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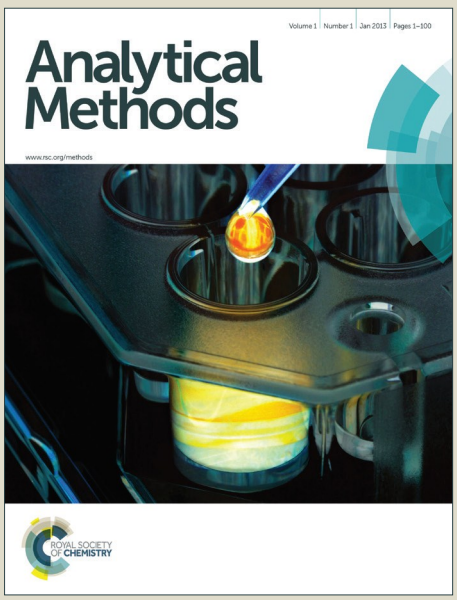

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Gel Electrophoretic and Liquid Chromatographic Methods for the Identification and Authentication of Cave and House Edible Bird's Nests from Common Adulterants

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Abstract

The increasing demands of edible bird's nest (EBN) have remarkably increased its price. Presently, several fake materials are being adulterated into EBNs for earning extra profits. In order to identify the adulterants and authenticate EBN; efforts were made to investigate and compare the protein and amino acid profiles of cave and house EBNs with white fungus, jelly, fish swimming bladder and egg white. The protein profiling indicated 10 bands for cave nests with two strong bands at 30 and 35 kDa. House nest proteins consisted of 9 bands with major bands at 120 and 140 kDa. White fungus displayed three dim bands at 22, 35 and 75 kDa whereas egg white was found to contain two predominant bands at 35 and 75 kDa. Fish swimming bladder showed substantial streaking of protein bands after dilution whereas protein profile of jelly was without any band. The amino acid analysis revealed EBN to consist of 17 types of amino acids. The major amino acids in cave and house nests were aspartic acid, arginine, histidine & leucine, and aspartic acid, glutamic acid, histidine & leucine, respectively. White fungus consisted of 16 types of amino acids in concentrations lower than that in EBN samples. Fish swimming bladder was rich in 6 amino acids. Egg white contained 16 types of amino acids with aspartic acid, glutamic acid, leucine and lysine in major quantities. However, jelly was found to contain no amino acids at all. Both the analytical procedures provided finger print profiles of the protein and amino acid compositions of the cave and house nests distinct from the adulterants. These results taken together can be used for the identification and authentication of any of these fake materials in EBN.

Keywords: Edible Bird's Nest, Protein analysis, Amino acid analysis, Gel electrophoresis, Liquid chromatography, EBN adulterants, EBN identification and authentication.

Introduction

Edible bird's nest (EBN) is a highly prized food produced by four swiftlet species *viz*. *Collocalia fuciphaga*, *Collocalia maxima*, *Collocalia germnis* and *Collocalia unicolor*.[1] The EBN producing swiftlets are particularly found in the Southeast Asian countries including Malaysia, Indonesia, Thailand and Vietnam.[2] EBN is known as delicacy and precious food in Southeast Asian countries especially among the Chinese communities. The main constituents of EBN are glycoproteins, carbohydrates, calcium, sodium and potassium.[3] EBN has been used as a health supplement in Traditional Chinese Medicines (TCM) from times immemorial. Besides, it is used in the treatment of malnutrition, boosting immune system, improving metabolism and enhancing skin complexion.[4]

The esteem of EBN as food with medicinal properties, and the requirement of highly skilled labourers for its collection and processing, makes it an expensive bioproduct. The cost of one kilogram of nests is approximately $6000 in China. The consumption of EBN products occurs in several Asian countries including China, Indonesia, Malaysia, Thailand, Vietnam and Philippines; in addition to its consumption in North America. This has led to rise in the trade scale of its global market from decades. The trade value of EBN rose from HK $1.3 billion to HK $3 billion in Hong Kong from 1989 to 2004.[5] Currently, there are much increasing demands of cave EBNs despite of their low production.[6] As a consequence of these facts, various fake materials such as Tremella fungus, karaya gum, pork skin, jelly, fish swimming bladder and egg white are being adulterated into EBN for increasing its weight before sale for higher profits.[7] Normally, the adulterants are quite difficult to identify without serious examination because of similar colour, physical appearance, taste and texture to that of the salivary nest cement. The

adulterants are generally added at levels around 10%.[6] These illegal adulterations leading to fake EBN have now become widespread.

Adulterated or fake EBN may be quite dangerous to the consumers. Therefore, the identification of fake materials and authentication of the quality of EBN is very important. Sophisticated techniques such as energy disperse X-ray microanalysis, electronic microscopy and spectroscopy have been used for the detection of plant based adulterants in EBN.[8] Joe Set[9] used Fourier Transform Infrared (FTIR) spectroscopy for the identification of genuine EBN. FTIR spectroscopy was also used by Hamzah et al.[10] to distinguish between EBN and adulterants like agar, starch, sodium alginate, carrageenan, pork skin and egg white. Zhang and co-workers[11] used a sandwich enzyme linked immunosorbent assay (ELISA) for identifying EBN. Yang and co-workers[12] used Gas Chromatography-Mass Spectrometry (GC-MS) fingerprint of oligosaccharides, environmental scanning electron microscopy (ESEM) of microstructure, and immunoblotting of epidermal growth factor (EGF) in a concerted approach for the authentication of EBN. Huang et al.[13] used spectrophotometry to differentiate EBN from saliva, pig's skin and Tremella fucifomis. Lin and co-workers[14] used stereoscopy to differentiate between white EBN, red EBN and EBN painted with colloid. Yang et al.[15] studied the sugar fingerprint spectrum, microscopic structure, and detected an epidermal growth factor (EGF) for the identification of true and fake EBN. Besides, some other methods including DNA-based polymerase chain reactions, protein-based two dimensional gel electrophoresis and genetic identification based on mitochondrial DNA have found applications in the authentication of EBN.[6,16]

Gel electrophoresis is a molecular biological analytical method that has been used to study EBN proteins.[17] Huang et al. successfully used gel electrophoresis for the purification and identification of proteins in EBN.[18] Chua et al.[19] carried out protein separations of EBN samples

using gel electrophoresis for distinguishing EBN samples from non-EBN ones. Therefore, gel electrophoresis is a preffered method for the identification of the protein profiles of EBNs.

HPLC (high performance liquid chromatography) is a fast and sensitive technique for the analysis of amino acids in EBN and other biological matrices.[20-22] Separation of analytes in HPLC is dependent on their flow in a proper and suitable solvent mixture (mobile phase) inside a packed bed or column (stationary phase). Generally, HPLC columns are packed with very small and homogeneous particles, which provide improved physical and chemical stability, better reproducibility, and faster separations. The basic advantages such as superior resolution and much shorter run times of HPLC over classical column chromatography make it as a choice instrument for the separation and identification of analytes in biochemical studies. Several chemical methods including gas chromatography and liquid chromatography coupled to different detector types have been used for the characterization of the chemical composition of EBN. Gas chromatography and liquid chromatography analyses have been reported to investigate amino acids, monosaccharides, fatty acids and hormones in EBN.[23-27] To the best of our knowledge, this is the debut approach utilizing the combined efforts of gel electrophoresis and reverse phase liquid chromatography for the identification of some common adulterants in EBN. Therefore, efforts were made to study the amino acid and protein profiles of cave and house nests and compare the results with white fungus, jelly, fish swimming bladder and egg white as a means for the identification and authentication of any of these fake materials in EBN.

Experimental

Sample Collection

Cave nests were obtained from the caves of Gua Niah (Niah National Park), Sarawak, Malaysia. House nests were collected from swiftlet premises (professional suppliers) in Batu

Pahat, Johor, Malaysia, respectively. Dried chips of tremella fungus, jelly and fish swimming bladder were procured from a local grocery store in Skudai, Johor, Malaysia. Pure egg white albumin was supplied by Promega Corporation, Madison, USA.

Chemicals and Reagents

All the chemicals and reagents were of analytical reagent grade and used without further purification. Electrophoresis buffer, acrylamide, bis-acrylamide (N,N´-methylenebisacrylamide), tris (2-hydroxymethyl-2-methyl-1,3-propanediol), SDS (sodium dodecyl sulphate or lauryl sulphate), TEMED (N,N,N´,N´-tetramethylenediamine-ethylenediamine), ammonium persulphate, 2-mercaptoethanol, glycerol, bromophenol blue, Gly (glycine), Coomassie Blue R-250 and Precision Plus Protein Dual Colour Standards were purchased from Bio-Rad Laboratories, Hercules, USA. Pierce 'H' standards, PITC (phenylisothlocyanate), acetonitrile, potassium dihydrogen phosphate and 10% phosphoric acid were procured from Waters Corporation, Milford, USA. Broad range protein molecular weight marker was purchased from Promega Corporation, Madison, USA. Hydrochloric acid and ethanol were purchased from Merck Chemical Company, Darmstadt, Germany. Methanol and glacial acetic were supplied by GCE Chemical Laboratory, Sweden, and Sigma-Aldrich, St. Louis, Missouri, USA, respectively.

Apparatus and Equipments

The apparatus and instruments used for the complete experimentation in this research work included Hamilton syringes, eppendorf tubes, pipettes, micropipettes, burettes, beakers, Whatman No. 1 filter paper, syringe filter 4.5 µm, 1 mm steel filter, air force oven (Memert, USA), water bath (Memmert, USA), rotary shaker, Minigel apparatus (Bio-Rad Mini-Protean III apparatus) connected to a power supply (capacity 200 V, 500 mA), HPLC instrument (model

710B, Waters Corporation, Milford, USA) Pico Tag with Waters Pico Tag 15×4.6 mm column connected to a UV detector at 254 nm range.

Preparation of Crude Protein Extracts from Raw Samples

The raw samples of cave and house nests were cleaned manually by removing dirt, feathers and egg shells. The cleaned samples (cave and house nests, white fungus, jelly and fish swimming bladder) were separately ground in a mortar and screened through a 1 mm steel filter. Crude egg white sample was prepared by dissolving 0.01 g pure egg white albumin with 20 ml distilled water. Crude proteins from the raw samples were extracted by aqueous extraction. Alkaline extraction and water extraction methods were applied for the extraction of proteins from the as-prepared samples.

Alkaline Extraction

1 g of each raw sample was immersed in 30 ml of 0.25 N NaOH solution for 48 h. This was repeated for 0.1 and 0.4 M NaOH solutions. Then, the aliquot of each extract was immersed in water bath at 65 ºC for 2 h. The extracted solutions were centrifuged and eventually the supernatant was obtained.

After the extraction processes, the suspensions were centrifuged at 18000 rpm for 20 minutes, and the supernatants were dialysed thoroughly against distilled water.

Water Extraction

This extraction method was adapted from Goh et al., 2001,[1] Oda et al., 1998[24] and Kong et al., 1987[28] with minor modifications. 1 g of each raw sample was suspended in 30 ml deionized water and allowed to elute for 48 h at 4 ºC. Then, the aliquot of each extract was immersed in water bath at 65 ºC for 2 h. The extracted solutions were centrifuged and eventually

the supernatant was obtained. The extraction process was repeated with changing the temperature of decoction to 75, 85 and 95 ºC.

Protein Profiling

Protein profiling was carried out by gel electrophoresis using the method of Bollag et al.[29].

Gel Electrophoresis

Acrylamide gel electrophoresis was used to separate proteins on account of the differences in their molecular weights. For the determination of the acrylamide percentage, the range of molecular weight was forecasted. For the present study, the range of molecular weight of bird's nest protein was approximately 14-97 kDa.[1] The fitting acrylamide percentage in separating gel was 12.5%.

Amino Acid Profiling

For the analysis of amino acids, it is very important to break peptide bonds of proteins and convert them into free amino acids. Therefore, the amino acid analysis was carried out by hydrolysis of proteins into their constituent amino acids followed by separation using HPLC.

Acid Hydrolysis

The acid hydrolysis method was adapted from Su et al.,[30] Oda et al.[24] and Alaiz et al.[31] The samples were ground into fine powder and then 100 mg of each sample was weighed and transferred into separate hydrolyzing bottles. 15 ml of 6 N HCl were added to each bottle and the bottles were tightly sealed. The samples were hydrolyzed at 110±1 °C in an air oven for 24 h. After hydrolysis, the hydrolysates were mixed with distilled water and made upto 50 ml and then filtered using Whatman No. 1 filter papers followed by filtration through 4.5 µm syringe filter.

Preparation of Amino Acid Standard

20 µl of Pierce 'H' standard were transferred into a test tube and dried by flowing N_2 gas. Subsequently, 20 µl PITC was added and the solution was dried again for 20 minutes. Finally, 200 µl of sample diluent was added to dissolve any dried particles and make the samples ready for injection into HPLC system.

For constructing standard curves for each amino acid type, the amino acid standard samples with different concentrations were prepared. The sample preparation process was repeated with changing volumes of Pierce 'H' standard as 25, 20, 15, 10 and 5 µl, respectively.

High Performance Liquid Chromatography

HPLC was used to determine the amino acid composition of the samples. The samples were derivatized with PITC before injecting into the HPLC system. 20 µl of each filtrate were transferred into test tubes and dried by flowing N_2 gas. Then 40 µl of redrying solution were added, and the samples dried again. Subsequently, 20 µl PITC were added and the solutions allowed to stand at room temperature for 20 minutes. Finally, 200 µl of sample diluent were added to dissolve any dried particles, and make the samples ready for injection into HPLC system.

4 µl of each injected sample were separated into constituent amino acids on Waters Pico Tag, 15 cm ×4.6 mm ID (3 µm particles) by elution in mobile phase of varying composition of components, A (25 mM KH_2PO_4, pH 7.0) and B (acetonitrile: methanol, 70:30, v/v) at 38 °C. The running time was set at 12 minutes with 8 minutes of equilibration time. The detection of the analytes was carried out at 254 nm. The setting details of the HPLC analytical procedure are given in **Table 1**.

Results and Discussion

Protein and amino acid constituents of foods and other materials are different from each other. Therefore, every such item should have different protein profiles and amino acid compositions. This stood out as basis of the analysis of protein profiles and amino acid compositions for the identification and authentication of EBN.

Basically, protein analysis involves two main steps, *viz.* sample extraction and gel electrophoretic analysis. Sample preparation is crucial to the clear and accurate resolution of protein bands[32]. For that reason, the raw materials were ground and sieved so that samples with large surface area were obtained. During the extraction process, the soluble proteins were dissolved leaving behind the insoluble ones. However, this procedure was excluded for the egg white sample because it was purchased in pure form.

Sample solutions for gel electrophoresis were prepared. 100 ml of solution A were prepared by dissolution of 29.2 g of acrylamide and 0.8 g of bis-acrylamide in 100 ml of distilled water. 100 ml of solution B were prepared by mixing together of 75 ml of 2 M Tris-HCl with pH 8.8, 4 ml of 10% SDS and 21 ml of distilled water. 100 ml of solution C were prepared by mixing together of 50 ml of 1 M Tris-HCl with pH 6.8, 4 ml of 10% SDS and 46 ml of distilled water. Finally, 5x sample buffer was prepared by mixing together of 0.6 ml of 1 M Tris-HCl with pH 6.8, 5 ml of 50% glycerol, 2 ml of 10% SDS, 0.5 ml of 2-mercaptoethanol, 1 ml of 1% bromophenol blue and 0.9 ml of distilled water. The separating gel (12.5% acrylamide gel) was prepared by mixing together 4.1667 ml of solution A, 2.5 ml solution B, 3.3333 ml distilled water, 50 µl of 10% ammonium persulphate and 15 µl of TEMED.

Solutions A and B, and distilled water were mixed in a small test tube. The mixture was added with ammonium persulphate and TEMED. The solution was inserted into the gel sandwich carefully using a pipette. Then a layer about 1-5 mm of water was added on top of the separating

gel solution which kept the surface wet. The gel polymerized for about 30-60 minutes. In the next step, loading samples were prepared by combining protein samples and 5x sample buffer in eppendorf tubes and heating at 100 °C for 2-10 minutes. The protein solutions were spun for 1 second in microfuge. Then the sample solutions and molecular weight standards were introduced into wells using Hamilton syringe. The power supply was turned on to 200 V constantly till the dye migrated to the bottom of the gel. 1g Coomasie Blue R-250, 450 ml distilled water, 450 ml methanol and 100 ml glacial acetic acid made Coomasie gel stain. On the other hand, 100 ml of methanol, 100 ml of glacial acetic acid and 800 ml of distilled water made Coomasie gel destain. The prepared gel was transferred to a small container containing 20 ml of Coomasie stain. The container was agitated for 10-20 min on slow rotary shaker. Stain was now poured off and rinsed a few times with distilled water. 50 ml Coomasie destain was added into the container subsequently and agitated for one hour. After complete destaining, the gel was rinsed with distilled water. The molecular weight determination was estimated by co-electrophoresis with the molecular weight markers.

Protein Profiles

Gel electrophoresis is one of the most common methods of choice for proteomic analysis[33]. Gel electrophoresis is suitable for the protein profiling of EBNs and adulterants using suitable protein extraction conditions. Appearance of prominent bands in gel electrophoresis depends upon the optimum protein concentration and the molecular structure of the proteins. A series of extraction processes was tried to find the most suitable results (clearly visible protein bands). Both alkaline and water extraction methods were used for protein extraction. Their effectiveness for extraction was compared, and the best method was selected to process the samples for ensuring a uniform standardization.

Alkaline Extraction

0.1, 0.25 and 0.4 M NaOH solutions were used for the alkaline extraction processes. The protein profiles observed from the results of SDS PAGE indicated smeared electrophoresis patterns for cave nest, house nest and fish swimming bladder [**Fig. 1**; (**a-c**]. However, no smeared electrophoresis pattern was observed for jelly and white fungus [**Fig. 1**; (**a-c**], which indicated that the proteins in cave nest, house nest and fish swimming bladder can be easily extracted out in NaOH solution. Contrary to this, the method is not suitable for the extraction of proteins from jelly and white fungus.

For 0.4 and 0.25 M NaOH extraction, the protein profiles showed smeared and distorted electrophoretic patterns for cave nest, house nest and fish swimming bladder lanes [**Fig. 1**; (**a**) and (**b**)]. This might be due to an overloading effect[34]. In general, if Coomassie blue stain is used, the amount of protein loaded should range from 0.4-4.0 µg for purified samples and 40-60 µg for crude samples.[27] The proteins of the samples placed in a well tend to diffuse into the stacking gel, both vertically and laterally with smaller proteins diffusing rapidly as compared to the larger ones. Therefore, overloaded wells cause the diffusion of denatured proteins into the stacking gel competitively and may spread out towards the edges, resulting into the smeared and distorted bands[34]. Additionally, an increase in NaOH concentration causes increase in the smearing effect.

For 0.1 M NaOH, a few bands surrounded by streaking were detected in the lanes of cave and house nest samples [**Fig. 1**; (**c**). These bands were located at 12, 21 and 50 kDa molecular weights for cave nests, and at molecular weights of 6, 12, 16, 20, 25, 42, 75 and 130 kDa for house nests. However, the smeared electrophoretic pattern still existed in the fish swimming bladder's protein profile [**Fig. 1**; (**c**)].

Water Extraction

The ability of water extraction mainly depends on the temperature used because different types of proteins get hydrolyzed or extracted at different temperatures[35]. Thus, every protein has its own maximum heat resistance level before it gets hydrolyzed. Therefore, using high temperature, means part of the protein may be hydrolyzed and not detected by SDS PAGE analysis. On the basis of this fact, a temperature gradient analysis was carried out to identify the best temperature for improving the protein extraction and reducing the possibility of protein hydrolysis in water extraction methodology.

After soaking for two days, the samples were decocted at 65, 75, 85 and 95 °C. Different protein profiles were produced at each specific temperature (**Fig. 2: a-d**). In general, no clear band was produced for the commercial jelly **[Fig. 2: (a):** lane 2, **(b):** lane 1, **(c):** lane 2, **(d):** lane 2] produced from seaweed, whereas for fish swimming bladder sample **[Fig. 2: (a):** lane 1, **(b):** lane 2, **(c):** lane 3, **(d):** lane 3], the streaking electrophoresis pattern was obtained. During the production process of commercial jelly from seaweed, the proteins may be destroyed or might prefer being in polymerized form and therefore, not detected by SDS PAGE. On the other hand, fish swimming bladder is mainly composed of animal type protein and so the molecular structure of fish swimming bladder's protein is relatively large and complex[36]. This might have resisted the protein structure to break into small polypeptides for sensing by SDS PAGE electrophoresis.

Fig. 2 (a) shows the protein profiles of the samples at 65 °C by water extraction. Cave nest proteins consisted of two major bands at 16 and 18 kDa and three intermediate bands with molecular weight ranging from 25-40 kDa. For house nest sample, two apparent bands appeared near 85 and 125 kDa. Besides, four intermediate bands were found in the molecular weight varying from 2-35 kDa. There were no protein bands detected in the protein profiles of white

fungus and jelly samples. These results indicated that proteins of white fungus cannot be extracted in water at 65 °C.

Fig. 2 (b) shows the protein profiles of the samples at 75 °C by water extraction. It can be seen that the protein profiles of cave and house nests have significant differences in comparison to the protein profile produced by 65 °C water extraction. There were thirteen bands for cave nest located at 13,15, 16, 17, 18, 21, 25, 29, 42, 45, 50, 68 and 125 kDa, whereas seven bands were observed for house nest at 230, 235, 75, 62, 45, 27 and 29 kDa. Three intermediate bands with molecular weights 10, 35 and 75 kDa were observed in the protein profile of white fungus. This showed that the proteins in white fungus could be extracted at 75 °C instead of 65 °C.

By comparing the protein profiles of the samples obtained by water extraction at 65 and 75 °C, it was evident that the bands in the protein profiles of cave and house nests were strong for photographic reproduction of the gel. For example, the bands obtained in cave nest profile at 25-40 kDa became more dense compared to its profile produced by water extraction at 65 °C, and this might be due to comparatively high protein concentration of the sample at 75 °C.

Fig. 2 (c) shows the protein profile of samples obtained by water extraction at 85 °C. The protein profile of cave nest consisted of 11 bands with the major bands located at 18 and 19 kDa. The protein profile of house nest composed of 4 bands at 22, 25, 37 and 42 kDa. Besides, a smeared electrophoresis pattern was observed in the upper part of house nest's profile. However, only three dim bands at 11, 18 and 25 kDa existed in the white fungus protein profile.

The protein profiles of the samples at 75 and 85 °C indicated that some bands (ranging from 25-125 kDa) in cave nest sample prepared by water extraction at 75 °C disappeared in 85 °C protein profile. This may be due to the fact that high temperature hydrolyzed some of the proteins in the cave nest. Hydrolysis might have dissociated the proteins into small peptides or

amino acids which cannot be trapped by the mesh formed by the SDS PAGE. Therefore, the small components at higher temperature diffused through the gel and eventually dissolved into the electrophoresis buffer.

Fig. 2 (d) shows the protein profile of the samples produced by water extraction at 95 °C. No clear and pronounced bands were detected in the protein profiles of samples. A substantial streaking and smearing was observed in the protein profiles of fish swimming bladder and house nest indicating overloading. High temperature conditions might have produced high protein concentrations of the samples, which induced clogging effect due to the diffusion of the denatured proteins into the gel. Besides, white fungus and cave nest protein profiles indicated that the bands become faint compared to **Fig. 2 (b)**, which might be attributed to protein hydrolysis at higher temperature.

Conclusively, the number of polypeptides and the molecular weight of the protein fractions varied between alkaline and water extraction method. Overall, water extraction was chosen as the standardized method to extract proteins of all samples under investigation. From the temperature gradient analysis, 75 °C was established as the best temperature for water extraction process because the protein profile of the samples produced by water extraction at this temperature showed more substantial bands except for fish swimming bladder and jelly.

After protein extraction, the second step of sample preparation is to adjust the sample concentration by dilution so that an appropriate amount of protein is loaded into the gel. A proper dilution adjusts the appropriate protein concentration for clear and accurate resolution of protein bands. Dilution helps in the solubilization and dissociation of proteins without affecting the peptide bonds.[37] Different dilution factors were used to dilute each of the concentrated samples. By comparing the protein profile produced for each dilution (**Figs. 3-7**), the most

suitable dilution factor was determined for each sample. The results of this analysis are presented in **Table 2**. Each sample possessed its own unique protein profile (except jelly). Besides, the protein profile of each sample except fish swimming bladder and jelly exhibited pronounced bands after dilution. On increasing the dilution gradually, the bands become faint and finally disappeared. As a result the best dilution conditions were achieved. The discussion of the protein profiles of the samples after suitable dilution are discussed as follows.

Fig. 3 shows that the proteins of cave nest consisted of 10 bands with strong bands at 30 and 35 kDa. Six more medium bands located at 12, 25, 28, 42, 50 and 60 kDa were also found; indicating that the cave nest protein contained various types of polypeptide chains at different proportions.

Fig. 4 shows that the protein profile of house nest consisted of nine bands, and two of the bands were identified at 120 and 140 kDa, which were absent in the protein profile of cave nest. The protein profile additionally displayed seven medium bands at 35, 45, 50, 60, 70, 80 and 100 kDa. **Figs. 3** and **4** show some common bands in both nest types at 50 and 60 kDa. Thus, it may be concluded that the cave and house nests consisted of different protein compositions though they have been produced by the same swiflet species (*Collocalia*).

Fig. 5 shows three dim bands at 22, 35 and 75 kDa for white fungus sample. The 35 kDa polypeptide of the white fungus is similar to one of the protein bands of cave nest. But the protein fraction of cave nest has been slightly stronger in comparison to the white fungus sample. Three bands were detected in the egg white sample, which were absent in other samples (**Fig. 6**).

Fig. 7 showed substantial streaking in the protein profile of fish swimming bladder. However, a band was detected after dilution at around 31 kDa. All the bands detected for each sample have been presented in **Table 3**.

Thus, a clear picture of the protein profiles of cave and house EBNs, white fungus, fish swimming bladder, egg white and jelly were obtained. A comparison of the protein profiles of both the types of EBNs with these materials (common adulterants) can be used for distinguishing between EBN and these materials.

Amino acid Profiling

Amino acid analysis was carried out by reverse phase HPLC using Waters Pico Tag 15×4.6 mm column. Before injecting into the HPLC instrument, the samples were subjected to pre-column treatment wherein the disulphide bonds were broken and the individual peptide chains separated. The peptide chains can be completely hydrolyzed by 6 N HCl at 110 °C. This was followed by derivatization of the released amino acids with PITC to produce PITC amino acids derivatives. The PITC amino acid derivatives can be analyzed by reverse phase HPLC in amounts as low as 1 picomole. Besides, PITC amino acids have a broad UV spectrum with maximum absorbance near 269 nm. A fixed wavelength detector operating at 254 nm produced excellent results.[38]

Basically seven samples were analyzed including amino acid standard, cave nest, house nest, white fungus, jelly, fish swimming bladder and egg white. Pierce 'H' standard was used as a standard in this analysis. The peak identity of each investigated sample was carried out by tallying with the authentic Pierce 'H' standard. The amino acid profile of Pierce 'H' standard is shown in **Fig. S1**. The types of amino acids and their corresponding retention times are given in **Table 4**. 18 peaks were detected in the amino acid standard profile along with their retention times ranging from 1.787-8.387 minutes. In addition, it was observed that each amino acid has its own particular retention time, though the retention times of some of the peaks were approximately same. For example, Gly and His (histidine), and Ala (alanine) and Pro (proline)

with retention times of 3.260 and 3.392, and 4.061 and 4.170 minutes, respectively. Besides, the amino acid standard profile was similar to the amino acid standard profile provided in Pico Tag Amino Acid System Handbook.[33] Nevertheless, the peak with retention time 4.591 minutes was due to ammonia, which was formed as a side product during derivatization of amino acids with PITC.

In general, the amino acid profile for each sample was virtually different. **Fig. S2** displays the amino acid profile of cave nest. A comparison of the amino acid profile of cave nest with the amino acid standard indicated that the cave nest protein consisted of 17 types of amino acids. Asp (aspartic acid), His, Arg (arginine) and Leu (leucine) were the major amino acids with concentrations of 4.98, 4.86, 4.63 and 5.47 %, respectively as shown in **Fig. 8**. On the other hand, Ser (serine), Met (methionine) and Cys (cystine) were identified in concentrations as low as 1.42, 1.40 and 0.74 %, respectively. The low concentrations of Ser, Met and Cys may be due to their progressive destruction during acid hydrolysis[29]. Besides, the peaks of Ser and Ala were noted as having incomplete separation at 3.169 and 4.115 minutes, respectively (**Table 5**). Moreover, some additional peaks were also found in the range of 4.829-5.017 and 7.514-8.333 minutes. These may be due to impurities such as small feathers and fine plumage.

The observations from **Figs. S2** and **S3** were precisely analogous. The proteins of house nests also contained 17 amino acids with Asp, Glu (glutamic acid), Leu and His as the major components with concentrations of 4.87, 4.58, 4.66 and 3.95 %, respectively. Ser, Met and Cys were detected in very small concentrations of 1.36, 1.18 and 0.95 %, respectively. The amount of each amino acid in house nest proteins was relatively lower than in cave nest protein (**Fig. 8**), except for Glu and Cys, which were reported with slightly higher concentrations of 4.58 and 0.95 % in house nest in comparison to their concentrations of 4.38 and 0.75 % in cave nests. Thus, it

can be seen that cave nests are more invigorative compared to house nests because of higher concentration of essential amino acids.

Fig. S4 showed that white fungus protein consisted of 16 types of amino acids with Glu, Asp, Leu and Lys as the predominant components with concentrations of 3.06, 2.76, 2.33 and 2.23 %, respectively. Ser was not detected in the amino acid profile of white fungus. Therefore, this can be used for distinguishing the amino acid profile of white fungus from bird nest. Comparatively, two additional impurity peaks at 9.11 and 2.74 minutes (**Table 5**) were found. It was quite interesting to note that the concentration of amino acids contained in the white fungus protein was significantly lower than bird's nest (**Fig. 8**).

Fig. S5 showed that the proteins of fish swimming bladder were composed of 15 types of amino acids. Six specific amino acids including Asp (6.61 %), Glu (12.18 %), His (50.11 %), Arg (10.92 %), Pro (13.42 %) and Lys (lysine, 6.97 %) were in higher concentrations (**Fig. 8**). However, Gly and Leu were not detected in the amino acid profile. Tyr (tyrosine) was found in significantly low concentration (1.19 %) as compared to its concentrations of 3.53 and 3.23 % in cave and house nests, respectively. Ser, Ile (isoleucine) and Met were more intense compared to bird's nest, which may be due to the fact that fish swimming bladder is composed mainly of animal type protein. Thus, the six concentrated amino acids can be used as a benchmark for fish swimming bladder sample in the comparison process.

Fig. S6 showed 16 types of amino acids in the amino acid profile of egg white. The major amino acids were Asp (5.49 %), Glu (7.58 %), Leu (4.93 %) and Lys (5.72 %). Gly was not detected. Moreover, several impurities were detected in the ranges of 7.427-8.232 minutes and 8.817-9.034 minutes (**Table 5**). Surprisingly, seven amino acids including Asp, Glu, Met, Ile,

Cys, Lys and Ala were found in higher amounts than in bird's nest. However, Pro was found in quite low amount compared to that in bird's nest.

No amino acids were detected in the jelly sample (**Fig. S7**) and this finding was consistent with the results of protein profiling experiments. Overall, each sample has its own unique amino acid profile. Besides, the amino acid composition of each sample was different. Taken together, the gel electrophoretic and liquid chromatographic analysis provides finger print profiles of the protein and amino acid composition of the cave and house nests distinct from the adulterants. By recognizing the amino acid profiles, the samples can be differentiated from each other. Since, the protein quality of a sample depends upon the essential amino acid composition, EBN can be esteemed as a high nutritional delicacy because it contains almost all the essential amino acids in good amounts.

In nutshell, the combination of gel electrophoresis and liquid chromatography ideally differentiates and distinguishes between cave and house EBNs from white fungus, fish swimming bladder, jelly and egg white. Several other studies reported previously were able to identify and authenticate EBN samples collected from different sources from the adulterants such as Tremella fungus, milk, soybean and rice[7], collagen, sodium alginate, Tremella fuciformis, polysaccharides, carrageenan and agar[39], taurine[40], calcium carbonate[9], cow's milk, chicken egg yolk, chicken egg white, quail egg yolk, quail egg white, fetuin, infant formula and human saliva[20]. All the methods were successful in authenticating genuine EBN, however, the methods involved sophisticated paraphernalia and extensive experimentation. The present study in a simple and reproducible fashion ideally identifies and authenticates, EBN samples from the common adulterants.

Conclusion

Gel electrophoretic and liquid chromatographic methods were used for protein profiling and amino acid studies of cave and house nests, white fungus, fish swimming bladder, jelly and egg white. Water extraction at 75 °C was chosen for protein profiling studies of the crude samples. Protein profiling of cave nest revealed 10 bands with two strong bands at 30 and 35 kDa. On the other hand, there were 9 bands with major bands at 120 and 140 kDa in the protein profile of house nest. Three dim bands at 22, 35 and 75 kDa were obtained in white fungus, whereas egg white was found to contain two predominant bands at 35 and 75 kDa. Substantial streaking was obtained in the protein profile of fish swimming bladder after dilution. The protein profile of jelly was without any band. The liquid chromatographic amino acid analysis indicated that both cave and house EBN samples consisted of 17 types of amino acids. Asp, Arg, His and Leu were the major amino acids in cave nest whereas Asp, Glu, His and Leu were the major ones found in house nest. White fungus composed of 16 types of amino acids in concentrations lower than that in EBN samples. Fish sample was extremely rich in 6 amino acids including Asp, Glu, His, Arg, Pro and Lys. Egg white was found to contain 16 types of amino acids with Asp, Glu, Leu and Lys being present in major quantities. However, jelly was found to contain no amino acid at all. The gel electrophoretic protein profiling results were well complemented by the results from HPLC amino acid analysis. Cave and house EBNs had the maximum number of bands in the electrophoretic analysis and also had the maximum types of amino acids as revealed by the chromatographic studies. Gel electrophoresis indicated no protein bands for jell. This was fully complemented from the chromatographic analysis, which showed no amino acids in jelly. Overall, each sample had a unique protein profile except jelly; with a characteristic amino acid composition. Thus, a clear picture of the actual composition properties of EBN and some commonly used adulterants has been presented. SDS PAGE adequately differentiated EBN from

other adulterants and HPLC facilitated the characterization of their compositions. The results of this analysis can be used for the identification and authentication of any of these fake materials in EBN.

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Conflicts of Interest

There are no conflicts of interest to declare.

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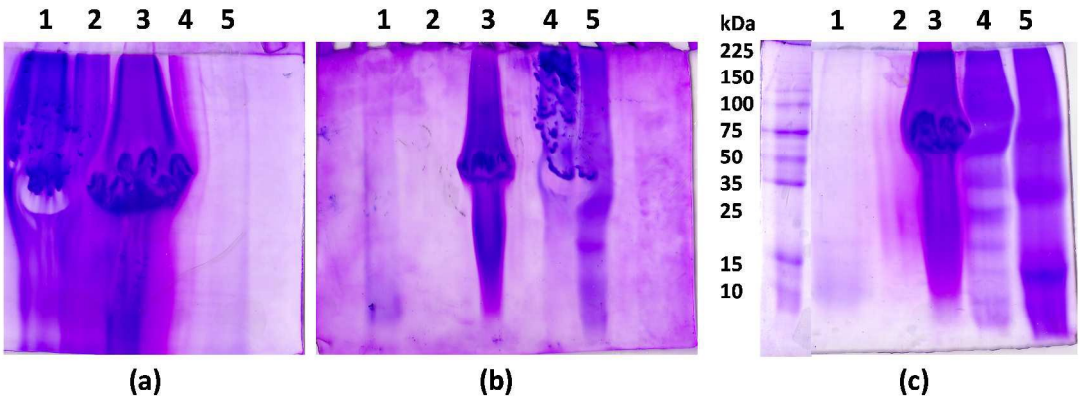

Fig. 1: Protein profiles for samples *via* alkaline extraction at different concentrations of alkali; **(a):** 0.4 M NaOH extraction; lanes 1, 2, 3, 4 and 5 represent protein profiles of cave nest, house nest, fish swimming bladder, jelly and white fungus, respectively; **(b):** 0.25 M NaOH extraction; lanes 1, 2, 3, 4 and 5 represent protein profiles of white fungus, jelly, fish swimming bladder, house nest and cave nest, respectively; **(c):** 0.1 M NaOH extraction; lanes 1, 2, 3, 4 and 5 represent protein profiles of white fungus, jelly, fish swimming bladder, house nest and cave nest, respectively.

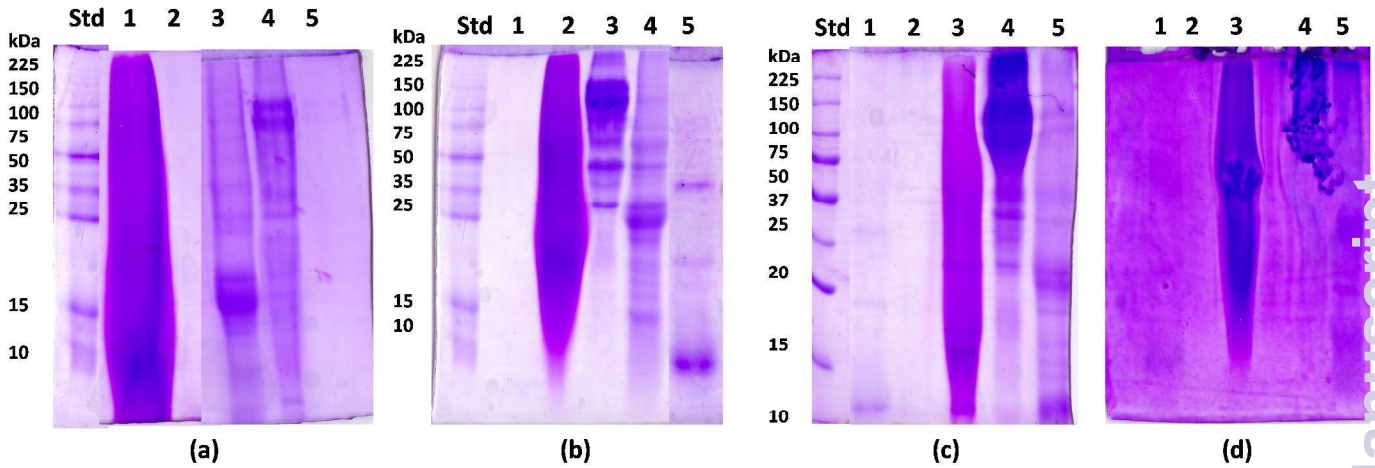

Fig. 2: Protein profiles for samples *via* water extraction at different temperatures; **(a):** Extraction at 65 °C; lanes 1, 2, 3, 4 and 5 represent protein profiles of fish swimming bladder, jelly, cave nest, house nest and white fungus, respectively; **(b):** Extraction at 75 °C; lanes 1, 2, 3, 4 and 5 represent protein profiles of jelly, fish swimming bladder, house nest, cave nest and white fungus, respectively; **(c):** Extraction at 85 °C; lanes 1, 2, 3, 4 and 5 represent protein profiles of white fungus, jelly, fish swimming bladder, house nest and cave nest, respectively; **(d):** Extraction at 95 °C; lanes 1, 2, 3, 4 and 5 represent protein profiles of white fungus, jelly, fish swimming bladder, house nest and cave nest, respectively.

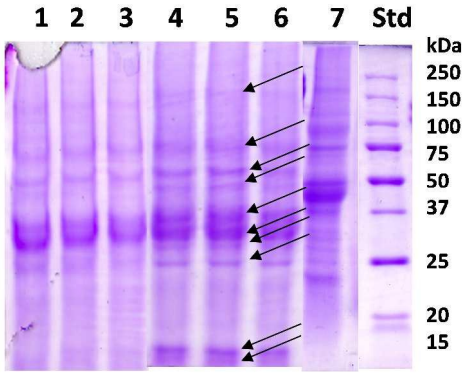

Fig. 3: Protein profile of cave nest sample obtained after dilution. Lane 1 (50:50), Lane 2 (60:30), Lane 3 (70:30), Lane 4 (80:20), Lane 5 (90:10), Lane 6 (95:5) and Lane 7 (No dilution).

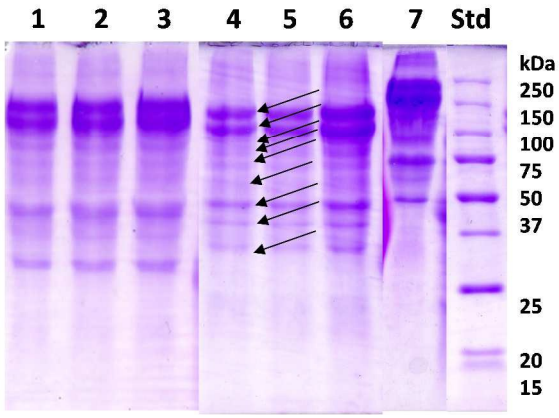

Fig. 4: Protein profile of house nest sample obtained after dilution. Lane 1 (30:70), Lane 2 (40:60), Lane 3 (50:50), Lane 4 (60:40), Lane 5 (70:30), Lane 6 (80:20) and Lane 7 (No dilution).

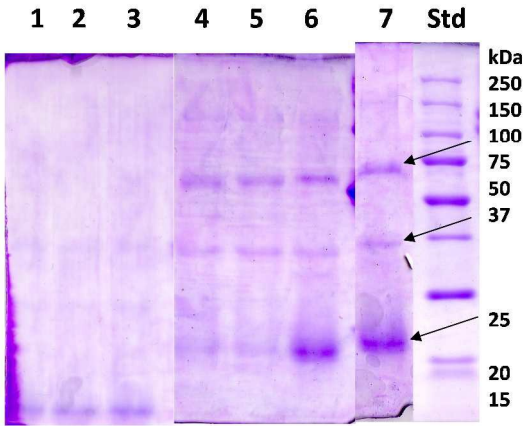

Fig. 5: Protein profile of white fungus sample obtained after dilution. Lane 1 (40:60), Lane 2 (50:50), Lane 3 (60:40), Lane 4 (70:30), Lane 5 (80:20), Lane 6 (90:10) and Lane 7 (No dilution).

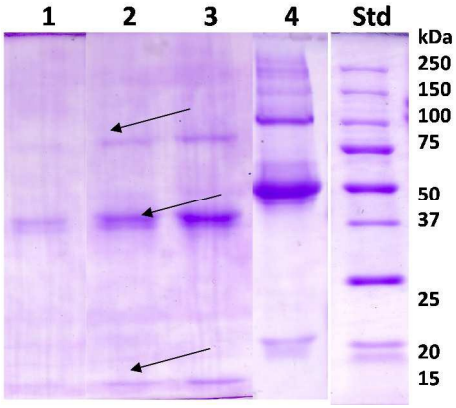

Fig. 6: Protein profile of egg white sample obtained after dilution. Lane 1 (50:50), Lane 2 (60:40), Lane 3 (70:30) and Lane 4 (No dilution).

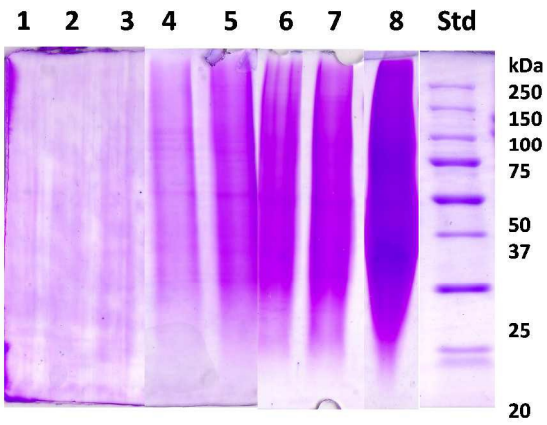

Fig. 7: Protein profile of fish swimming bladder sample obtained after dilution. Lane 1 (5:120), Lane 2 (5:110), Lane 3 (5:100), Lane 4 (5:95), Lane 5 (10:90), Lane 6 (20:80) Lane 7 (30:70) and Lane 8 (No dilution).

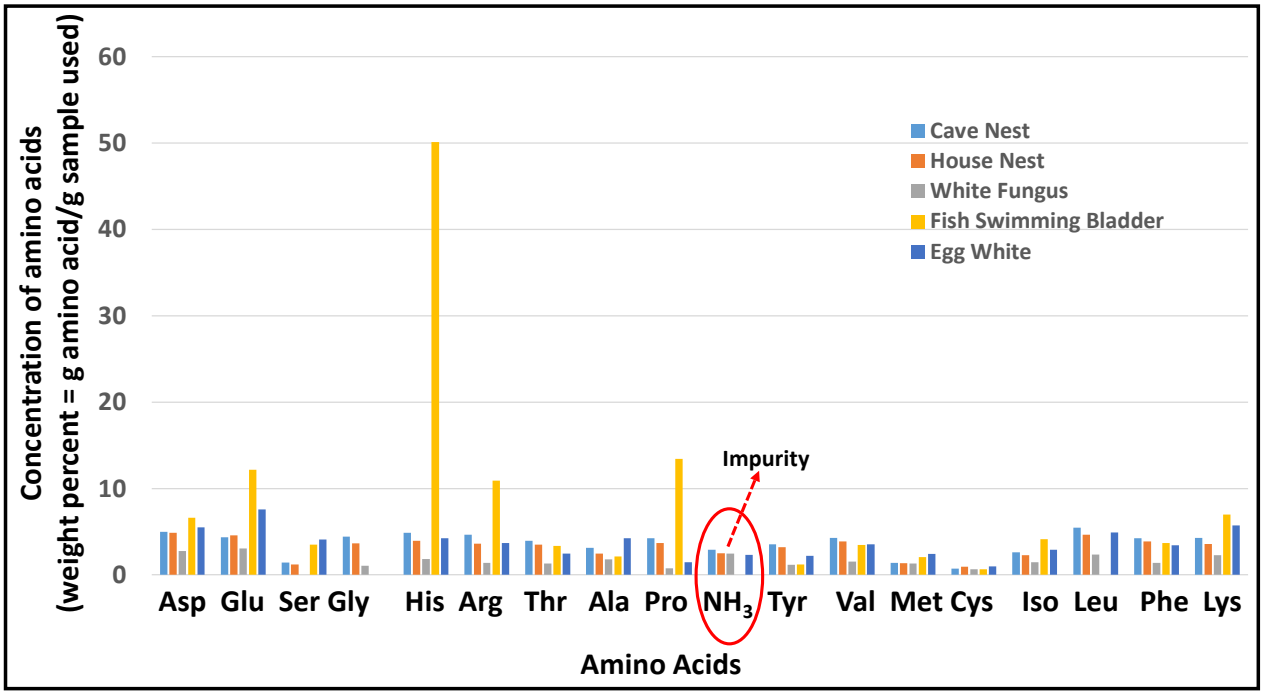

Fig. 8: Concentrations profiles of amino acids in cave nest, house nest, white fungus, fish swimming bladder and egg white.

Table 1: Gradient flow rate.

Time	Flow	%A	%B	Curve
Initial	1.0	100	0	-
10	1.0	54	46	5
10.5	1.0	0	100	6
11.5	1.0	0	100	6
12.0	1.5	0	100	6
12.5	1.5	100	0	6
20.0	1.5	100	0	6
20.5	1.0	100	0	6

Table 2: The most suitable dilution used for each sample.

S. No.	Sample	Dilution (volume of sample: volume of water)
1	Cave nest	90:10
2	House nest	60:40
3	White fungus	-
4	Fish swimming bladder	20:80
5	Jelly	-
6	Egg white	60:40

Table 3: Protein bands detected for the samples under study.

Sample / Molecular weight (kDa)	Cave nest	House nest	White fungus	Fish swimming bladder	Jelly	Egg white
140	-	yes	-	-	-	-
120	yes	yes	-	-	-	-
100	-	yes	-	-	-	-
80	-	yes	-	-	-	-
75	-	-	yes	-	-	yes
70	-	yes	-	-	-	-
60	Yes	yes	-	-	-	-
50	yes	yes	-	-	-	-
45	-	yes	-	-	-	-
42	Yes	-	-	-	-	-
35	yes	yes	yes	Yes (31)	-	Yes (37)
30	yes	-	-	-	-	-
28	yes	-	-	-	-	-
25	yes	-	-	-	-	-
22	-	-	yes	-	-	-
12	yes	-	-	-	-	-
11	yes	-	-	-	-	-
10	-	-	-	-	-	yes

Table 4: Amino acids along with their retention times detected in the HPLC chromatogram of Pierce 'H' standard.

S. No.	Amino acid	Retention time (Minutes)
1	Aspartic acid	1.787
2	Glutamic acid	2.034
3	Serine	3.057
4	Glycine	3.260
5	Histidine	3.392
6	Arginine	3.753
7	Threonine	3.929
8	Alanine	4.061
9	Proline	4.170
10	Ammonia (side product of derivatization reaction)	4.591
11	Tyrosine	5.361
12	Valine	5.757
13	Methionine	6.059
14	Cystine	6.497
15	Isoleucine	6.789
16	Leucine	6.913
17	Phenylalanine	7.572
18	Lysine	8.387

Table 5: Retention times of the peaks detected in cave nest, house nest, white fungus, fish swimming bladder, jelly and egg white.

Amino acid		Retentions times for each peak (Minutes)				
	Cave nest	House nest	White fungus	Fish swimming bladder	Jelly	Egg white
Aspartic acid	1.787	1.785	1.789	1.727	-	1.732
Glutamic acid	2.033	2.031	2.037	1.951	-	1.956
*	-	-	-	2.680	-	-
*	-	-	2.740	-	-	-
Serine	3.169	3.169	-	3.187	-	3.196
Glycine	3.255	3.261	3.266	-	-	-
Histidine	3.411	3.414	3.418	3.338	-	3.348
Arginine	3.782	3.786	3.790	3.707	-	3.717
Threonine	3.951	3.954	3.958	3.877	-	3.886
Alanine	4.115	4.115	4.083	3.998	-	4.011
Proline	4.149	4.155	4.198	4.123	-	4.082
#Ammonia	4.603	4.605	4.609	-	-	4.543
*	4.829	4.830	4.832	-	-	-
*	5.017	5.018	5.016	-	-	-
Tyrosine	5.282	5.283	5.286	5.202	-	5.208
Valine	5.678	5.680	5.684	5.595	-	5.601
Methionine	5.983	5.981	5.998	5.903	-	5.909
Cystine	6.429	6.428	6.435	6.462	-	6.349
Isoleucine	6.722	6.722	6.729	6.761	-	6.642
Leucine	6.849	6.849	6.854	-	-	6.766
*	-	-	-	-	-	-
Phenylalanine	7.513	7.513	7.518	7.418	-	7.427

*	-	-	-	-	-	7.856
*	8.124	8.125	-	-	-	-
Lysine	8.333	8.333	8.360	8.228	-	8.232
*	-	-	-	-	-	8.817
*	-	-	9.110	-	-	9.034

* Impurities.

* # Side product of derivatization reaction.

Graphical Abstract

Gel Electrophoretic and Liquid Chromatographic Methods for the Identification and Authentication of Cave and House Edible Bird's Nests from Common Adulterants