and prepared for sale (Babji *et al*., 2015).

Five different types of EBN with different colors and qualities are arranged in an ascending order: feather nests, yellow nests, white nests, silver nests and red nests (Manan & Othman, 2012). This grading system is less popular wherein people usually differentiate the nests only to either white nests or black nests, if they were to grade them based on colors. According to Marcone (2005), red nests or blood nests are much sought after premium nests of superior quality than white nests. There is legend which postulates that exhausted swiftlets rushing in completing the nest with blood in their saliva yielded blood nest with red color. There were investigators who suggested nesting materials were secreted by the swiftlets' own bodies which may be mixed with blood (Koch, 1909). Certain groups linked the red color to the oxidation of iron in the cave percolation water or swiftlets' saliva (But *et al.*, 2013; Lim, 2006), mollusks and seaweeds foraged from the seacoast areas, and artificial dyes (But *et al*., 2013). However, a research conducted by But *et al*. (2013) has proved that the red color is induced by the vapors from guano droppings or sodium nitrite.

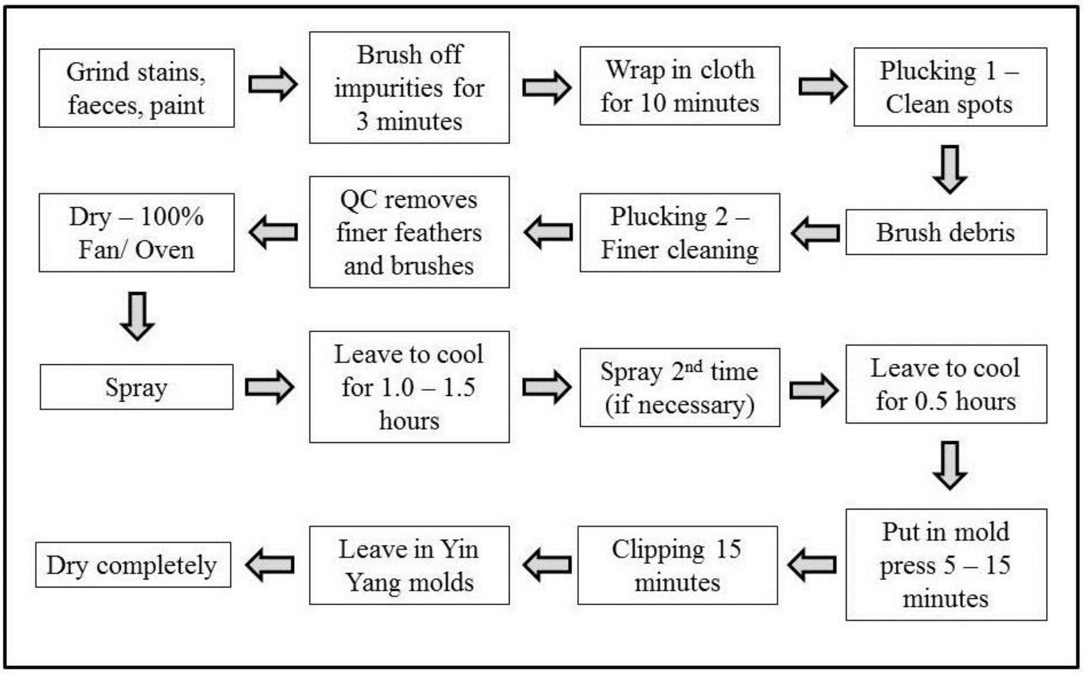
2.5 House-farmed edible bird's nest (EBN)

The idea of setting up swiftlet farms which could fetch lucrative returns is initiated in 1880 by the discovery of swiftlets in abandoned houses in East Java, Indonesia. Tremendous efforts have been put in to modify and improve the house conditions to mimic a cave-like environment conducive for swiftlets to visit and settle, which leads to a new era of semi-intensive farming in the 1970's in Indonesia (Koon & Cranbrook, 2002; Lim, 1999). Collective efforts with trial and error yield encouraging and positive results where many wild swiftlets have been attracted to build their nests inside the house farm. The employability of cross-fostering technique by swapping the other species of swiftlets' eggs with the eggs of the White-nest swiftlets is practiced to ensure a pure breed of desired species with premium nests quality is fostered (Lim, 2006).

Similar spontaneous colonization of swiftlets in old shop houses in Peninsular Malaysia around 1940's was evidenced in a study on White-nest swiftlets in Penang (Langham, 1980). The spread of intensive farming techniques in Peninsular Malaysia have encouraged the growth of EBN industry by the burgeoning of swiftlet farms in Penang, Perak, Kelantan, Pahang, Terengganu, Malacca and Johor in 1995. The success in Indonesia and Peninsular Malaysia has motivated people in Sabah and Sarawak to invest and venture into this wealth-creating business. Conversion of shop lots to swiftlet houses occurred following the discovery of White-nest swiftlets' nests in a shopping complex (Koon & Cranbrook, 2002; Lim, 1999). According to Koon & Cranbrook (2002), the swiftlet houses were first built near the coast but they are now setting up inland such as in paddy fields, oil palm plantations, highways and even in town areas. For a newly established swiftlet farm to attract swiftlets effectively, there are points to take note such as temperature and humidity control, and internal cleanliness monitoring. To facilitate the monitoring of swiftlet farms' conditions, a wireless sensor networks was developed and the monitoring system could be accessed by remote control provided there is internet connectivity (Othman *et al.*, 2009). The internal environment should be maintained as closely as the cave-like conditions and guanos with foul odor which attracts flies should be cleaned regularly to avoid spread of potential diseases and breeding of mosquitoes (Alias *et al.*, 2013). Farmers might be able to turn the swiftlets faeces into gold in the future as there has been a study on its nutritional composition to look into the guanos' potential as fertilizer and protein or nitrogen source for livestock (Azizon *et al*., 2013). Swiftlets' chirping sounds recorded from caves are used as external and internal sound to first attract the swiftlets to enter the farms and secondly encourage them to build their nests inside (Lim, 2006). Characteristics of swiftlets' attraction sounds that invite swiftlets have been identified and are expected to benefit the swiftlet farming industry in the future by attracting swiftlets more effectively (Zaini *et al*., 2013). Wooden planks are usually set up to maximize the nest building sites to accommodate more swiftlets, with Light Red Meranti (*Shorea acuminata*) as a preferable choice by farmers (Manan & Othman, 2012). To assure a better quality of nest, it is essential to avoid the Meranti wood which comes with odor, unnatural dampness and discoloration that indicates possible contamination (Lim, 2006). Every step from cleaning of the nests to the point of export has to follow procedures which comply to the Malaysian Standards set by Ministry of Health, Malaysia.

People who don't have sufficient knowledge of swiftlets' foraging behavior and how the swiftlets farming operate might make a bad impression towards this activity. It is not surprising to know that they are misled by the common misconceptions and assume that the swiftlets in the houses are captured and their activities are completely restricted within the houses. Potential farmers will have to swear and obey the "Hippocratic" Oath prior to enroll into this profession and they are responsible of protecting the nests with offspring and ensuring the swiftlets are free from any physical or psychological harm (Lim, 2006). For easy understanding of how it works, swiftlet farming is associated with apiculture like bees farming, whereby swiftlets are completely free to fly and forage outside the purpose-built houses and back to roost at night. Contrary to common misconceptions, swiftlet farm served as an alternative or optional roosting and breeding place for swiftlets. Unfortunately, house farming does not help to reduce extinction risk of swiftlets in natural habitats due to excessive harvesting practices (Koon & Cranbrook, 2002; Sankaran, 2001).

As the process to retain the soaked EBN in their original half-cup shaped is quite a challenging step, premium grade white nests are being excluded from cleaning by some processors (Ma & Liu, 2012b). This problem is solved with the development of cleaning protocol but the steps in cleaning swiftlet nests might be varied according to different processing centres or cleaning houses which their practices and routines are normally not disclosed to the public. One of the cleaning process with the steps described in detail is showed in Figure 2.1. Effective cleaning ensure final products which are presentable on table for consumption besides meeting consumers' expectation of safe foods with minimal nutrient loss.



(Adapted from: Manan & Othman, 2012)

Figure 2.1 Swiftlet nests cleaning process.

Apart from the systems that classify and categorize EBN into white nests and black nests; or cave nest and house nests, there is another classifying system commonly practiced by the swiftlet farming industry wherein cleaned house-farmed nests are sorted and categorized into different grades based on their colors and shapes. The criteria for each grade are tabulated in Table 2.1. Current EBN grading system judges and inspects the quality of nests according the shape, size and weight by a group of panels. Realizing the inconsistency occurs in human judgment, an approach that applied Fourier descriptor and Wilk's lambda based discriminant analysis has been proposed and this quality assessment based on shapes could differentiate them into different groups accurately (Syahir *et al*., 2012). According to Ma & Liu (2012b), determination of grades for EBN is based on its dry mass, the duration of nest building and its fat and protein content. Recently, there is also an approach introducing the implementation of the fuzzy Failure Mode and Effect Analysis (FMEA) methodology to EBN processing (Jong *et al*., 2013). Two enhanced model, i.e. clustering-based FMEA (Tay *et al*., 2015) and single input rule modules connected fuzzy FMEA (Jong *et al*., 2014) were then introduced. The methodology is expected to serve as a quality and assessment tool for the production of EBN from swiftlet farming to the packaging of EBN, where the causes and effects of failure are identified and the risks of failure could be minimized or eliminated.

Table 2.1 Different grades of edible bird's nest.

Grade	Color	Remarks
A	Clear, pearl-like	• Perfect shape (like the letter "D") • Dense structure/ pattern • Less feathers
B	Light yellowish	• A bit of feathers • Imperfectly shaped
C	Whitish yellow	• Many feathers • Imperfectly shaped
D	Not specified	• Spoilt/ crumbly nests • Different shape from Grades A, B or C • Includes nests which have been eaten by ants etc

(Adapted from: Manan & Othman, 2012)

2.6 Market price

In the old days, traders classified EBN into white nest of premium quality and black nest of inferior quality. In 1845, the prices for one kilogram of raw pre-processed EBN at different grades were: RM58-66 for white nests, approximately RM46 for second grade white nests and as low as RM0.50-1.75 for black nests. The prices (per kg) increased steadily over years but dropped during world wars and revived to RM5000-6800 for raw white nests and RM400-1500 for raw black nests during 1996-2001. It is not surprising to find that larger and whiter pieces of nests were sold at RM7000-12000 per kilogram during that period (Koon & Cranbrook, 2002). According to Lim (2006), the selling prices of raw pre-processed EBN were RM3500-5500/kg and the export prices were RM8000-12000/kg of processed EBN in year 2006. In the period of 2010-2011, market price of raw pre-processed nests and processed nests were RM3000-7500/kg and RM10000-18000/kg, respectively. The average price of raw uncleaned cave EBN (black nest) was RM2500/kg while raw uncleaned house EBN was priced at RM3867/kg (Manan & Othman, 2012).

Current market prices of cleaned cave EBN are sold at RM19000-30000/kg and the prices of cleaned house EBN were in the range of RM4000-9000/kg. Renowned for its nutritional and medicinal merits, and challenging harvesting process, EBN could be the most expensive animal product (Ma & Liu, 2012b). Chinese consumers from China (especially Hong Kong), Taiwan, Singapore and North America makes up the primary market for EBN and there is a growing interest among the consumers from Middle East, Japan and Korea (Babji *et al*., 2015). It is expected to generate revenue from the trade of EBN to achieve USD $3.6 billion in year 2020 from USD $0.5 billion (Sharifuddin *et al*., 2014).

2.7 Preparation and cooking of edible bird's nest

EBN is a restorative dish and is always associated with the social status, wealth, power and prestige (Marcone, 2011). To ensure that consumers are benefited from the consumption of EBN, "mild cooking" should be employed to avoid loss of nutrient and functional bioactive compounds. "Mild cooking" refers to the double boiling of EBN using the stewing principle. According to Lim (2006), house nest and cave nest are normally double boiled for 30 minutes and 3 hours, respectively before consumption. Koch (1909) mentioned different ways of cooking EBN where the Chinese normally boiled it gently together with capon or duck for 25 hours, the Japanese served it cold after boiling it into slimy mass and mixed with sugar and the European epicures preferred to have it boiled in a strongly spiced broth that could stimulate their appetites. EBN by itself has no distinctive taste (Ismail, 2004). Nowadays, the common cooking practice is to double-boil the EBN together with rock sugar (Marcone, 2011) and is served as either hot or cold bird's nest soup depending on individual preferences. It is also advisable to consume the bird's nest soup at bed-time for health enhancing purposes (Koon & Cranbrook, 2002). EBN has also been bottled and marketed as ready-to-drink instant product which could save up the hassle for preparation and cooking of this health food.

2.8 Proximate and nutritional composition

Instead of serving as pleasant food to savor, EBN is deemed as catholicon which is believed to contain nutritional values which could benefit the consumers. Despite renowned as an expensive Traditional Chinese medicine, EBN is still vastly consumed especially by the Chinese community. They uphold the belief handed down based on anecdotal evidences that EBN is beneficial in enhancing immunity and body energy restoration. Nevertheless, scientific research on the chemical composition of EBN which justifies the function of nutritive compounds is still in paucity and the underexplored area needs further investigations.

According to Ma & Liu (2012b), the proximate composition of EBN arranged in a descending order is: protein (42-63%), carbohydrate (10.63-27.26%), moisture (7.5-12.9%), ash (2.1-7.3%) and fat (0.14-1.28%). Moisture content often serves as index of stability and quality and EBN with high protein content signifies the availability of good feeding environment for swiftlets (Hamzah *et al*., 2013a). White nests were lighter than black nests and 8% of the black nest total protein was attributed by the presence of feathers. White nests were found to contain 4% and 7% more of lipid and protein content, respectively as compared to black nests (Kang *et al*., 1991). The composition implies that EBN is largely constituted of glycoproteins, the proteins with sugar units attached, which possesses both protein and carbohydrate properties and play a remarkable role in biological systems (Cole & Smith, 1989; Wang, 1921). Protein characterization conducted by Utomo *et al*. (2014) has discovered the presence of glycoprotein only in white EBN and not in either black or swallow EBN. For research studies characterizing the nest composition or investigating the bioactivities using glycoproteins, *Collocalia* mucoid (approximately 50% carbohydrate) is usually obtained from EBN using the aqueous extraction method proposed by Howe *et al*. (1961), often with slight modifications or at different extracting temperatures (Ma & Liu, 2012b). A study on *Collocalia* mucoid was carried out and this glycoprotein was found to contain approximately 9% sialic acid (probably *N*-acetyl-4-*O*-acetylneuraminic acid), 16.9% galactose, 7.2% galactosamine, 5.3% glucosamine, 0.7% fucose and high amount of amino acids such as serine, threonine, aspartic acid, glutamic acid, proline and valine (Kathan & Weeks, 1969). All essential amino acids present in EBN (Ma & Liu, 2012b) and white EBN contains higher aromatic amino acids content (tyrosine and phenylalanine) as compared to red EBN (Marcone, 2005). Tyrosine and phenylalanine are associated with their effects as antidepressant and pain reliever, respectively. Hence, EBN could be a choice of supplement for consumers for stress effect alleviation and increase in their pain threshold (Young, 2007; Walsh *et al*., 1986). EBN is recognized as popular highly nutritious food but its amino acids content was found to be actually quite low (Ang *et al*., 1984). The nest protein is also claimed as of inferior quality and it is definitely not an option as staple foods or source of complete protein if taken alone, but as a supplementary constituent (Ma & Liu, 2012b; Koon & Cranbrook, 2002; Wang, 1921).

The elemental composition of white nest and red nest is significantly different, albeit the ash content is the same. Via elemental analysis, Marcone (2005) discovered that the content of calcium is significantly higher in white nest while red nest is significantly richer in sodium, magnesium, potassium and iron. The iron content originates either from cave wall or saliva itself is suggested to be responsible in the red color of the nest (Lim, 2006). Some hazardous elements (heavy metals) such as lead, cadmium and mercury are listed to be present in EBN which is suggested to be incorporated during nest processing (Ma & Liu, 2012b). However, the health-harmful elements are not reported in subsequent elemental analysis by different researchers and this indicates that the elements are probably not present in EBN. Not only that, both mono- and di-glycerides are reported to be present in high amount despite of low content of lipid obtained. This requires further investigation and exploration as the origin and function remains unclear. Two assumptions postulated are: 1) they are produced during hydrolytic cleavage of triacylglycerol owing to the high cave humidity, 2) they are products of enzyme's action in EBN. Study on freshly weaved nest could be the solution to this problem (Marcone, 2005). Unlike the analysis conducted by Marcone (2005) with only four fatty acids found in EBN, Nurul Huda *et al*. (2008) discovered eleven fatty acids and they are all Omega-6 fatty acids. This is associated to the diets of insectivorous swiftlets which are fed on insects that take up plants (sources of Omega-6) as foods (Nurul Huda *et al*., 2008).

Five types of vitamin were also determined in EBN, namely vitamin A, D, C, H (biotin) and B1 (thiamine) (Teo *et al*., 2014; Teo *et al*., 2012; Lu *et al*., 1995). The content of vitamin A and D were related to the previous belief that swiftlets were fed on small fishes and prawns which were then proved not accurate by research studies (Lu *et al*., 1995). In view of this, it is possible to differentiate EBN samples of different breeding sites based on their nutritional compositions (Saengkrajang *et al*.,