EFFECT OF EXTRACTION CONDITIONS ON PROTEIN CONCENTRATION DURING EDIBLE BIRD'S NEST ALKALINE HYDROLYSIS

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Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
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SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).

Signature	:
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Date	: JUNE 2014

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

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Dedication

I would like to dedicate my thesis to my beloved supervisor, friends and family who supported me each step of the way.

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ABSTRACT

Edible bird's nest (EBN) is originated from the saliva of swiftlet. There are four species of swiftlet which are *Collocalia fuciphaga*, *Collocalia germanis, Collocalia maxima* and *Collocalia unicolor*. *Collocalia fuciphaga* is the common species which can be found in Malaysia. EBN consists mainly of amino acids, carbohydrates and mineral salts but it major ingredients were protein. Protein from EBN can be extracted by water, acid, alkaline and enzymatic hydrolysis method. This study focused on alkaline hydrolysis using sodium hydroxide (NaOH) at 50°C. Prior to alkaline hydrolysis, pre-treatment method (EBN cleaning by soaked with water, feathers removal and fine plumage from EBN sample) has been carried out. The effect of extraction time, NaOH concentration and liquid solid ratio (LSR) on protein concentration have been determined during alkaline hydrolysis. The optimum condition of alkaline hydrolysis has been identified using response surface methodology (RSM). From the experimental result, it showed that the maximum protein concentration was at 25 minutes of extraction time, 30:1 of LSR and 100 g/L NaOH concentration. However, increased in LSR and NaOH concentration were increased the protein concentration. The optimum condition of protein concentration was 1.260 g/L during alkaline hydrolysis using RSM were found at 25 minutes of extraction time, 100g/L NaOH concentration and 30:1 of LSR. From the ANOVA result, the significant factor was extraction time ($p=0.0187$) and was clearly showed that further increased in extraction time (25 minutes) resulted in decreased in protein concentration due to the degradation of amino acid structure at long duration of extraction time. The FTIR spectrums of the untreated and treated sample resulted in the different trend of spectrum. In the treated sample after pre-treatment process and alkaline hydrolysis, it is showed that N-H and C=O stretching peak is increased. Besides, it was confirmed that the material was correspond to amides as it consists of C=O stretch. While, from the analysis using the ICP-MS, amount of cadmium, arsenic and lead in the sample was 0.002mg/L, 0.00006mg/L and 0.262mg/L respectively after pre-treatment. It also showed that percentage decreasing of argentums, cadmium and magnesium is the highest compared to others metal which were 99%, 97.70% and 97.24% respectively. It can be concluded that EBN can be performed as an alternative source for protein.

ABSTRAK

Sarang burung berasal dari air liur burung walit. Terdapat empat spesies burung iaitu *Collocalia fuciphaga, Collocalia germanis, Collocalia maxima dan Collocalia unicolor*. *Collocalia fuciphaga* adalah spesies yang biasa didapati di Malaysia. EBN terdiri daripada asid amino, karbohidrat dan garam mineral tetapi bahan utamanya adalah protin. Protin dari EBN boleh diekstrak dengan air, asid, alkali dan kaedah enzimatik hidrolisis. Kajian ini tertumpu kepada hidrolisis alkali menggunakan natrium hidroksida (NaOH) pada 50°C. Sebelum hidrolisis alkali, kaedah rawatan awal (pembersihan EBN dengan merendam sampel dengan air, penyingkiran bulu kasar dan bulu halus dari sampel EBN) telah dijalankan. Kesan masa pengekstrakan, kepekatan NaOH dan nisbah pepejal cecair (LSR) kepada kepekatan protein telah ditentukan semasa proses hidrolisis alkali. Keadaan optimum hidrolisis alkali telah dikenal pasti menggunakan balas metodologi permukaan (RSM). Dari keputusan eksperimen, ia menunjukkan bahawa kepekatan protein maksimum dicapai pada 25 minit masa pengekstrakan, 30:1 nisbah pepejal kepada air (LSR) dan 100g/L kepekatan NaOH. Peningkatan dalam LSR dan kepekatan NaOH telah menyumbang kepada peningkatan kepekatan protein. Keadaan optimum kepekatan protein adalah 1.260g/L semasa hidrolisis alkali menggunakan RSM ditemui pada 25 minit masa pengekstrakan, 100g/L kepekatan NaOH dan nisbah 30:1. Dari keputusan ANOVA, faktor penting didapati adalah masa pengekstrakan ($p = 0.0187$) dan telah jelas menunjukkan bahawa peningkatan terus menerus pada masa pengekstrakan (25 minit) menyebabkan penurunan kepada kepekatan protein disebabkan oleh kemusnahan struktur asid amino oleh signal masa pengekstrakan. Spektrum FTIR sampel yang tidak dirawat dan dirawat menunjukkan trend spektrum yang berbeza. Dalam sampel yang dirawat selepas proses rawatan awal dan hidrolisis alkali, ia menunjukkan bahawa N-H dan C=O regangan puncak bertambah. Selain itu, ia telah mengesahkan bahawa bahan tersebut dikategorikan sebagai amida kerana terdiri daripada C =O regangan. Selain itu, dari analisis ICP-MS, jumlah kadmium, arsenik dan plumbum dalam sampel adalah 0.002mg/L, 0.00006mg/L dan 0.262mg/L masing-masing selepas rawatan awal. Ia juga menunjukkan bahawa peratusan penurunan argentums, kadmium dan magnesium adalah yang tertinggi berbanding dengan logam lain yang masing-masing 99%, 97.70% dan 97.24%. Ia boleh membuat kesimpulan bahawa EBN sesuai digunakan sebagai sumber alternatif untuk protein.

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Ppb	parts per billion
Ppm	parts per million

LIST OF ABBREVIATIONS

EBN	Edible bird’s nest
FTIR	Fourier transform infrared spectroscopy
ICP-MS	Inductively coupled plasma-mass spectrometry
LSR	Liquid solid ratio
NaCI	Sodium chloride
NaOH	Sodium hydroxide
RSM	Response surface methodology

1 INTRODUCTION

1.1 Background of the study

Protein is a complex macromolecules made up of amino acids which are covalently bonded together through substituted amide linkages called peptide bonds (Rosenberg, 2005). The bonded is in head-to-tail arrangement. Each protein molecule is arranged in a linear that is unbranched fashion. Protein also known as the combination of amino acids in the peptide linkage containing carbon, hydrogen, oxygen, nitrogen and sulfur (Yada, 2004). There are some unfavoured conditions that tend to made protein molecules become unfolded and fully denatured. The unfavoured conditions are high temperature, an acidified condition, excessive shear and high pressure. Instead of being aggregated and/or crosslink to form larger cluster, denatured protein will form a three-dimensional solid-like network (or gel) at high concentration. Besides, protein is also be known as main classes of building blocks used in semi-solid food that acts for conferring mechanical properties (Linqiang *et al*., 2008)

Ebru *et al.*, (2010) has said that the standard used in order to quantify the amount of protein is bovine serum albumin (BSA). The definition of protein yield and extraction rate that assessed extraction method is the percentage ratio of the protein quantity extracted to the total amount of the protein. Method that is widely used to determine the protein content is known as The Lowry method. A modified cupric sulphate-tartrate reagent has been develop by Pierce,1996 (from the instruction manual) that acted to places two of the three reagents in the original established Lowry method with one stable reagent. This is due to the interest in avoiding the necessity to prepare the fresh reagent daily. About 100% correlation of the colour response curves with various proteins has been observed between the Pierce modified Lowry protein assay reagent and the original Lowry method.

Apart from that, there are three general methods that were widely used to hydrolyze protein into its composition which is amino acids. There are acid hydrolysis, alkaline hydrolysis and enzymatic hydrolysis. This study used alkaline hydrolysis based on its advantages. Among the advantages of alkaline hydrolysis included the time taken to complete hydrolysis. Relatively long

periods required for the complete hydrolysis of bonds using acid hydrolysis. Besides, alkaline hydrolysis is more simples compared to enzymatic hydrolysis. This is because, most enzymes attack only specific peptide bonds rapidly which difficult the whole process. One of the important groups of emulsifying agents used in the food industry is protein. Hence, protein extraction method is suggested in order to isolate and purified protein in large amount.

Alkaline hydrolysis is a simple natural process where complex molecules are broken down into their constituent building blocks by the insertion of water (Gordon *et al.,* 2004). On the previous years, alkaline hydrolysis has been used to study chemical structure of biological molecules, make soaps from animal fats and to prepare skeletal remains for study. Alkaline hydrolysis is acted as improved alternative to incineration for disposing of waste biologic tissues and animal carcasses based on the same chemical reaction, with strong alkali and heat acts in increased speed the process (Thacker, 2004). This method usually used water solutions of alkali metal hydroxides such as sodium hydroxide (NaOH) or potassium hydroxide (KOH). The advantages of alkaline hydrolysis included combination of sterilization and digestion into one operation, reduction of waste volume and weight by as much as 97% complete destruction of pathogens including prions, production of limited odour or public nuisances, and elimination of radioactively contaminated tissues. In addition, heating the reactants dramatically accelerates hydrolysis.

In this study, EBN from *Collocalia Fuciphaga* is used as the raw material. The entire length and weight measured of *C.fuciphaga* is about 12cm and 15-18gm respectively. Besides, in many years, mitogen and avian epithelial growth factor that is known as hormone like substances is found in the *C.fuciphaga* (Jie *et al.*, 2009). EBN has been used for health and tonic as it often offers a good effect on curing tuberculosis, dry coughs, suppressing coughs and phlegm-dyspnea (breathing difficulty), treating consumptive diseases, alleviating hemoptysis (blood's cough), asthma, improving the voice, relieving gastric troubles, stomach ulcer, asthenia and main or common weakness of bronchial ailments. In traditionally, EBN also used to nourish the lungs heart, kidneys and stomach to aid renal functions, promoting growth, strengthen and enhance the immune system, improve concentration, regulate circulation and increase energy and metabolism of body (Kong *et al.*, 1987 and Chan, 2006).

Malaysia is currently one of the largest producers of EBN in the world and it publication can be hypothesized based on the enduring qualitative reasons below that:

1. Consumption of EBN is considered as a status symbol
2. The health giving properties of consuming EBN
3. Strong economic growth rates experienced by Hong Kong, China and Taiwan
4. Potential of EBN as a base mineral to be used in the production of herbal and vitamin supplement, the international market for EBN will grow at double-digit rates for the next two decades or so on.

1.2 Motivation

Every property that characterizes a living organism is affected by proteins. There are many function of protein that made it useful and needed in great amount so that the nutrients from the food can be obtained by all people who in need. Some of the function of protein are to store and transport a variety of particles ranging from macromolecules to electron as they guide the flow of electrons in the vital process of photosynthesis. Protein also acts as hormone, they transmit information between specific cells and organs in complex organism and in between proteins control the passage of molecules across the membranes that compartmentalize cells and organelles. Others than that, protein function in the immune systems of complex organisms to defend against intruders and control gene expression by binding to specific sequences of nucleic acids, thereby turning genes on and off (Lauritzen, 1992). One of the most crucial problems of the world is deficiency of food especially, in protein. About 500 million people suffer from severe-protein-calorie malnutrition. Furthermore, the increase of human population has caused serious problem (Lasztity *et al.*, 1983). In order to obtain protein in great amounts, people may extract protein from any resources including marine life and porcine sources which sometimes do not suit to be consumed in large amounts by certain people. This is because, protein from marine life and other animal may contains high fat that will attributed heart diseases and blood pressure (Hoffman *et al.*, 2004), while protein from porcine sources are not Halal so this protein cannot be consumed by Muslim because of religious barrier. By then, in order to compete with protein extracted from animal and marine life, the percentage of protein extracted from EBN should be optimized. In addition, as there are many disadvantages of protein extraction from

others sources, so, this study will focus on the extraction of protein from EBN via alkaline hydrolysis. In this study, the optimum condition for the protein extraction from EBN that are not been done before was being investigated.

1.3 Statement of problem

In order to produce the high yield of protein, the optimum condition for EBN extraction is determined instead of protein extraction of other sources. Previously, EBN has been extracted using water and enzymatic method which resulted 0.354 g/L (Suzana, 2012) and 0.493 g/L (Afifi, 2013) protein extract respectively. Thus, in this study the optimum condition of protein extracted from EBN using alkaline method will be determined by manipulating the extraction time, sodium hydroxide concentration and solid liquid ratio as there was lacking of research on the alkaline hydrolysis of EBN.

1.4 Objectives

The main objectives of this research are to extract protein from EBN using alkaline hydrolysis. This research also consists of a few specific objectives which are:

- To determine the effect of liquid solid ratio (LSR), extraction time and sodium hydroxide (NaOH) concentration on protein extract.
- To determine the optimum condition of protein extract during alkaline hydrolysis of EBN.

1.5 Scope of this research

- This research will be focusing on analyze EBN using UV-Vis Spectrophotometer
- This research also focus on characterization of EBN using Fourier Transform Infrared Spectroscopy (FTIR) and Inductively Coupled Plasma – Mass Spectroscopy (ICP-MS)
- Study the effects of three parameters, LSR (30:1 to 70:`1), reaction time (5 minutes to 25 minutes) and sodium hydroxide (NaOH) concentration on extraction protein concentration (40g/L to 120g/L)
- Response Surface Methodology (RSM) is used to determine the optimal extraction condition for the protein extraction using alkaline hydrolysis

1.6 Rationale and significance

Analysis of EBN chemical constituents can be traced back to 1921 (Wang, 1922). It is known that EBN is mainly composed of proteins. EBN has the properties of carbohydrate besides protein. As protein are the highest content in the EBN and it has valuable function to people, hence, the study of the extraction protein from EBN with the optimum condition is conducted so that the nutrients on the protein in the EBN is preserved as well as produced high amount of protein extract. However, it is not known yet which method of extraction protein will yield the large amount of total protein. So, this research is revealed that alkaline hydrolysis produced the highest protein yield compared to the acid and enzymatic hydrolysis.

2 LITERATURE REVIEW

2.1 Edible bird's nest (Collocalia Fuciphaga)

Around world, there are more than 24 species of ecolocating and insectivorous swiflets being distributed, but only a few produce nests that are deemed as 'edible' (Koon, 2000). Marcone (2005) stated that 'Edible bird's nest (EBN)' refers to the nest produced by several different swiftlet species which are *C. fuciphaga*, *C. germanis*, *C. maxima* and *C. unicolor* (Goh *et al*., 2001). This research is focused to *C. fuciphaga species* (Figure 2.1) which only be found in the Southeast Asian region. The consumption of these nests by human has been a symbol of wealth, power, and prestige, as well as being used medicinally in traditional Chinese medicine dating as far back as the Tang and Sung dynasties (Koon and Cranbrook, 2002).

Figure 2-1: *Collocalia Fuciphaga* swiflet

2.2 Types of edible bird's nest (EBN)

Nowadays, classification of EBN is usually based on the original place they are built (Goh *et al*., 2001). There are two main types of EBN which are House nest (Figure 2.2) and Cave nest (Figure 2.3). The House nest refers to nests that are built in wooden-roofed swallow farmhouses which are humid and dark. The interior temperature (28°C - 30°C), brightness and relative humidity (90%) are relatively suits to the bird's natural cave habitat. The swiftlet leaves the farmhouse to find food in the wild in the morning and returns in the evening. House nest consists of extremely less impurities and feathers since it farmhouse provides a better habitat. One of the characteristic of house nest is cleaner. House nest looks like a spoon and have a high swelling capacity. Besides, the House nest consists of three different colors which are white, yellow and bloody-red nests. The white nests are smooth contrast to yellow and bloody-red nests that are crunchier due to existences of high amount of minerals. The nest that was constructed in caves and on cliffs is known as cave nest. It is firm, hard and deep in color due to owing to the harsh natural climate and environment. It is not as well shaped as the house nest and exhibits higher impurities. The cave nest is crunchy with low abilities to swell that extends the time for it to swell when preparing, (Sam *et al*., 1991).

Figure 2-2: House nest of Edible bird's nest (EBN)

Figure 2-3: Cave nest of Edible bird's nest (EBN)

2.3 Nutritional value of edible bird's nest (EBN)

Due to the highly evaluated function both nutritiously (water-soluble protein, carbohydrate, iron, inorganic salt and fibre) and medically (anti-aging, anti-cancer, immunity-enhancing, etc), EBN has been esteemed a precious food tonic by Chinese people ever since the Tang dynasty and it was referred as "Caviar of the East". There is current scientific study confirmed that EBN has haemagglutination inhibiting activity against the influenza virus (Howe, 1961; Howe, Lee, and Rose, 1960). An update discovery demonstrated that partially purified swiftlet nest extracts possess the first known avian epidermal growth factor (EGF) (Kong *et al*., 1987; Ng, Chan, and Kong, 1986). Nowadays, EBN was developed into various food products including drink, food additive, and even cosmetic ingredient with the help of modern commercialization and technology. It was also found that both nests share a common 77 KDa protein that has properties similar to those of the ovotransferrin protein in eggs. This protein may responsible for the severe allergic reactions that sometimes occur among young children who consume EBN products (Marcone, 2005). EBN is not the only commercially available food product highly esteemed or priced for human consumption processed through an animal but also includes argan oil made

from the argan nut that has passed through the digestive track of a goat and Kopi Luwak which is the most expensive and rarest beverage/coffee. More scientific work should be done in the future to fully elucidate the biological and medicinal functions of the EBN.

2.4 Origin of edible bird's nest (EBN)

The nests are built mainly by male swiftlet. It is made almost entirely from the saliva secreted by the swiftlet's two sublingual glands. The swiftlet's sublingual salivary glands increase their weight from 2.5 mg to 160 mg and reach maximum secretory activity during nesting and breeding season (Medway, 1962). The birds use saliva to bind materials together and attach them to the vertical walls of inland or seaside caves in order to construct their nests, (Kang *et al*., 1991). The weight of the nest can be 1–2 times the body weight of the swiftlets and the nests can only support the mother and the nestlings. Besides, the nest is looks like half-bowl (Figure 2.4) and 35 days may be needed for the construction process (Marcone, 2005.) The nests are built almost exclusively by the 7–20 g male swiflet over this period of time. Grade of EBN is determined by observing three parameters including dry mass, the duration of time the swiftlets spend in order to construct the nest, the fat and protein content of hardened saliva. Majority of EBN come from two exploited species which is White-nest swiflet and the Black-nest swiflet, whose habitats range from the Nicobar Islands in the Indian Ocean to sea-aves in the coastal regions of Thailand, Vietnam, Indonesia, Borneo and the Palawan Islands in the Philippines (Koon, 2000; Koon and Cranbrook, 2002). The white nest is made almost entirely from saliva (Sims, 1961) while the black nest contains about 10% feathers which contribute 8% of the protein content in the nest (Kang *et al*., 1991).

Figure 2-4: Edible bird's nest (EBN)

2.5 Contents of edible bird's nest (EBN)

There are some properties of proteins, peptides, amino acids, and nitrogen in the EBN as for example, the composition of nitrogen consists in the EBN is about 10.29% (Chen *et al*.,1996 ; Wang, 1921). For two types of nests, the white nest and the red blood nest, there is compositional properties of lipid (0.14 – 1.28%), ash (2.1%), carbohydrate (25.62 - 27.26%) and protein (62 – 63%)(Marcone, 2005). It has confirmed that EBN contains a higher percentage of humin nitrogen and cysteine nitrogen than for pure proteins which may be because of the carbohydrate radical and fine feathers present in the nest (Wang, 1921). It was known that EBN rich in protein which are composed of 20 amino acids. EBN contains all the essential amino acids as the most abundant amino acids in the EBN are serine, threonine, aspartic acid, glutamic acid, proline, and valine (Kathan and Weeks, 1969). While, the white nest, it is rich in two aromatic amino acids such as phenylalanine and tyrosine (Marcone, 2005).

2.6 Market of edible bird's nest (EBN)

Hong Kong is world's largest importer and consumer of the processed nests, while North America being the second largest market (Goh *et al*., 2001). 17–20 million nests were estimated are harvested each year with the total weight approximately about 2 metric tonnes (Goh *et al*., 2001). Depending upon grade, the EBN retails for anywhere from $2000.00 (for white nests) to $10,000.00 (for "red blood" nests) Canadian per kilogram (Koon and Cranbrook, 2002)

2.7 Pre-treatment of edible bird's nest (EBN)

EBN need to be cleaned right after the collection and the procedure is known as pre-treatment. It is a long and time-consuming process. First of all, the raw EBN is soaked in water for 6 to 48 h for them to absorb the water and expand. Then, the removal of the feathers is performed in which the larger feathers are removed by using tweezers while fine feathers are removed with a floatation technique using vegetable oil as this procedure is depends on the processors. In order to produce premium grade nests, the long strands of nest cement are carefully been isolated. Instead of that, the broken strands are meshed and used to constitute the base of the cleaned nests. The color of processed nests resembles that of the raw nests. Besides that, the processors do not normally clean the good quality white nests because it's difficult to restore them to their original shape after they have been soaked in water and for the information, the process may take a person 8 h to clean about 10 nests (Koon and Cranbrook, 2002; Leh, 2001).

2.8 Swiflet farming in Malaysia

There are fundamental and long-standing industries in Malaysia such as rubber, palm oil, oil and gas, timber and financial services, but since 5 years ago, swiflet farming industry being new development in this country. Such good news, due to the industry's relatively profitable risk-return profile followed by a continuously growing demand for EBN by wealthy overseas countries, the swiftlet farming industry has potential to grow into a multi-million ringgit industry. In order to produce natural and organic health supplement products for local and overseas consumption, there is also a discernable world-wide trend pursued by international as well as home grown pharmaceutical and herbal products companies using EBN as base materials. As the swiftlet farming industry continues to expand and grow, more and more

supplies of EBN sourced from purpose-built farms that are constructed specially to house EBN swiftlets find their way into the supply chain (Hameed, 2007).

After the Asian Economic Crisis (1997-1998), the swiftlet farming industry in Malaysia has began to gather momentum. At this time, many businesses, excluding larger business experienced hard times and a great number of them closed down throughout the country. Over the last 8 years, the swiftlet farming industry in Malaysia has been growing and before 1998, it has been estimated that there are 900 plus swiftlet farms throughout the country. There are number of businessmen, landlords and investors has began to realize that the financial viability of the swiftlet farming industry in Malaysia right after the first generation of swiftlet farms that are established after 1998 started to produce commercially harvestable quantities of EBN. By the end of 2006, the estimation of swiftlet farms throughout the country close to 36,000 units, with an average annualized growth rate of 35% per year (Hameed, 2007).

Nowadays, the business of swiftlet farming essentially involves the conversion of people-centric buildings into buildings used to house and protect a certain species of swiftlets that can be found in the South East Asian region as well as the design and construction of purpose-build buildings for the purposes of accommodating such swiftlet populations as well. Each and every day, a continuous vocalization of swiftlet chirps and mating sounds are played using speakers and audio systems installed within such buildings in order to lure the swiftlets that are flying overhead to fly into the said buildings to mate and make the buildings their new home. There are almost 99% of all swiftlet farms in Malaysia are geared towards the production of white edible birds' nests (Hameed, 2007).

Mostly, secondary and tertiary townships is often being a choice of major swiflet farming areas due to the abundance of food source is and pollution levels are at their relative minimum. These secondary and tertiary townships include Kampong Tebing, Kampong Tasoh, Kampong Banat Bawah, Jampong Bakan, Kuala Nerang, Pokok Sena, Kampong Tanjung Radin, Kuala Ketil, Lunas, Kulim, Sungai Petani, Jitra, Bukit Mertajam, Nibong Tebal, Kepala Batas, Cangkat Kledang, Legong, Jelai, Cangkat Jering, Bruas, Pantai Remis, Lumut, Teluk Intan, Setiawan, Bagan Serai, Parit Buntar, Selama, Tanjung Malim, Kuala Kubu Bahru, Rawang, Kepong, Cheras, Slim River, Kulai, Kanpong Bahru Paroi, Alor Gajah, Ayer Pasir, Durian Tunggal,

Tangkok, Pagoh, Bukit Pasir, Kampong Machap, Ulu Tiram, Tai Hong Village, Senai, Pontian Kecil, Jemaluang, Kampong Seri Pantai, Mersing, Kampong Sawah Datuk, Kampong Air Papan, Kuala Besut, Tok Soboh, Kampong Pinang, Rompin, Pekan, Kuala Terengganu and Pasir Mas (Hameed, 2007). In this study, the raw material has been taken from Pekan, Pahang Darul Makmur.

Currently, behind Indonesia (60%) and Thailand (20%), the third largest producer of EBN (7% of gross supply value) in the world is comes from Malaysia. In 2006, about 90 to 120 of unprocessed white EBN is able to fetch production level prices of RM$4500 to RM$6000. A kilogram of processed white EBN is able to fetch retail level prices of RM$15000 to RM$25000 in Hong Kong and China (Hameed, 2007).

Hong Kong (50% of world trade), China (8%), Taiwan (4%) and Macau (3%) has been categories as the main export markets for EBN with a consumption weight value of approximately 160 tons for 2006. The total consumption value of EBN was estimated to be in the range of RM8 billion to RM12 billion. In 2004, Ministry of Housing and Local Government with the gazzetting of the "Guidelines on Swiftlet Farming" recognized the swiftlet farming industry in Malaysia as a valid contributor of important foreign exchange currency for the country (Hameed, 2007).

The Guidelines require registered premises with relevant council that have not been designated as a Class 1 Heritage Building converted into a swiftlet farm. The Guidelines also require that certain standards and levels of premises upkeep must be adhered to in areas of noise, health, pollution, scope of renovation works, building façade rendition and lighting be adhered to before swiftlet farming licenses can be issued by the relevant council (Hameed, 2007).

A swiftlet farm can yield returns that are competitive with other types of profitable industries if it been constructed properly. There are many swiftlet farms throughout the country yield 1kg to 10kgs (RM4000 to RM40000) of collected white EBN per month based on perceptive and cognitive observations. On average, an average yield of around 1kgs to 3kgs (i.e. RM4000 to RM12000) of collected white EBN per month can be obtained from a 2 storey swiftlet farm that had been properly converted from a conventional shop-lot or shop-house in Malaysia (Hameed, 2007).

2.9 Protein

The bioactive components contains in the EBN (protein) need to be isolated and purified as it consists of many functions that benefits human being. Protein acts as an essential nutrient for human body which is not only as an energy source but also as a building agent. Protein is used to build and maintain body tissues, produce neurotransmitter for brain, produce amino acids and hormones, maintain immunity system and also maintain acid-base balance in cell fluid. Apart from that, protein also functioned well in body growth. In addition, protein also plays important roles in phenomena such as signal transduction, gene expression, metabolism, cellular and extracellular structures, etc. Denise *et al*., (2001) revealed that promotion of cell division and epidermal growth-factor like proteins are capable in the presence of protein. Many proteins are also useful for therapeutic or diagnostic applications. Besides, any property on which the utility of food proteins depends on the food ingredients (Cepeda *et al*., 1998). Many reasons food proteins are hydrolyzed such as for the improvement of nutritional and functional properties, texture characteristics to the removal of odour, flavour, and toxic or anti-nutritive components. In hydrolysis treatments, general proteins had used are casein, whey and soy protein (Periago *et al*., 1998). Therefore, it is still necessary to prepare the protein of interest in a pure form without contaminants that may potentially put a human being's health at risk. Separation and purification of proteins constitute a major bottleneck of modern process biotechnology.

2.10 Source of protein

Commercially available protein foods are obtained from a range of animal and plant sources and are used as functional ingredients (Periago *et al*., 1998). Due to the increasing costs and limited supplies of animal proteins, and since vegetable protein is the most abundant source of protein on the Earth, a number of vegetable proteins have been investigated for possible incorporation into formulated foods (Achouri *et al*., 1999; Sathe and Sze-Tao 2000). The examples of vegetables protein are alfalfa leaf, cottonseed, winged bean, peanut and soya.

2.11 Amino acid

Each protein is made up from various types of amino acids that are associated together in varying order or arrangement (Lauritzen, 1992). Protein is also origin from large molecules that consist of several hundred amino acids arranged in branches and chain. Amino acid has general classification (Table 2.1) according to the different functional group attached to it with different location with one another. Based on the statement of the requirement on dietary scope of amino acid, the types of amino acid can be classified and categories. However, the classification may be hard to be made as the required amount of the amino acids is different.

Table 2-1: Classification of twenty types of amino acid

ESSENTIAL	NON-ESSENTIAL
Histidine	Alanine
Isoleucine	Arginine
Leucine	Aspartate
Lysine	Asparagine
Methionine	Glutamic acid
Phenylalanine	Glutamine
Threonine	Glycine
Valine	Serine
	Tyrosine

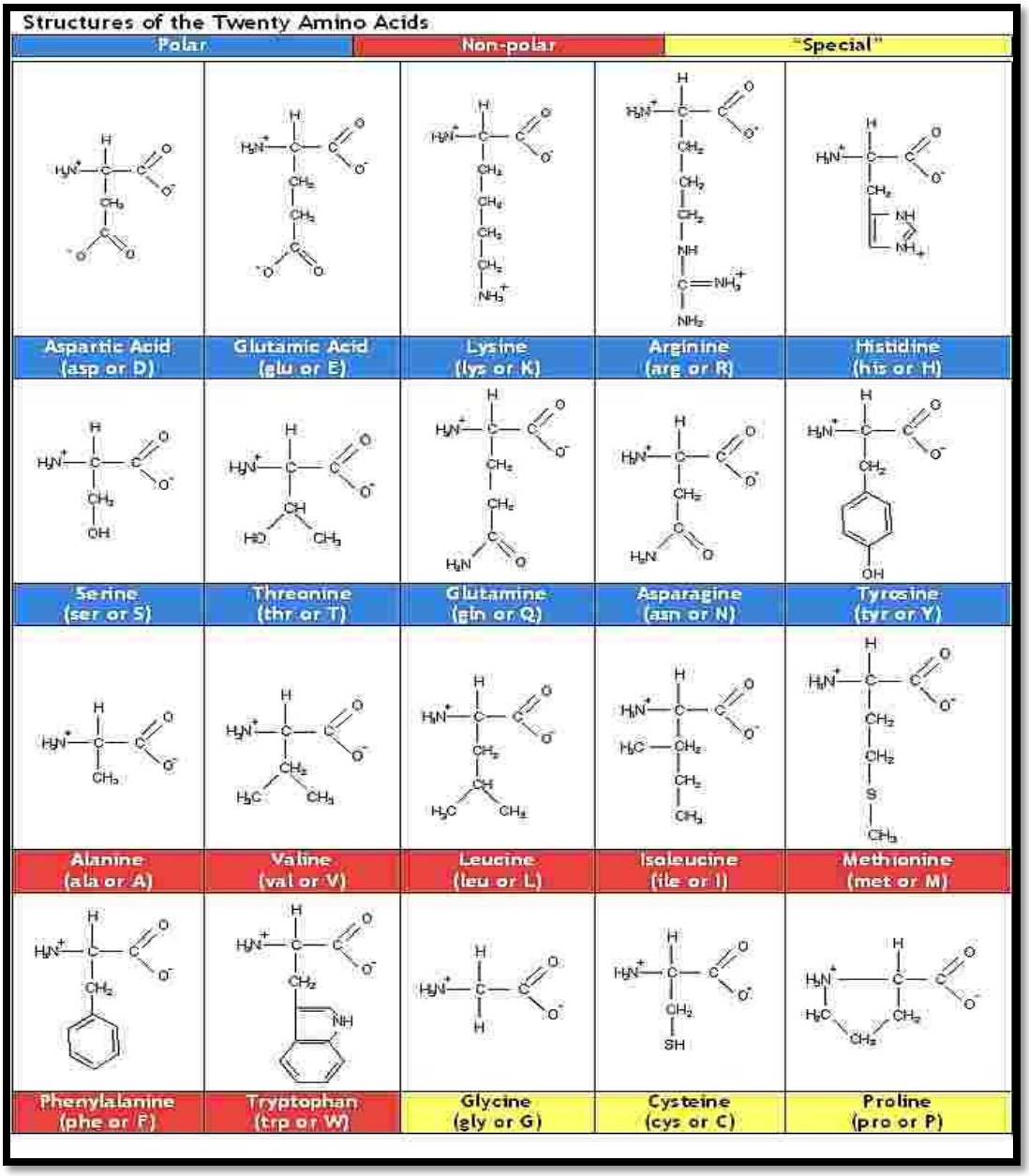

Figure 2-5: Structure and classification of twenty amino acids

(Source: http://www.bing.com/images/search?q=20+amino+acids&FORM=HDRSC2#view=detail&id=0690722C0F6619C8155ADA8C8D2570AD54E1D0B5&selectedIndex=2)

2.12 Alkaline hydrolysis concept

Alkaline hydrolysis uses sodium hydroxide or potassium hydroxide to catalyze the hydrolysis of biological material such as protein, nucleic acids, carbohydrates, lipids and others into a sterile aqueous solution consisting of small peptides, amino acids, sugars, and soaps. In order to significantly accelerate the process, heat is applied at about 150°C, or ~300°F (Wilson, 2003).

2.13 Protein extraction

Liu (1997) stated that various parameters such as pH, temperature, ionic force, salt or solvent type, extraction time, LSR, and presence of components that causing linking will give an affect to protein solubility. As nutritional ingredient, the protein solubility also may be affected by extraction conditions, solvent type and heat treatment. Besides, the procedures of the isolation, extraction and fractioning were differed as it depends on the end-use. When the isolation and fractionation was carried out for application in the food industries, an extraction method in alkaline aqueous solution will be chosen. In addition, Sath *et al.,* (1984) stated that the precipitated proteins may be separated by heat coagulation, filtration, centrifugation or ultracentrifugation. Wani *et al.,* (2006) and Liadakis *et al.,* (1995) reported that temperature was not gave significant factor to the protein extraction.

2.14 Liquid Solid Ratio (LSR)

According to Stella *et al.,* (2010), liquid solid ratio (LSR) is a ratio of the solvent volume per gram of raw material. Since the LSR is a ratio, then, to cancel out the unit of volume per gram of LSR, the volume will be change to unit of gram by also weighing it on the analytical balance. Besides, the effect of the LSR shows "saturation" on the both ends (Edward, 2013). The LSR is an important factor in determining the extraction efficiency. Generally, in conventional extraction techniques, a high solvent volume will increase the extraction efficiency, and a low LSR will result in the incomplete extraction of the analytes and low extraction efficiency. However, larger solvent volumes could complicate the procedure and result in unnecessary waste (Lei *et al*., 2013). The extraction resulted from the study of LSR affect is also depended on the raw materials it used.

2.15 Alkaline hydrolysis of protein extraction

Based on Table 2.2 below, there are six previous studies that focused on the alkaline hydrolysis method in order to extract protein from different type of raw materials. In this research, sodium hydroxide (NaOH) is chosen as a solvent to extract protein because of high protein extracted are obtained (80.71%) by Wani *et al*., (2008). The parameter studied in this study are temperature (40ºC - 60ºC), concentration of NaOH (0.03g/L – 0.15g/L), duration of extraction time (5 – 25 minutes) and LSR (30:1 – 70:1). At the end of this study, it concluded that the optimum condition of the extraction protein is 50ºC, 0.12 g/L NaOH, 15 minutes and 70:1 respectively. In this study, it was proved that alkali concentration and LSR have significant effect while temperature and extraction time will gave slight effect on protein yield after all the responses been considered.

According to the previous research on the extraction of pigeon pea protein, the product yield is only about 75% which is lower than the results obtained from extraction using NaOH. Ivone *et al*., (2000) only investigate three parameters if compared to the study on extraction protein from watermelon seed. The three parameters involved were NaCI concentration (M) (0.000 - 0.100), LSR (v/w) (5:1 - 25:1) and pH (2.5 - 8.5). The percentage of protein yield obtained when the pH used approximately 8.5 without the addition of NaCI regardless of the LSR under the conditions investigated.

High protein yield also is observed in two other research that also using NaOH in their method of extraction. This two research is investigated the protein extraction from lupine seed and beef bones. Although the protein yield on the beef bones is only 17.2% but it still higher if compared to the results of extraction using only NACI (Rathgeber *et al.*, 2000). While the protein yield obtained in the research on the isolation of protein from lupine seeds is 79.7% (Khalil *et al*., 1990). As conclusion, there are slightly differences in the amount of protein yield between three researches that using NaOH but it still more higher when compared to the NACI. The results obtained is different may be due to the different type of sample material, type of equipment used and other agriculture practices during growing conditions.

Table 2-2: Alkaline hydrolysis method in extraction of protein in different type of samples.

EXPERIMENT	HYDROLYSIS CONDITION	REFERENCES	NOTES
Extraction and isolation of protein from lupine (Lupinus termis L.) seeds	Three extractants : Distilled water, 5% NACI and 0.1N NaOH. For 0.1N NaOH : Extraction time(min) : 10, 20, 30, 40, 50 LSR : 100 :1	M. Khalil and Simon- Sarkadi (1990)	Protein yield for Distilled water : 28.5% 5% NACI : 43.5% 0.1N NaOH : 79.7% Optimum condition ; -Extraction time : 30 minutes -LSR : 100 :1
The production of protein from germinant pumpkin seeds	NACI concentration : 4% ; 6% ; 8% LSR (v/w) : 10:1 ; 20:1 ; 30:1 Reaction time (min) : 10 ; 20 ; 30	Quanhong and Caili (2005)	Protein yield : 7.84 g protein extraction / 100g seed Optimal condition ; NACI concentration : 4.26% LSR : 30.2 :1 (v/w) Reaction time(min) : 18.1 Most relevant variable : LSR
Recovery of proteins from beef bone and the functionality of these proteins in sausage batters	4% NACI (control) 0.3% STP 0.3% TTP 0.05M NaOH	Boles, Rathgeber and Shand (2000)	Protein yield NaCI : 11.4% NaCI & STP : 14.3% NaCI & TTP : 13.2% NaOH : 17.2%

			Optimum condition: LSR : 10:1
Extraction condition for max protein recovery from watermelon	Temp (ºC) : 40; 45; 50; 55; 60 NaOH concentration (g/L) : 0.03; 0.06 ; 0.09; 0.12; 0.15 Reaction time (min) : 5; 10; 15; 20; 25 LSR (v/w) : 30:1; 40:1; 50:1; 60:1; 70:1	Wani *et al*., (2008)	Optimum condition : NaOH concentration (g/L) : 0.12 Reaction time (min) : 15 LSR (v/w) : 70:1 Temp : 50ºC Product yield : 80.71g/100g seed
Extraction optimization of pigeon pea protein	NACI concentration (M) : 0.000; 0.025; 0.050; 0.075; 0.100 pH : 2.5 ; 4.0 ; 5.5.; 7.0 ; 8.5 LSR (v/w) : 5:1; 10:1; 15:1;20:1; 25:1	Ivone *et al*., (2000)	Optimum condition : pH : 8.5 Product yield : 75%
Protein extraction optimization of red pepper seed	Extractor : Deionized water Temp (°C) : 30; 35; 40; 45; 50 pH : 7.0; 7.5; 8.0; 8.5; 9.0 Reaction time(min) : 20; 30; 40; 50; 60 LSR (v/w) : 10:1; 15:1; 20:1; 25:1; 30:1	Ebru and Ozgul (2010)	Optimum condition: Temp (°C) : 31 pH : 8.8 LSR (v/w) : 21:1 Protein yield : 12.24g/100g seed

2.16 Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy (Figure 2.6) is often used as a tool to gain average structural information on proteins. FTIR can be used to derive accurate information on isolated amino acids with the advent of stable isotope editing. It is possible to measure the orientation of the peptidic carbonyl groups in an anisotropic sample. The example of anisotropic is membrane layers (Manor *et al*., 2013).

Figure 2-6: Fourier Transform Infrared Spectroscopy (FTIR)

An IR-absorbance spectrum of amide vibrational modes from peptides separately from the lipid vibrations which represented by the FTIR spectroscopy has allow the study of peptides embedded in their native environment. Moreover, it is a technique that is particularly suited for the study of membrane proteins due to the water absorbs dramatically in the infrared regions in which proteins absorb. The reason being, is that without any deleterious effects, bulk water can normally be removed when studying membrane proteins, but not from their water-soluble counterparts (Manor *et al*., 2013).

In traditionally, based on a correlation between the peak frequencies and specific structural elements, FTIR spectra have been used to analyze secondary structure content of the protein. With this respect, in terms of information, FTIR is similar to circular dichroism (CD) spectroscopy. However, when examining large particles (proteins) that is embedded in lipid vesicles, CD only able to employs short wavelengths (ca. 200 nm) which may result in differential absorption flattening (Manor *et al*.,2013).

Besides, FTIR spectra is sensitive to is H^+/D^+ exchange which enabling the determination of the solvent accessibility of the protein. In order to yield information on the polarity of the surrounding area, line width information in FTIR has also been used. The principle of the method is accordingly to the correlation between the in-homogenous line width of the electrostatic field and vibrational mode (Manor *et al*., 2013).

Apart from that, FTIR has also been used as universal instrument in order to analyze varieties of samples due to its ability to identify functional group of chemical compounds, such as carbohydrate and ester, as well as inter atom chemical bonds. It has high accuracy level in identifying process instead of safe, fast, and sensitive. In addition, FTIR has also is used to analyze protein plasma in blood, breast cancer diagnosis and chemical components of gallstone. The presence of reproductive hormones also can be detected by FTIR due to the presence of specific functional groups, such as ketone (=O), carboxyl (COOH), and methyl (CH3) (Sjahfirdi *et al*., 2004).

In order to identify the presence of protein, FTIR method is used to indicating amide groups and measuring its level in food through specific groups of protein. The wave number 400—4000 cm-1 is used during the scanning process. The determination of functional group was being done by comparing wave number of amide functional groups of the protein samples to existing standard. By comparing absorbance of protein specific functional groups to absorbance of fatty acid functional groups, protein level can be measured (Sjahfirdi *et al*., 2004).

At last but not least, based on absorbance spectroscopy according to infrared radiation absorbance difference by molecules of a matter, this method will be works. Specific functional groups that build a compound will be identified by the FTIR. As the protein consists of amino acids which is each has specific chemical structure such as aliphatic, hydroxyl, amide, etc, FTIR can be used to detect those functional groups to indicate protein presence as well. Amide is one of specific functional groups that can be easily detected (Sjahfirdi *et al*., 2004).

2.17 Inductively coupled plasma-mass spectrometer (ICP-MS)

Recently, inductively coupled plasma (ICP) mass spectrometry (MS) is used in many diverse research fields such as earth, environmental, life and forensic sciences and in food, material, chemical, semiconductor and nuclear industries. The high ion density and the high temperature in plasma provide an ideal atomizer and element ionizer for all types of samples and matrices introduced by a variety of specialized devices (Adrian, 2007).

ICP-MS is a type of mass spectrometry which is capable of detecting metals and several non-metals at concentrations as low as one part in 1012 (part per trillion). This is achieved by ionizing the sample with inductively coupled plasma and then using a mass spectrometer to separate and quantify those ions (Adrian, 2007).

After introduction of the first commercially available instrument in 1983, the technique has continuously improved. Several manufacturers produce reliable and robust instruments with very low detection limits (ppt) and high spectral resolution (10 000) for multi element isotope detection. On the basis of the broad range of applications and its indispensable role demonstrated in the investigation of numerous devastating health crises (such as nerve degeneration by methyl mercury, degeneration of male sexual organs in animals by organotin compounds, brain damage by organolead compounds, poisoning by arsenicals in drinking water, etc.), the technique is further expanding in life science research since it is well established in sensitive heteroelement detection and ease of quantitation (Adrian, 2007).

Outstanding properties such as high sensitivity (ppt–ppq), relative salt tolerance, compound-independent element response and highest quantitation accuracy lead to the unchallenged performance of ICP MS in efficiently detecting, identifying and reliably quantifying trace elements. The increasing availability of relevant reference compounds and high separation selectivity extend the molecular identification capability of ICP MS hyphenated to species-specific separation techniques. While molecular ion source MS is specialized in determining the structure of unknown molecules, ICP MS is an efficient and highly sensitive tool for target-element orientated discoveries of relevant and unknown compounds (Adrian, 2007).

Plasma source mass spectrometry (PS MS) has a long-lasting, unbroken and still increasing record of excellent performance. However, in most publications, MS automatically is associated

with a ‘soft’, low-temperature ion source, as though MS would exclusively be performed by low-temperature ion sources for organic molecular ion formation and fragmentation. This is not justified, especially because the performance of PS MS is undoubtedly superior in useful aspects of analytical chemistry. Inductively coupled plasma (ICP) MS, the most widely applied PS MS, has played and is still playing an important role in many fields of applied science and research. The complementarily of ICP MS with other types of ion source MS (such as electro spray ionization MS) and the recent tremendous progress made in the development of these for bioinorganic analytical chemistry have been well documented in an excellent review. The information that can be recovered from the application of the two types of ion sources is quite different but ultimately complementary, which is affirmed by the efforts to develop modulated ion sources capable of generating either elemental ions or molecular fragment ions within the same ion source device (Adrian, 2007).

ICP MS has also become the method of choice in elemental speciation, covering a broad field of covalently bound elements, coordinated metals, metalloids and organometallic metabolites. A large number of proteins bearing heteroelements such as S, P, Se natively binding metals (Zn, Fe, Mn, Cu, Ni, Mo, Cr) have been detected and quantified10 by ICP MS, emphasizing its potential in life science research.11 Parallel to proteomics research, the activity in bioinorganic speciation has rendered metallomics studies accessible, a new research field linked to proteomics, since more than 25% of all proteins contain metals (Adrian, 2007).

Figure 2-7: Inductively coupled plasma-mass spectrometer (ICP-MS)

2.18 Heavy metals

Heavy metals constitute a very heterogeneous group of elements widely varied in their chemical properties and biological functions. It are kept under environmental pollutant category due to their toxic effects on plants, animals and human being. Heavy metals are persistent in nature, therefore get accumulated in soils and plants. Usually, heavy metals interfere with physiological activities of plants such as photosynthesis, gaseous exchange and nutrient absorption, and cause reductions in plant growth, dry matter accumulation and yield. Besides, it also interferes with the levels of antioxidants in plants, and reduces the nutritive value of the produce. Dietary intake of many heavy metals through consumption of plants has long term detrimental effects on human health (Sharma and Agrawal, 2005).

The main threats to human health from heavy metals are associated with exposure to lead, cadmium, mercury and arsenic. These four heavy metals need to taken seriously as they are most dangerous between others. Table 2.3 below shows the effect of others heavy metals towards human and mammals. International bodies such as the WHO have been regularly viewed the effects of these metals towards human. Heavy metals have been used by humans for thousands of years. Although several adverse health effects of heavy metals have been known for a long time,

exposure to heavy metals continues, and is even increasing in some parts of the world, in particular in less developed countries, though emissions have declined in most developed countries over the last 100 years (Järup, 2003).

Currently, cadmium compounds are used in re-chargeable nickel–cadmium batteries. During the 20th century, cadmium emissions have increased dramatically, one reason being that cadmium-containing products are rarely re-cycled, but often dumped together with household waste. Cigarette smoking is a major source of cadmium exposure. Somehow, in non-smokers, food is the most important source of cadmium exposure. Recent data indicate that adverse health effects of cadmium exposure may occur at lower exposure levels than previously anticipated, primarily in the form of kidney damage but possibly also bone effects and fractures (Järup, 2003).

The general population is exposed to lead from air and food in roughly equal proportions. Lead emissions to ambient air have caused considerable pollution, mainly due to lead emissions from petrol. Children are particularly susceptible to lead exposure due to high gastrointestinal uptake and the permeable blood–brain barrier. Blood levels in children should be reduced below the levels so far considered acceptable, recent data indicating that there may be neurotoxic effects of lead at lower levels of exposure than previously anticipated. Although lead in petrol has dramatically decreased over the last decades, thereby reducing environmental exposure, phasing out any remaining uses of lead additives in motor fuels should be encouraged. The use of lead-based paints should be abandoned, and lead should not be used in food containers. In particular, the public should be aware of glazed food containers, which may leach lead into food (Järup, 2003).

While, exposure to arsenic is mainly via intake of food and drinking water, food being the most important source in most populations. Long-term exposure to arsenic in drinking-water is mainly related to increased risks of skin cancer, but also some other cancers, as well as other skin lesions such as hyperkeratosis and pigmentation changes. Occupational exposure to arsenic, primarily by inhalation, is causally associated with lung cancer. Clear exposure–response relationships and high risks have been observed (Järup, 2003).

Table 2-3: The effect of heavy metals towards human and mammals

Element	Effects	References
Arsenic	*Acute*: nausea, vomiting, "rice-water" diarrhea, encephalopathy, multi-organ dysfunction syndrome, long QT syndrome, painful neuropathy	Soghoian and Sinert (2008)
	Chronic: diabetes, hypopigmentation/hyperkeratosis, cancer: lung, bladder, skin, encephalopathy	Soghoian and Sinert (2008)
	Toxic concentration: 24-h urine: ≥50 μg/L, or 100 μg/g creatinine	Soghoian and Sinert (2008)
	Other effects: promotes bladder, lung, skin, and prostate cancer	Salgado-Garcia *et al*., (2006)
Cadmium	*Acute*: pneumonitis (oxide fumes)	Soghoian and Sinert (2008)
	Chronic: proteinuria, lung cancer, osteomalacia	Soghoian and Sinert (2008)
	Toxic concentration: proteinuria and/or ≥15 μg/g creatinine	Soghoian and Sinert (2008)
	Other effects: kidney and bone damage	WHO (1992)
	Inhibition of progesterone and estradiol	Zhang *et al*., (2008)
	Alterations in uterus, ovaries and oviduct	Massanyi *et al*., (2007)
	Progesterone synthesis of ovaries	Zhang and Jia (2007)
	Endocrine disruption	Henson and Chedrese (2004)
	Act as estrogen in breast cancer	Brama *et al*., (2007)
	Excess risk of cardiovascular mortality	Jarup (2003)

Element	Effects	References
Chromium	*Acute*: gastrointestinal hemorrhage, hemolysis, acute renal failure (Cr^{6+}ingestion)	Soghoian and Sinert (2008)
	Chronic: pulmonary fibrosis, lung cancer (inhalation)	Soghoian and Sinert (2008)
	Toxic concentration: no clear reference standard	Soghoian and Sinert (2008)
	Chromium(VI): potential lungs, liver, and kidney cancer producer; DNA damages	Dong *et al*., (2007)
Lead	*Acute*: nausea, vomiting, encephalopathy (headache, seizures, ataxia, obtundation)	Soghoian and Sinert (2008)
	Chronic: encephalopathy, anemia, abdominal pain, nephropathy, foot-drop/wrist-drop	Soghoian and Sinert (2008)
	Toxic concentration: Pediatric—symptoms or [Pb] ≥ 45 μ/dL (blood); Adult—symptoms or [Pb] ≥ 70 μ/dL	Soghoian and Sinert (2008)
	Other effects: damages central nervous system, excretory system, circulatory system, and cardiovascular system	Ma (1996)
Mercury	*Acute*: Elemental (inhaled)—fever, vomiting, diarrhea, acute lung injury. Inorganic salts (ingestion)—caustic gastroenteritis	Soghoian and Sinert (2008)
	Chronic: nausea, metallic taste, gingivo-stomatitis, tremor, neurasthenia, nephrotic syndrome; hypersensitivity (Pink disease)	Soghoian and Sinert (2008) Soghoian and Sinert (2008)
	Toxic concentration: Background exposure "normal" limits: 10 μg/L (whole blood); 20 μg/L (24-h urine)	Soghoian and Sinert (2008)

Element	Effects	References
	Other effects: cough, dyspnea, fever, tremor, malaise, motor neuropathy, gum disease, delusions and hallucinations	Guzzi and La Porta

2.19 Scanning electron microscope (SEM)

The SEM is the most crucial electro-optical instrument for the investigation of bulk specimens. An electron probe is produced by two- or three stage demagnification of the smallest cross section of the electron beam after acceleration. The diameter of the electron probe is about 2-10nm. This electron probes is scanned in a raster over a region of the specimen. Possibility of direct surface profiling is the striking advantage of SEM which restricted to the imaging and analysis of surface (Ludwig and Helmut, 2008). Figure 2.8 below is the schematic ray path for a SEM.

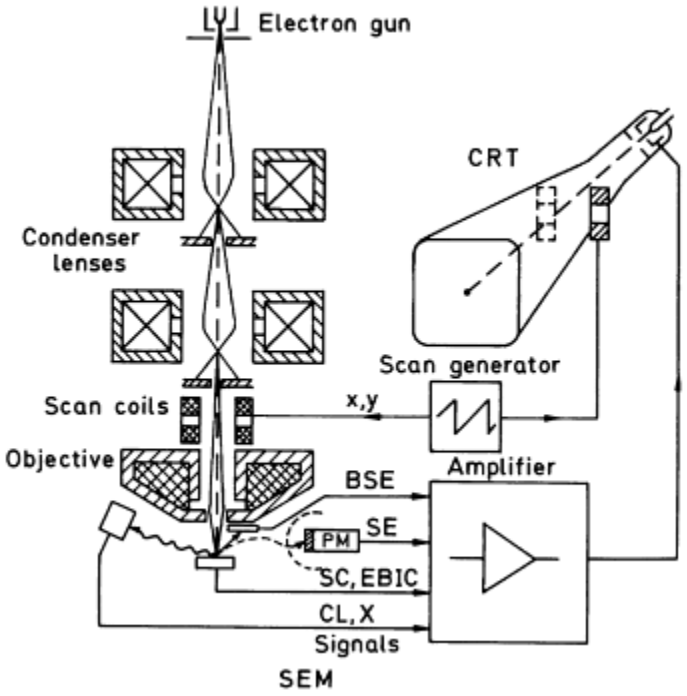

Figure 2-8: Schematic ray path for a SEM (Source: Ludwig and Helmut, 2008)

2.20 Scanning electron microscope and its field of application

SEM has ability to examine detail on a wide range of materials in an easily interpreted manner, from high to low magnification with an exceptional depth of focus (Carl Zeiss, 2013). SEM has distinct advantages over the conventional transmission microscope and the reflexion electron microscope. For example, there are specimens who are too thick to be viewed by direct transmission and which nevertheless do not lend themselves readily to the construction of replicas, either because they are too fragile, or because their surfaces are undercut so that the replica would be keyed to them or because observation of the specimen at high temperatures is desired. In particular, a pronounced three-dimensional effect is observed by using SEM. Besides, bombardment of the SEM is much less severe than with other electron microscopes (Smith and Oatley, 1955).

Primarily, the SEM is a tool for the observation of surfaces, with the advantage of a depth of focus approximately an order of magnitude greater than that of the optical microscopes and with the possibility of direct viewing of complicated surfaces without replication. The facility of experimental study of phenomena occurring at surfaces, or features of surfaces characteristic of flow and fracture, appear to indicate the importance of the microscope in studying oxidation and corrosion phenomena and in diagnosing mechanical failure modes. In the field of semiconductors, application includes the fabrication and inspection of devices and the detection of defects. While, the applications also extend across the field of materials and include metals, oxides, glass, textile, fibers, wood and paper (Minkoff, 1967).

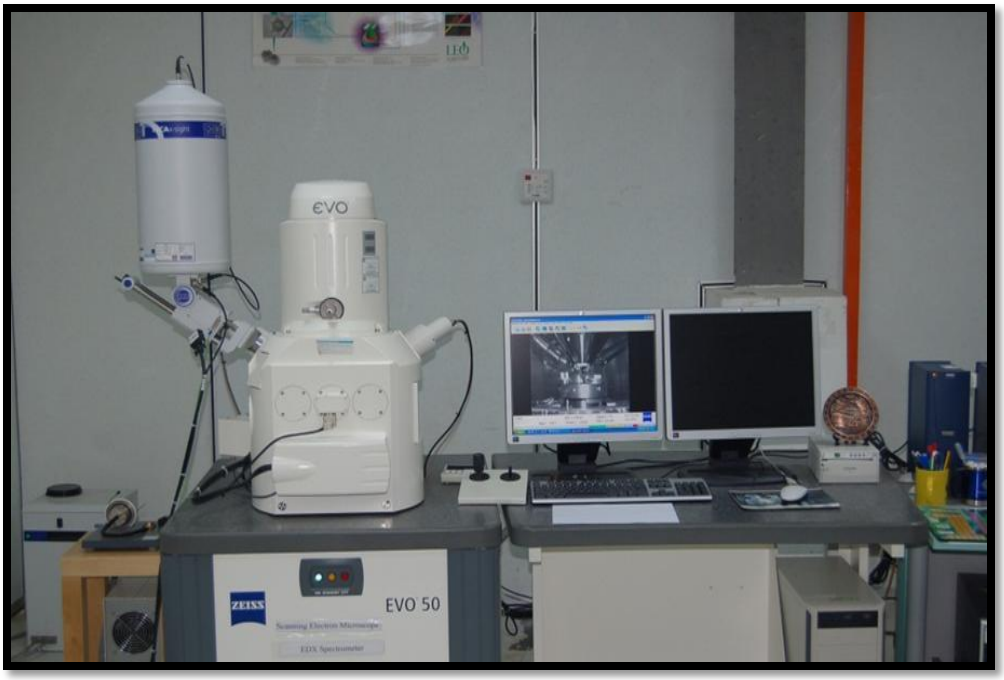

Figure 2-9: Scanning electron microscope (SEM)

2.21 Response surface methodology (RSM)

According to Myers, R.H. *et al*., 2002, Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes. RSM also is extremely useful as an automated tool for model calibration and validation especially for modern computational multi-agent large-scale social-networks systems that are becoming heavily used in modeling and simulation of complex social networks. The RSM can be integrated in many large-scale simulation systems such as BioWar, ORA and is currently integrating in Vista, Construct, and DyNet.

In the particular situations, the most extensive applications of RSM are where several input variables potentially influence some performance measure or quality characteristic of the process. Thus performance measure or quality characteristic is known as a response. The input variables are sometimes called independent variables, and they are subject to the control of the scientist or engineer. The field of RSM consists of the experimental strategy for exploring the space of the process or independent variables, empirical statistical modeling to develop an appropriate approximating relationship between the yield and the process variables, and

optimization methods for finding the values of the process variables that produce desirable values of the response (Kathleen *et al*., 2004).

The first-order model is likely to be appropriate when the experimenter is interested in approximating the true response surface over a relatively small region of the independent variable space in a location where there is little curvature in f.

The second-order model is widely used in RSM for several reasons:
1. The second-order model is very flexible. It can take on a wide variety of functional forms, so it will often work well as an approximation to the true response surface.
2. It is easy to estimate the parameters (the ß's) in the second-order model. The method of least squares can be used for this purpose.
3. There is considerable practical experience indicating that second-order models work well in solving real response surface problems.

Quanhong and Caili (2004) stated that RSM is employed in order to study the effect of LSR, NaCl concentration and reaction time on the production of protein from germinant pumpkin seeds. In this study, the most relevant variable identified was LSR. The coefficient determination (R2) was good for the second-order model. A LSR of 30.2: 1 (v/w), a NaCl concentration of 4.26% and a reaction time of 18.1 min were found to be optimal for protein extraction from germinant pumpkin seeds.

When many factors and interactions affect desired response, RSM is an effective tool for optimizing the process (Triveni, Shamala, and Rastogi, 2001). As the needed information about the shape of the response surface is applied, RSM is an effective statistical method that uses a minimum of resources and quantitative data from an appropriate experimental design to determine and simultaneously solve a multivariate equation (Kalaimahan and Tapobrata, 1995).

In order to identify the response that can be thought of as a surface over the explanatory variables, response surface experiments is been attempt first. An experimental design such as central-composite experimental design (CCED) is usually used to fit an empirical, full second-order polynomial model. A central-composite experimental design, coupled with a full second-order polynomial model, is a very powerful combination that usually provides an adequate

representation of most continuous response surfaces over a relatively broad factor domain (Deming, 1990). The corresponding variables will be more significant if the absolute t value becomes larger and the p-value becomes smaller (Nor Aishah and Didi Dwi, 2004).

There was another study which aqueous extraction of hydrocolloidal gum from flaxseed been optimized by using RSM. Extraction temperature (25-100°C), pH (2.0-7.0) and water:seed ratio (6.0-20.0) were the factors investigated with respect to yield, apparent viscosity and protein content of the final gum extracts. Temperature and pH were found to have a significant influence on both yield and quality of the extracted crude gum while the water:seed ratio had only minor effects. Optimum conditions for the extraction were a temperature of 85-90°C, a pH 6.5-7.0 and a water:seed ratio of 13 (Cui *et al*., 1994).

RSM was also used to study the effects of pre-treatment variables of Viscozyme L concentration (6–30 FBG), pH (3.0–5.0), incubation time (0.5–2.5 h) and temperature (35–55 °C) on protein extraction from oat bran. Protein extraction from oat bran was mainly affected by pH and incubation temperature. From the RSM-generated model, the optimum conditions of enzymatic pre-treatment were identified as Viscozyme L concentration 30 FBG/10 g of oat bran, pH 4.6, incubation time 2.8 h and temperature 44 °C. The RSM-predicted and experimental extracted proteins were not significantly different from each other (Xiao and Yao, 2008).

The advantages of using RSM are reported to be reduction in the number of experimental trials needed to evaluate multiple parameters, and the ability of the statistical tool to identify interactions (Chen et al., 2004 and Lee et al., 2000). In addition to analyzing the effects of the independent variables, the experimental methodology also generates a mathematical model that accurately describes the overall process. RSM was successfully utilized for optimization of enzymatic reactions (Ansharullah *et al*., 1997 and Shieh *et al*., 1995).

Optimization for pigeon pea protein extraction (*Y*) (*Cajanus cajan* (L) Millsp was investigated using RSM. A compound central design was used with variables (X_1) NaCl concentration (0.000; 0.025; 0.050; 0.075 and 0.100M); (X_2) pH (2.5; 4.0; 5.5; 7.0 and 8.5) and (X_3) LSR (5:1; 10:1; 15:1; 20:1; and 25:1, v/w). The following equation: $\hat{Y} = -19.3733 + 8.6004x_2 - 0.508526x_2^2$ shows optimum conditions for protein extraction of about 75% yield, at pH 8.5 without NaCl regardless of the LSR (v/w) under the experimental conditions studied (Ivone *et al*., 2000).

Ebru and Ozgul (2010) identified that RSM was used to determine optimum conditions for extraction of protein from red pepper seed meal. A central composite design including independent variables such as temperature (30, 35, 40, 45 and 50 °C), pH (7.0, 7.5, 8.0, 8.5 and 9.0), extraction time (20, 30, 40, 50 and 60 min) and solvent/meal ratio (10:1, 15:1, 20:1, 25:1 and 30:1 v/w) was used. Selected response (dependent variable) which evaluates the extraction process was protein yield and the second-order model obtained for protein yield revealed coefficient of determination of 96.7%. Protein yield was primarily affected by pH and solvent/meal ratio. Maximum yield was obtained when temperature, pH, mixing time and solvent/meal ratio were 31 °C, 8.8, 20 min, 21:1 (v/w), respectively.

3 METHODOLOGY

3.1 Raw materials

3.1.1 Edible bird's nest (EBN)

EBN from species *C.Fuciphaga* was purchased from Pekan, Pahang. The sample consists mainly of protein. The carbohydrate component found in the sample are 9% sialic acid, 7.2% galactosamine, 5.3% glucosamine, 16.9% galactose, and 0.7% fucose (Noraini, 2012).While the most abundant amino acids consist in the sample are serine, threonine, aspartic acid, glutamic acid, proline and valine (Kathan, 1969).

3.2 Method

3.2.1 Characterization of the sample using Fourier Transform Infrared Spectroscopy (FTIR)

The formation of functional group in the sample was confirmed by FTIR spectroscopic analysis of the untreated and treated sample in order to characterize the element contains in the sample meet the standard requirement of the study. The trend of spectrum of untreated and treated sample is observed to come out. FTIR device use supernatant liquid of the sample to be analyzed. From the analyzed sample, the graph obtained was being compared with the table of the element wavelength (Mariey *et al*., 2001).

3.2.2 Inductively coupled plasma- Mass Spectrometry (ICP-MS)

ICP-MS fitted with a dynamic reaction cell (Elan DRC-e, PerkinElmer SCIEX, CT, USA) was employed for multi element analyses. Instrument performance and operational parameters are summarized. In order to obtain the best sensitivity (115In 25,000 cps/ppb), Perkin recommend to optimized the minimal formation of oxides (156CeO +/ 140Ce + < 1%) and doubly charged ions (138Ba 2+/ 138Ba + < 3%) and the source parameters (gas flows and ion lens voltages). 103Rh (10lg/L) was used as an internal standard. Interference correction equations for measurement of 138Ba and 60Ni, afforded through the software, were: 138Ba = 138Ba 0.000901 / 139La 0.002838 /140Ce and 60Ni = 60 Ni 0.0022 / 40Ca, respectively (Hua et al., 2014).

3.2.3 Scanning electron microscope (SEM)

Uncoated samples of EBN were attached to carbon stubs and were scanned at a high-voltage setting of 15 kV using a Hitachi S-4500 Field-Emmission Scanning Electron Microscope equipped with "Quartz One" energy dispersive X-ray spectrometer. Analyses were performed essentially as described by Houston, Moore, Favrin, and Hoff (2004). All data analysis was performed by software supplied by Hitachi. While, Scanning Electron Microscope (SEM) with brand Zeiss Evo 50 is used for surface imaging, provide surface topography and elemental mapping on sample's surface with best magnification ranging from 15x-10,000x. Before analysis, all solid samples must be in dry condition with sample dimension of not more than 20x20x20 (mm). Best images produced on conductive, powdered form and steel samples.

3.2.4 Standard protein curve

Bovine Serum Albumin (BSA) was used as a standard in order to quantify the amount of protein. Preparation of different dilution of BSA solutions (Figure 3.1) was made by mixing the BSA with water in the test tube. The final volume in each test tubes are 5ml and the BSA range used is 0 to 1.0 g/L (Suzana, 2012).

Figure 3-1: The BSA solution for the determination of protein standard curve

Table 3-1: Dilution from the BSA solution (1.0g/L) for the standard curve

Volume distilled water, L	Mass of BSA, g	Final concentration, g/L
0.01	0	0
0.01	0.002	0.2
0.01	0.004	0.4
0.01	0.006	0.6
0.01	0.008	0.8
0.01	0.010	1.0

3.2.5 Pre-treatment of edible bird's nest

The sample was cleaned by soaking it in water (Figure 3.3) and then it was filtered to remove excess water. After that, manually, removal of feathers and fine plumage from sample is done (Figure 3.4). Next, again the sample was filtered (Figure 3.5) and transferred into universal bottle (Figure 3.6). The sample was kept at ambient temperature (Norhayati, 2010).

Figure 3-2: The nests

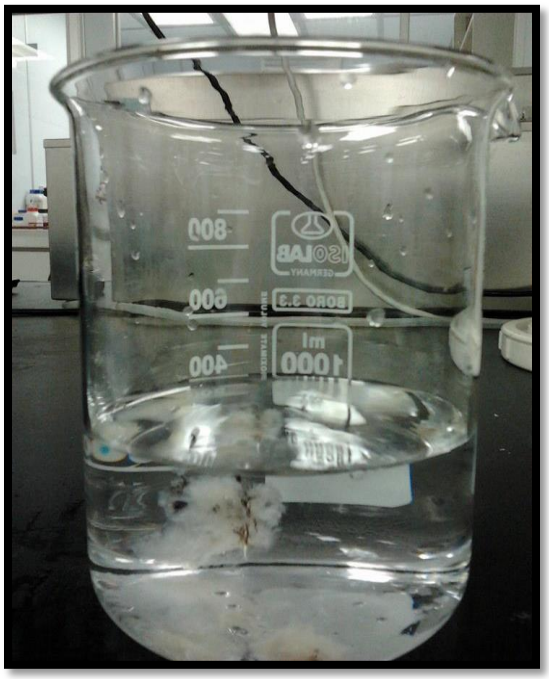

Figure 3-3: Soaking the EBN sample

Figure 3-4: The small feather was removed from the sample

Figure 3-5: The EBN sample with excessive distilled water before filtration

Figure 3-6: The EBN sample after pre-treatment

3.2.6 Alkaline hydrolysis

Three parameters are controlled in this research which reaction time, liquid solid ratio (LSR), and concentration of sodium hydroxide (NaOH). The temperature used is 50ºC while the reaction time required about 5 minutes to 25 minutes. Besides, LSR and NaOH concentration was varying from 30 to 70 and 40g/L to 120g/L respectively. According to Wani *et al.*, (2008), the molarities of NaOH must not exceed 3M. The extraction process was occurred inside the test tube (Figure 3.7) and the test tube was shaken in incubator shaker (Figure 3.8) for about 1 hour or based on the extraction time (Rosenthal *et al*., 2001).

Figure 3-7: The extraction process occurred in the test tube

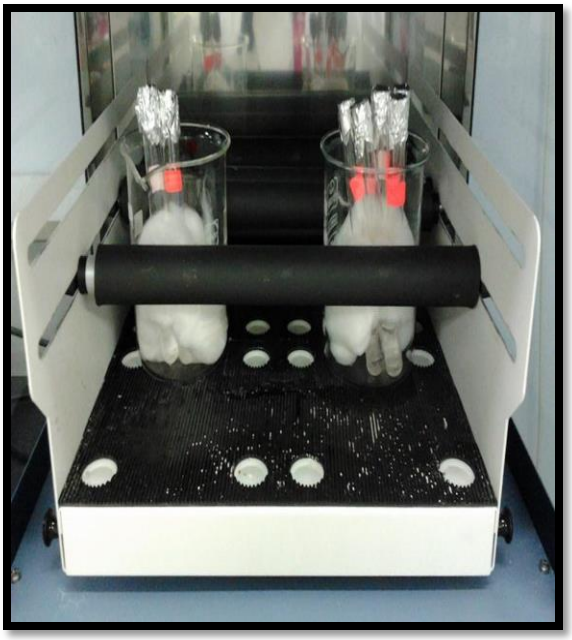

Figure 3-8: Incubator shaker used in the extraction process

3.2.7 Modified Lowry and Folin-Ciocalteu reagent

This reagent was prepared before protein assay preparation. The reagent needed to prepare Modified Lowry reagent are Reagent A (20g Na_2CO_3 + 4g NaOH dissolved in 1L distilled water) and Reagent B (2.5g $CuSO_4$. $5H_2$ 0 + 5g sodium citrate dissolved in 1L distilled water) in the proportion of 50:1. While Folin – Ciocalteu reagent was prepared by diluting 2X reagent (2N) 1:1 with distilled water. Next, 1g of sample was added into different test tube for different buffer solution. 1.0 ml of the Modified Lowry reagent was mixed with the sample and incubated at room temperature for 10 minutes. After that, 0.1 ml of 1 N Folin – Ciocalteu reagent was added into the reaction mixture at the end of the incubation period and left at room temperature for 30 minutes (Lowry *et al*., 1951).

3.2.8 Solid liquid separation

Centrifugation of suspension under 3000 rpm for 15 minutes at 20ºC was performed to further extraction process in a Sorvall RC5C centrifuge. In determination of moisture content, supernatant was taken as it is needed instead of precipitate (Rosenthal *et al*., 2001).

3.2.9 Determination concentration of protein

After 30 minutes, in order to determine the concentration of protein in the sample, UV –Vis spectrophotometer model Hitachi U-1800 (Figure 3.11) was used to analyze the sample. Distilled water is used as a blank and the absorbance of the sample is 743nm. Preparation of standard curve was needed by plotting the average blank corrected 743 nm reading for each BSA versus its concentration in g/L. The protein concentration of the unknown sample was determined by using standard curve (Palupi *et al*., 2010)

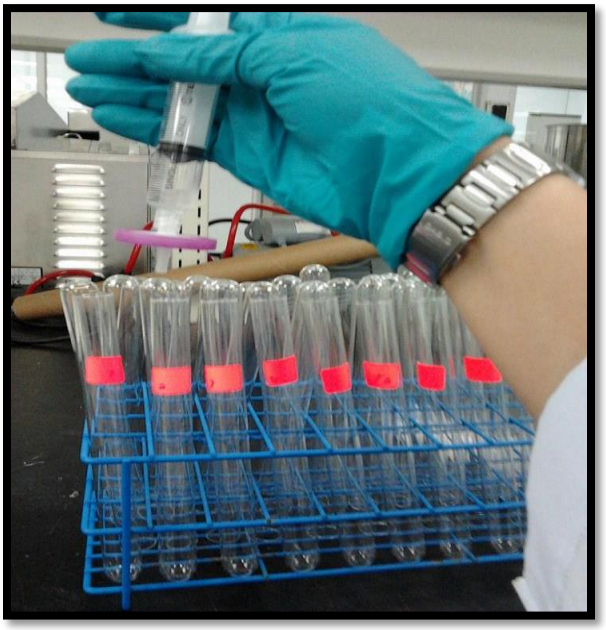

Figure 3-9: The BSA solution for the determination of protein standard curve

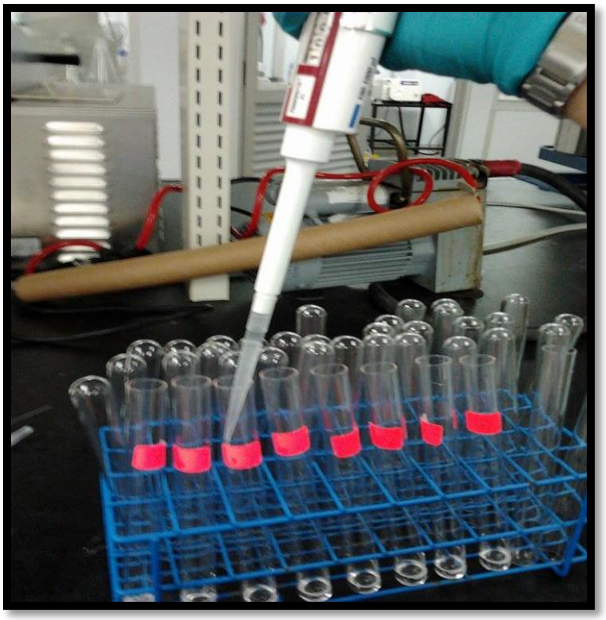

Figure 3-10: Lowry method before analysis of sample

Figure 3-11: UV-vis spectrophotometer used to analyze sample

Figure 3-12: Samples that need to be analyzed

3.2.10 Response surface methodology (RSM)

RSM is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data which must describe the behavior of a data set with the objective of making statistical previsions. Before applying the RSM methodology, it is first necessary to choose experimental design which experiments should be carried out in the experimental region being studied (Bezera *et al*., 2008).

The Design Expert Version 6.0.8 software was used to develop the experimental plan for RSM. The data collected was analyzed by performing analysis of variance (ANOVA) using this software. The three-dimensional graphs and contour plots are plotted for interpretation if the model looks good. Briefly, a good model must significant while the lack-of-fit must be insignificant. Besides, the various coefficient of determination, R^2 values should be close to 1 and the diagnostic plots should exhibit trends associated with a good model and these been elaborated subsequently.

Based on central composite design (CCD), the response surface design is developed. Table 3.2 shows the processing parameters involved in the study. 20 runs involved in the design and the response variables measured were the extraction time, NaOH concentration and LSR. The design and the parameter used in this study were shown in Table 3.2 and 3.3 below.

Table 3-2: Parameter and levels for response surface study

Name	Units	Low	High
Time	Min	20	30
NaOH concentration	g/L	80	120
LSR	-	25	35

Table 3-3: Design layout

Std	Run	Block	Factor 1	Factor 2	Factor 3
			A : Time (min)	B : NaOH concentration (g/L)	C : LSR
15	1	Block 1	25.00	100.00	30.00
10	2	Block 1	35.00	100.00	30.00
7	3	Block 1	20.00	120.00	35.00
9	4	Block 1	15.00	100.00	30.00
4	5	Block 1	30.00	120.00	25.00
14	6	Block 1	25.00	100.00	40.00
6	7	Block 1	30.00	80.00	35.00
13	8	Block 1	25.00	100.00	20.00
2	9	Block 1	30.00	80.00	25.00
16	10	Block 1	25.00	100.00	30.00
8	11	Block 1	30.00	120.00	35.00
17	12	Block 1	25.00	100.00	30.00
1	13	Block 1	20.00	80.00	25.00
20	14	Block 1	25.00	100.00	30.00
19	15	Block 1	25.00	100.00	30.00
11	16	Block 1	25.00	60.00	30.00
12	17	Block 1	25.00	140.00	30.00
18	18	Block 1	25.00	100.00	30.00
5	19	Block 1	20.00	80.00	35.00
3	20	Block 1	20.00	120.00	35.00

3.3 Flow Diagram

Figure 3.13 below show the overall flow diagram of the methodology in this study.

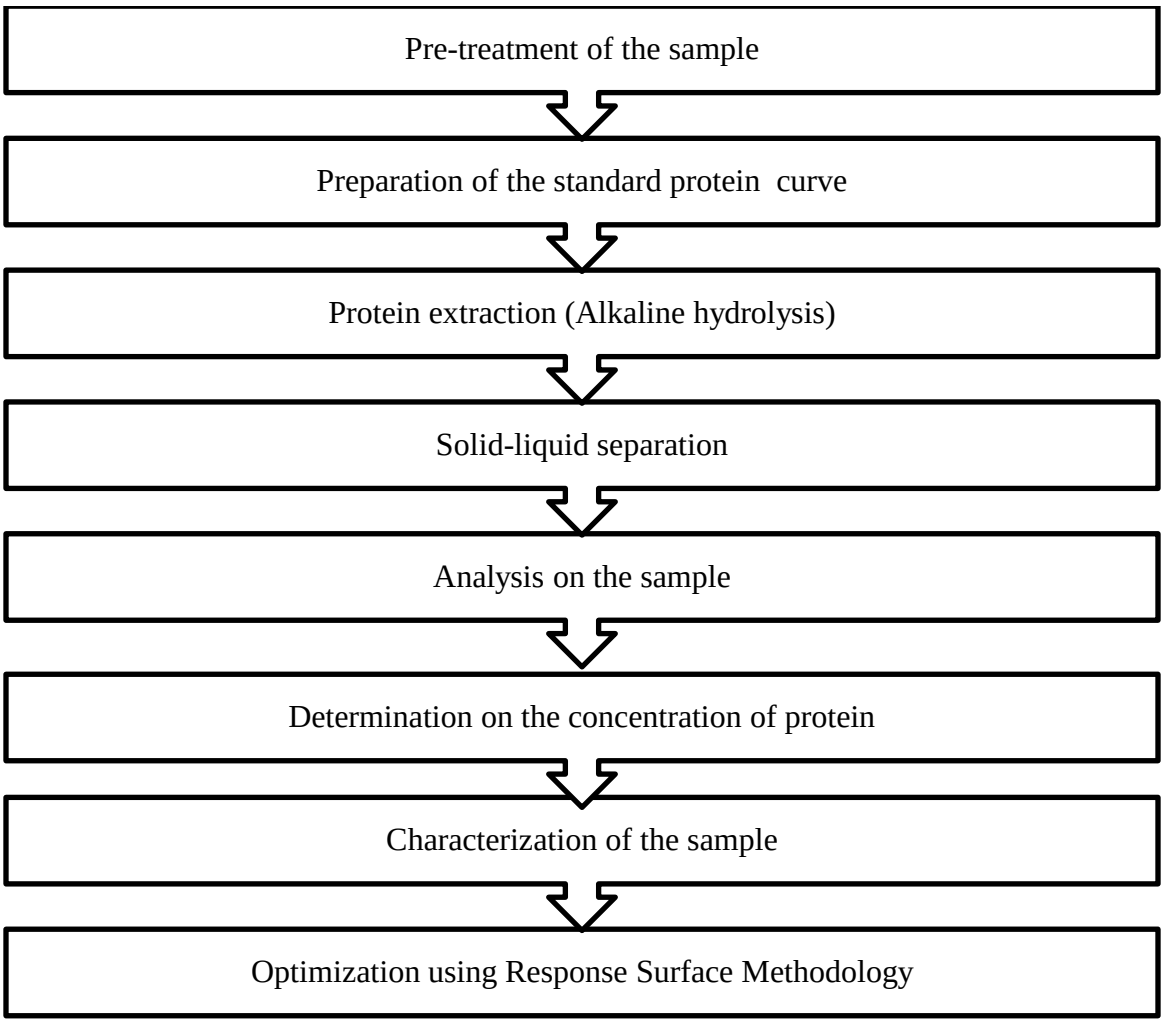

Figure 3-13: Flow diagram of the methodology

4 RESULTS AND DISCUSSION

4.1 Experimental results

Based on the experiment that has been done in this research, the data was analysis and collected was shown and discuss in this chapter.

4.2 Characterization of sample

4.2.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopic analysis is confirmed the formation of the functional group in the EBN of the untreated and treated sample as shown in Figure 4.1, 4.2 and 4.3 respectively. The FTIR spectra identified functional groups of organic materials depositing on the EBN. Figure 4.1 illustrates the IR peaks of the untreated sample of EBN in solid phase. While figure 4.2 and 4.3 shows the IR peaks of the treated sample after pre-treatment (gel phase) and alkaline hydrolysis (liquid phase) was performed respectively. In the untreated sample (solid phase) exhibited significant FTIR peak as shown in Table 4.1. The significant FTIR peak at wave numbers of 1697.36, 3151.06, 3079.48, 2871.56, 3419.68, 3384.43, 3326.84, 3243.10, 3207.92, 3586.92, 3521.51, 2952.97, 2792.24, 2624.33cm-^{1} were indicating possible organic materials such as proteins, and/ or amino sugars. It was observed that the absorption bands is due to O-H stretching vibration, N-H stretching vibration, C=O stretching vibration and C-H stretching vibration. Based on peptide bond of the protein (Anchouri *et al*., 2011), the absorption bands shown were due to hydroxyl group and amines bonds in the samples. Peptide link is known as four-atom functional group -C (=OH) NH and this peptide bonds are metastables which is meant to break spontaneously in the presence of water.

Based on the Table 4.1, it was showed that the functional group contained in the untreated and treated EBN samples was not identical. When comparing the untreated sample and treated sample (gel) after pre-treatment, the untreated EBN samples consists of four functional group which C=0, C-H, O-H and N=H, while the treated samples (gel) only consists of two functional group which is N-H and C=O. Moreover, while comparing the treated sample (gel) and alkaline hydrolysis sample, the absorption bands is identified to be increased in value. From the Table

4.1, the value of absorbance is observed and being classified according to the functional group. From Figure 4.2 and Figure 4.3, it is showed that N-H (v = 3500 ~ 3300; 3500 ~ 3180) and C=O (v = 1680~1630) stretching peak is obviously increased. N-H stretch peak increased from 3339.25 to 3362.53 while C=O stretch peak was increased from 1639.86 to 1643.39. This is due to the achievement of optimum condition of protein extraction on EBN using alkaline hydrolysis. The comparison that can be made between these two samples is the sample from Figure 4.2 only performed pre-treatment process while the sample from Figure 4.3 was further analyses by alkaline hydrolysis. The alkaline hydrolysis is performed at optimum condition which is in 25minutes, 30 and 100g/L of extraction time, LSR and NaOH concentration respectively.

In addition, further investigation of others regions of spectrum could identify whether the organic material is in group of amines or amides of protein. The absorption peak at 3339.25cm^{-1} which correspond to N-H stretch in probably due to amines groups or amides groups, but the absorption peak at 1639.86cm^{-1} was confirmed that the material was correspond to amides which consists of C=O stretch. According to Ng *et al*., (2012), peptide group that is known as structural repeat unit of proteins has nine characteristic bands namely amide A, B, I, II, III, IV, V, VI and VII. Amide I is the major bands of wavelength detected on the protein infrared spectrum. The amide I band (v = 1600 ~ 1700) can be associated with the C=O stretching vibration in the group.

The FTIR technique is a useful tool and rapid technique for the determination of purity of EBN and thus as a deterrent to the commercial adulteration of EBN based products. Due to its high demand and undoubtedly one of the most expensive food ingredients in the world as well as rare product and limited supply of natural authentic EBN, this in turn has led to upsurge of a lot of fake and shoddy commodities edible bird nest products in global market.

A number of methods had since been explored in the past to determine the purity of EBN. The method can be categorized into two categories, such as empirical identification method and chemical method. FTIR spectroscopy is becoming an attractive alternative to the existing analytical techniques in food analysis because it is simple and rapid, low in cost, environmental friendly and non-invasive. IR spectroscopy measures the covalent chemical bonds, creating a molecular and fingerprint of the chemicals present. This fingerprint can be used to identify and quantify chemicals present in a sample. The FTIR spectroscopy region 4000 – 600 cm -1 in

particular, is able to identify a large number of components and the absorption bands are sensitive to the physical and chemical states of individual constituents. Recently, a handheld FTIR was invented to determine and evaluate the presence of additives present in EBN product like, salt, sugar and mono sodium glutamate (MSG). In addition, the conventional FTIR method was used in past to identify the quality of EBN from *Collocalia esculenta* of swiftlet species. Therefore, there is an urgent need to identify the purity of edible bird nest. The increasing trend in fake and adulterated EBN products in the world market is disturbing. Therefore, this study was undertaken to determine the purity of EBN products using the FTIR spectroscopy technique.

Table 4-1: The functional group of unprocessed and processed EBN samples

No	**Functional group**	**Absorption range (cm^{-1})**		
		Unprocessed	**Processed (gel)**	**Processed (liquid)**
1	**C=0**	1697.36	1639.86	1643.39
2	**C-H**	3151.06	-	-
		3079.48		
		2871.56		
3	**N-H**	3419.68	3339.25	3362.53
		3384.43		
		3326.84		
		3243.10		
		3207.92		
4	**0-H**	3586.92	-	-
		3521.51		
		2952.97		
		2792.24		
		2624.33		
		2573.32		

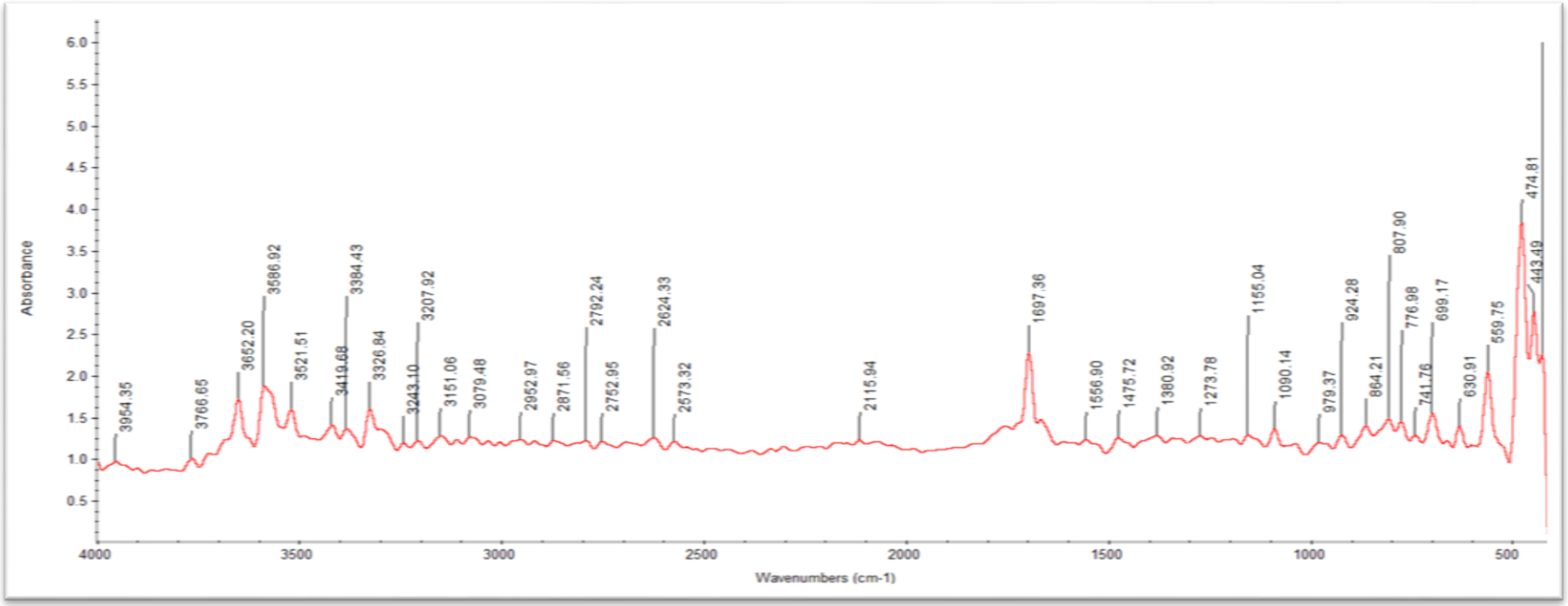

Figure 4-1: FTIR analysis of untreated sample (powder form)

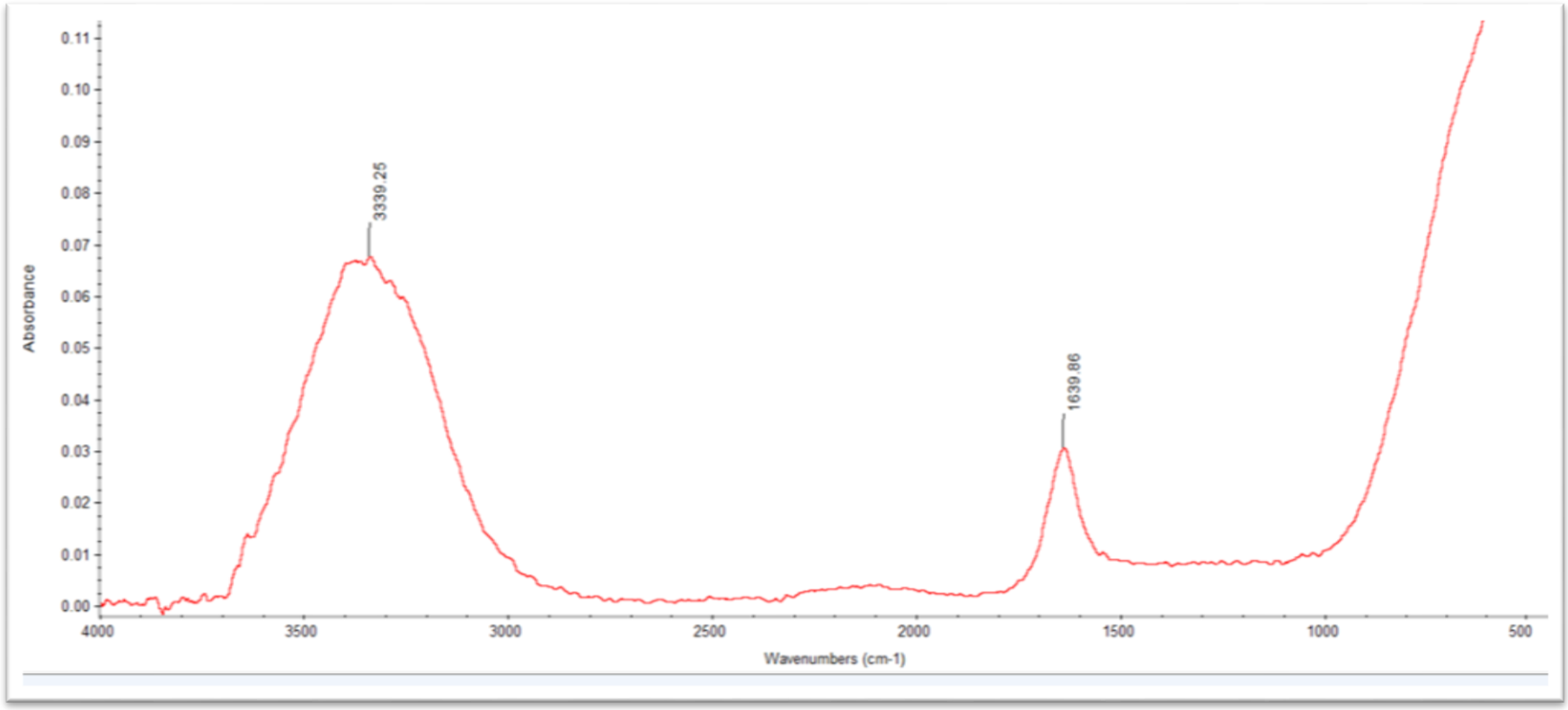

Figure 4-2: FTIR analysis of treated sample after pre-treatment process (gel form)

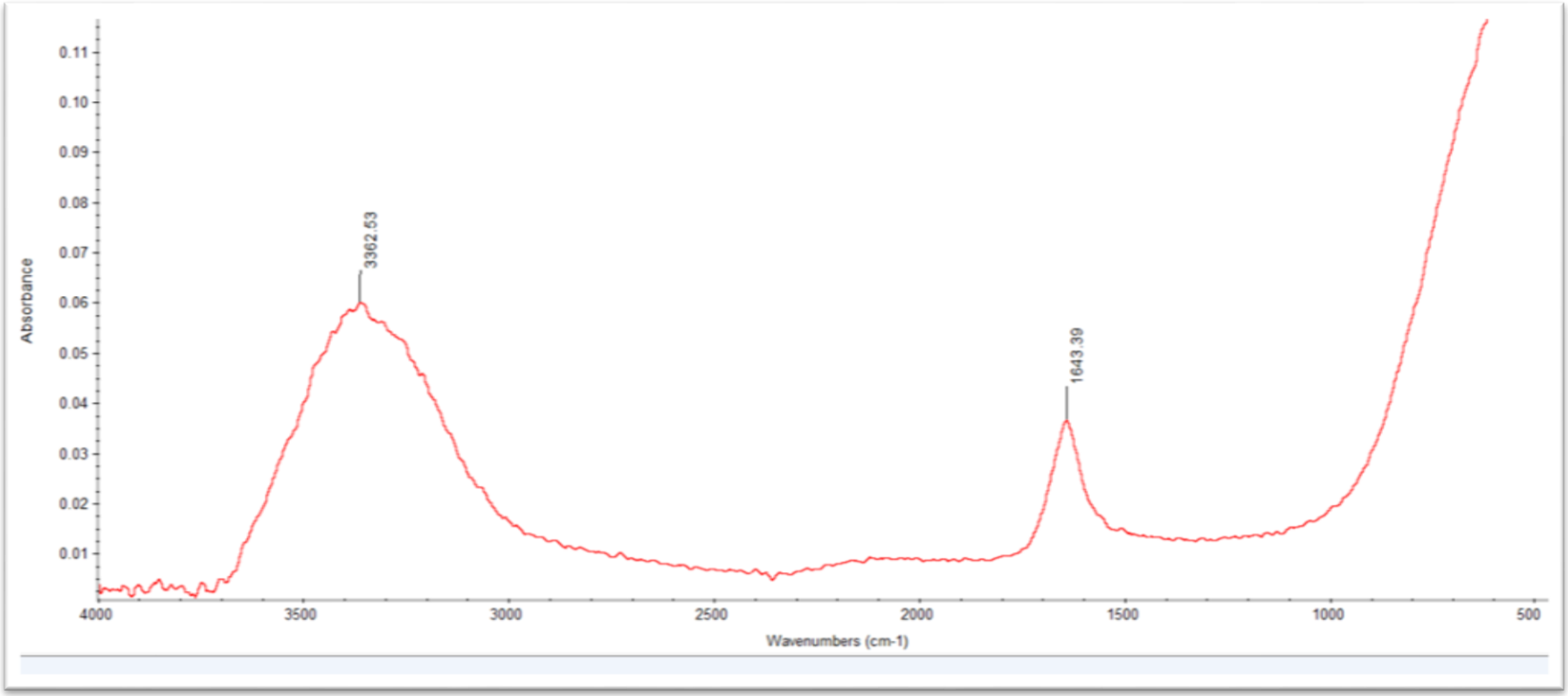

Figure 4-3: FTIR analysis of treated sample after alkaline hydrolysis (liquid form)

4.2.2 Inductively Coupled Plasma (ICP)

Heavy metals are defined as metallic chemical element that has a relatively high density and are toxic or poisonous at low concentrations (Connel, 1984). Living organisms require trace amounts of some heavy metals, including calcium, copper, iron, manganese, molybdenum, vanadium, strontium, and zinc. However, heavy metals are also dangerous because they tend to bioaccumulate.

Potassium, chlorine, phosphorus, calcium, iodine, iron, copper, zinc and manganese are essential metals while, mercury, lead and cadmium are toxic metals. Besides, cadmium, chromium, mercury, lead, arsenic, cadmium and antimony are non-essential heavy metals of particular concern to the surface water systems (Kennish, 1992).

As a consequence of heavy metal toxicity, and of the serious contamination of food that occurs from time to time during commercial handling and processing, most countries monitor the levels of toxic elements in foods. However, the potential hazards of metals transferred to humans are probably dependent on amount of muscles consumed by an individual (Irwandi *et al*., 2009).

According to Warasri *et al*., (2013), sodium (Na) was the most abundant mineral in the EBNs, followed by calcium (Ca), magnesium (Mg) and potassium (K). The presence of iron (Fe), selenium (Se), copper (Cu) and manganese (Mn) was little. Major minerals found in the EBNs were similar to those of seawater (Castro and Huber, 2005 and Bardi, 2010). A bird food such as an insect that lives near the seashore may contain high content of Na, Ca, Mg and K according to their plant feed (Lee *et al*., 2007).

The result from Table 4.3 shows the amount of minerals found in the EBN before treated (dry) and after treated (gel) process. By using ICP-MS spectroscopy, the most abundant mineral found in the EBN in this study was same as the previous study accept for the calcium. When observing the value of EBN dry, it revealed that the abundant mineral found was sodium (Na), followed by magnesium (Mg), potassium (K) and then calcium. This may be due to the slightly technical error when conducting the experiment. Besides,

the presence of others mineral such as iron (Fe), selenium (Se), copper (Cu) and manganese (Mn) was little exactly actual from the previous study.

Dietary reference intake (DRI) values have been established for Ca and Mg, with the daily adequate Ca intake for adults at 1000 mg/day and Mg at 310–420 mg/day, respectively (Institute of Medicine, 2004). From Table 4.3, it showed that the value of Ca and Mg after pre-treatment process meets the adequate intake for adults of previous study. All of the EBNs (100 g) from different regions of Thailand can supply 70% of the DRI for Ca and 40% of the DRI for Mg. However, consumption of the EBN (100 g) would meet approximately 70–100% of the DRI for Na. High Na content may be not beneficial to consumers who prefer lower sodium products.

Table 4-2: Concentration of heavy metals contain in the sample

No	Parameter	Before Pre-treatment	After Pre-treatment	Unit
1	Cadmium (Cd)	0.087	0.002	mg/L
2	Arsenic (As)	Less than 0.00006	Less than 0.00006	mg/L
3	Lead (Pb)	0.292	0.262	mg/L

Based on Table 4.2, it was shown that the cadmium concentration in the sample was 0.087mg/L and 0.002mg/L before and after pre-treatment respectively. Food is the most important source of cadmium exposure in the general non-smoking population in most countries. Cadmium is present in most foodstuffs, but concentrations vary greatly, and individual intake also varies considerably due to differences in dietary habits (Jarup *et al*., 1998). Women usually have lower daily cadmium intakes, because of lower energy consumption than men. People living in contaminated areas have higher urinary cadmium concentrations. Cadmium exposure may cause kidney damage. WHO estimated that a 200 mg/L would constitute a ‘critical limit’ below which kidney damage not occur. Thus, the concentration amount of cadmium in the EBN sample was not being concerned by the people that consumed it.

For arsenic, the standard daily uptake of a person can consume is below 2mg/L (Lau *et al*., 1987). In this study, the concentration of arsenic found in the EBN was below 2 mg/L which was not harmful towards the person that consumed it. Arsenic is known as a natural chemical element that is found throughout environment and its living systems. Arsenic can be classified into two groups which are inorganic and organic arsenic. Both of this type of arsenic can be found in the food as the inorganic arsenic was toxic to human health while organic arsenic was not harmful to human health.

The very common and toxic heavy metal is lead. It can damage nervous connection in young children while can cause blood and brain disorders to all ages due to it poisonous character. From the table above, the concentration of lead is 0.292mg/L and 0.262mg/L before and after pre-treatment. Average people can consume maximum value of lead concentration about 3.402 mg/L, thus this sample is safe from the most significant of all heavy metals.

Table 4-3: The ICP-MS analysis of EBN dry and EBN gel sample

Parameter	Results EBN Dry	Results EBN Gel	Percent (%) of decreases
Sodium (Na)	11171.975*	308.831*	97.24
Magnesium (Mg)	994.684*	52.450*	94.73
Aluminium (Al)	59.522*	11.526*	80.64
Potassium (K)	261.122*	72.780*	72.30
Manganase (Mn)	0.113*	0.015*	86.73
Cobalt (CO)	0.039*	0.039*	-
Nickel (Ni)	0.034*	0.012*	64.71
Copper (Cu)	0.720*	0.095*	86.81
Zinc (Zn)	2.918*	0.279*	90.44
Silver (Ag)	0.015*	Less than 0.00015**	99.00
Cadmium (Cd)	0.087*	0.002*	97.70
Barium (Ba)	0.073*	0.029*	60.27
Lead (Pb)	0.292*	0.262*	10.27
Beryllium (Be)	Less than 0.00029**	Less than 0.00029**	-
Calcium (Ca)	72.780*	Less than 0.0040**	99.99
Vanadium (V)	Less than 0.00095**	Less than 0.00095**	-
Chromium (Cr)	Less than 0.390**	Less than 0.390**	-
Iron (Fe)	Less than 0.013**	Less than 0.013**	-
Arsenic (As)	Less than 0.06363**	Less than 0.06363**	-
Selenium (Se)	Less than 0.4568**	Less than 0.4568**	-
Molybdenum (Mo)	Less than 0.00353**	Less than 0.00353**	-
Uranium (U)	Less than 0.00002**	Less than 0.00002**	-

*ppm
**ppb

4.2.3 Scanning electron microscope (SEM)

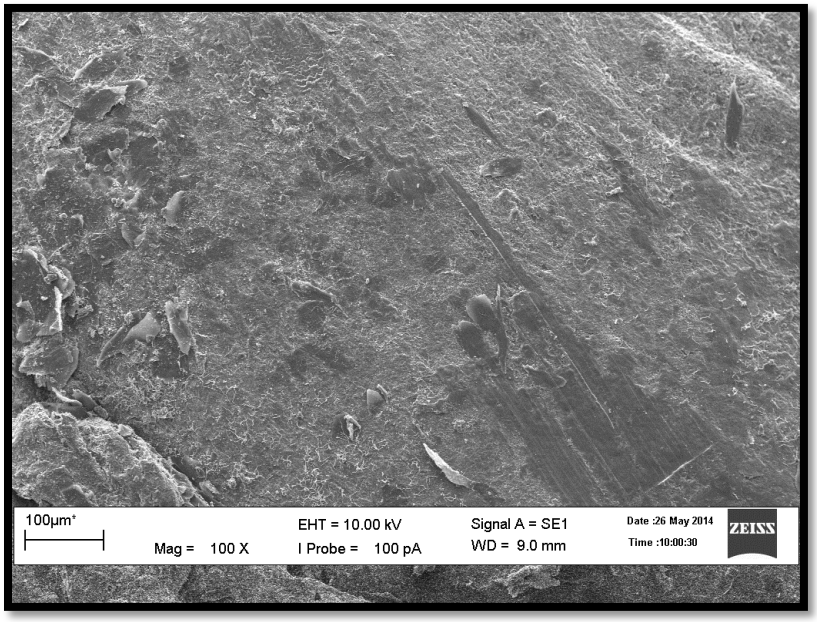

Figure 4-4: Surface morphology of raw edible bird's nest (100 X)

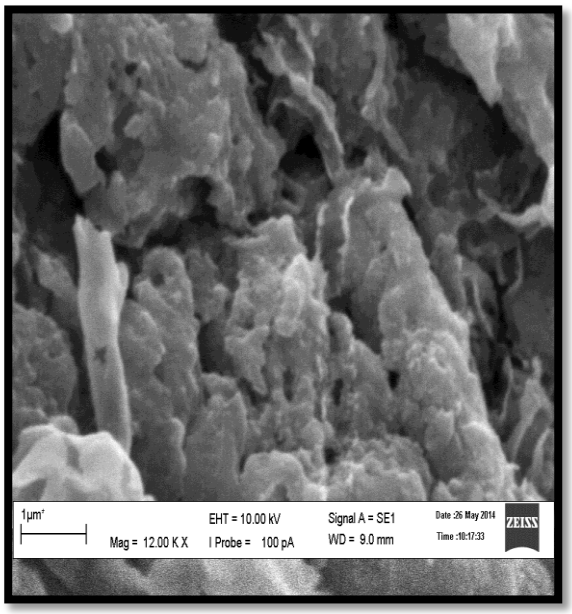

Figure 4-5: Surface morphology (12 KX)

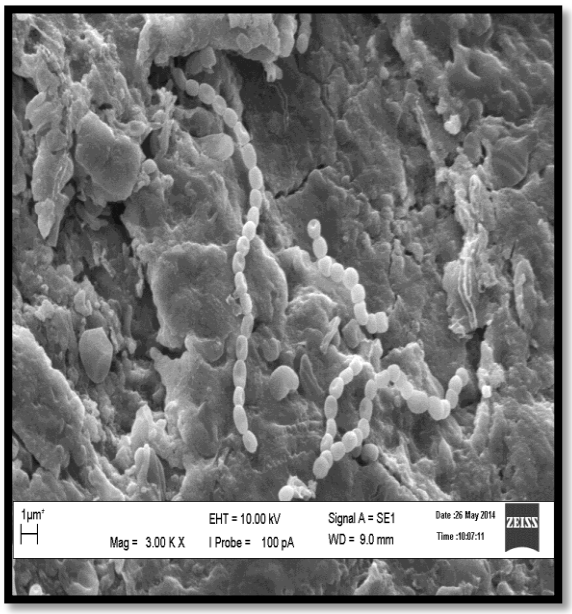

Figure 4-6: Surface morphology (3 KX)

Based on the Figure 4.4, 4.5 and 4.6 above, the surface morphology of the EBN samples was observed by SEM in this study. There were previous studies which also observed surface morphology of EBN, but then, used different type of EBN sample to ensure the quality control of EBN for being consumed by people. The result of the previous study was obtained as Figure 4.7 below. According to Yang *et al*., (2014), the micrographs of authentic raw EBN samples displayed the crater-like structure of irregularly shaped three-dimensional networks, measuring from less than one, up to several microns. The imperial EBN exhibited a well-defined rough network with interspaces of different shapes, while the surface morphology of grass EBN was glossier with fuzzy boundaries as observed in Figure 4.7c below. Yang et *al*., (2014) also stated that the unique three-dimensional network structure was also observed in instant EBN samples shown in Figure 4.7d and 4.7e below. Notably, the counterfeit instant EBN samples revealed granule- or cudgel-like structures rather than network structures (Fig. 4.7f–4.7h).

By observing the structure and surface morphology of the sample used in this study, the result obtained is assumed same with the authentic raw EBN. This is because, as seen in Figure 4.4 and 4.5 above, the surface of EBN is identified to be irregular in shape and somehow a quit rough. Hence, it is assumed that the sample was not from fake sample of EBN. While, from Figure 4.6, it is observed that there are one place that shows chain-likely structure. This is maybe due to the impurities contain in the sample. The different surface morphology is maybe also due to different type of species of sample obtained. Hence it was revealed that the counterfeit materials possessed different physical properties, with respect to surface morphology, and that SEM analysis can be employed as one of the criteria for the quality control of EBN.

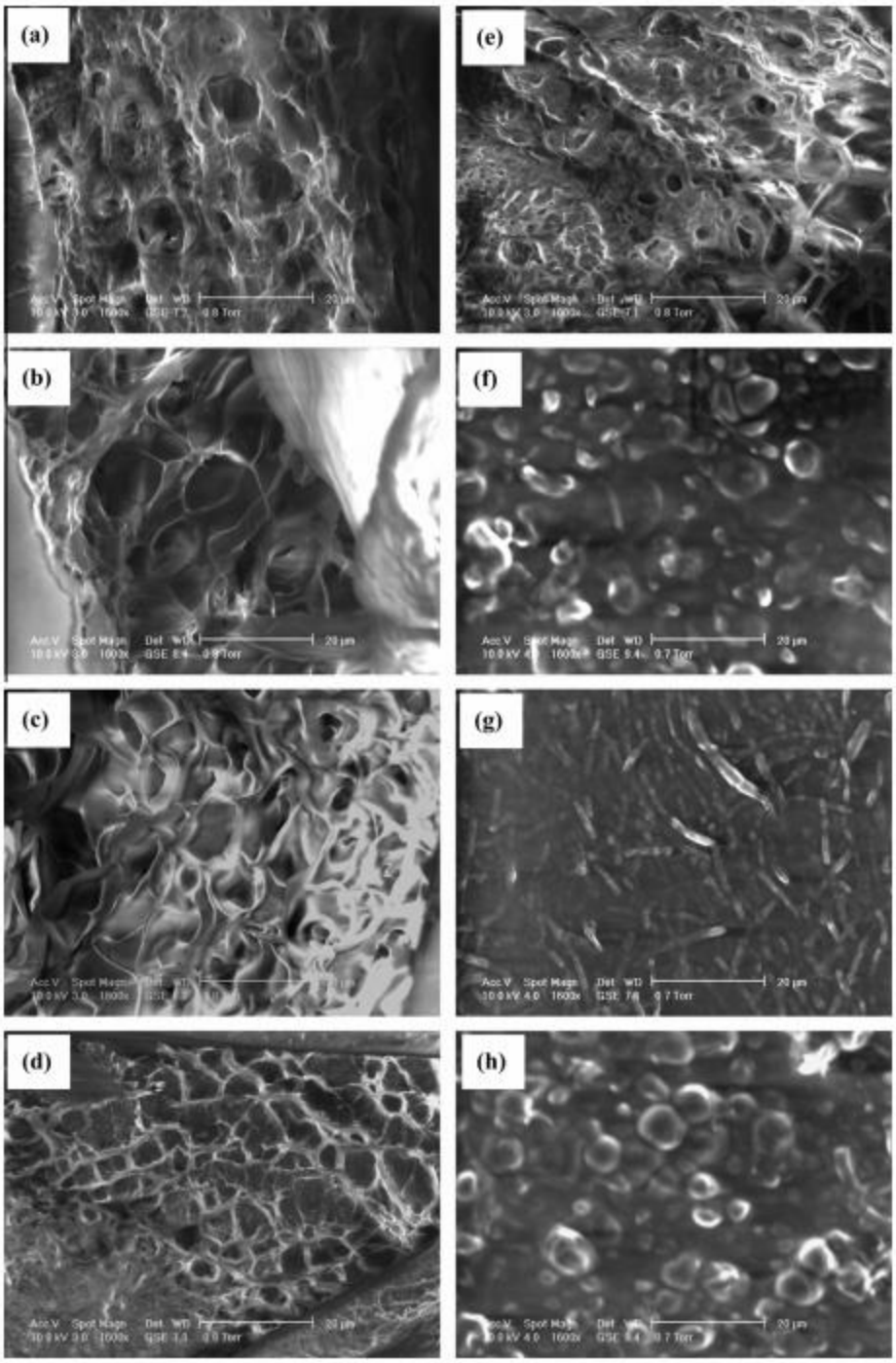

Figure 4-7: SEM photos of representative raw samples and instant samples. (a) Imperial EBN sample, (b) feather EBN sample, (c) grass EBN sample, (d) instant EBN sample (Imperial), (e) instant EBN sample (Feather), (f) fake instant EBN sample (Jelly fungus), (g) fake instant EBN sample (Agar) and (h) fake instant EBN sample (Pigskin)

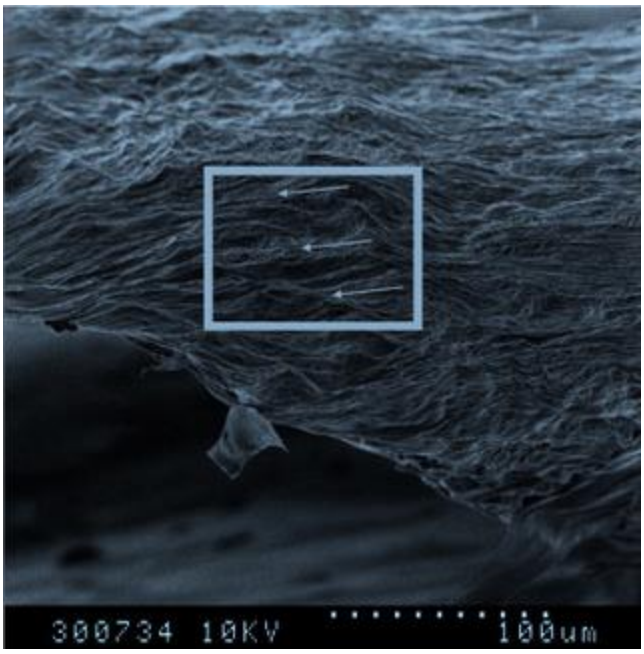

Figure 4-8: SEM of seaweed cross-section in adulterated nest taken by Marcone (2005)

Furthermore, the difference between chemically treated processed EBN and untreated EBN can be easily discovered by the shine of the cement strands. Chemical additive may lead to nest cement that is too shiny. When observing the nest with an optical microscope, genuine nest is semitransparent and has a lot of fine texture while *Tremella* fungus is non-transparent and has only coarse textures (Wu *et al.*, 2007). Thus, according to this statement, the EBN sample used in this study was not being exposed to the chemical additive as it surface morphology was not shine.

4.3 One factor at a time

4.3.1 Effect of extraction time to protein concentration

Extraction time is one of the parameters that influence in the production of protein concentration. At 50ºC, extraction time is ranging from 5 minutes to 25 minutes while NaOH concentration and LSR is constant at 60g/L and 60:1 respectively. The effect of extraction time on the protein concentration is shown in Table 4.4 below. In Table 4.4, a higher extraction time resulted in the higher protein concentration until it reached the optimum condition. This result was meeting to the previous study which according to Zhang *et al*., (2009), increasing of time, extraction rate is increased. This is because, the higher the extraction time, the higher the availability of the alkaline to extract the protein. However, further increased in extraction time beyond the optimum resulted in decreased in protein concentration due to the degradation of amino acid structure at long duration of extraction time (Zhang *et al*., 2009). Thus, in order to find the optimum extraction time, the further investigation was made, and the effect of extraction time towards protein concentration at 30 minutes was performed as shown in Figure 4.9. From this figure, the protein concentration is observed has been decreased in value after 25 minutes (optimum condition). From Table 4.4, it was showed that at 25 minutes, the extraction amount was increased by 213%.

Table 4-4: Results of protein concentration (g/L) with varying extraction time (minutes)

Time (min)	**Protein concentration (g/L)**			
	Run 1	**Run 2**	**Run 3**	**Average**
5	0.100	0.087	0.092	0.093
10	0.100	0.091	0.109	0.100
15	0.109	0.124	0.118	0.116
20	0.122	0.137	0.158	0.139
25	0.289	0.291	0.296	0.292
30	0.197	0.188	0.190	0.190

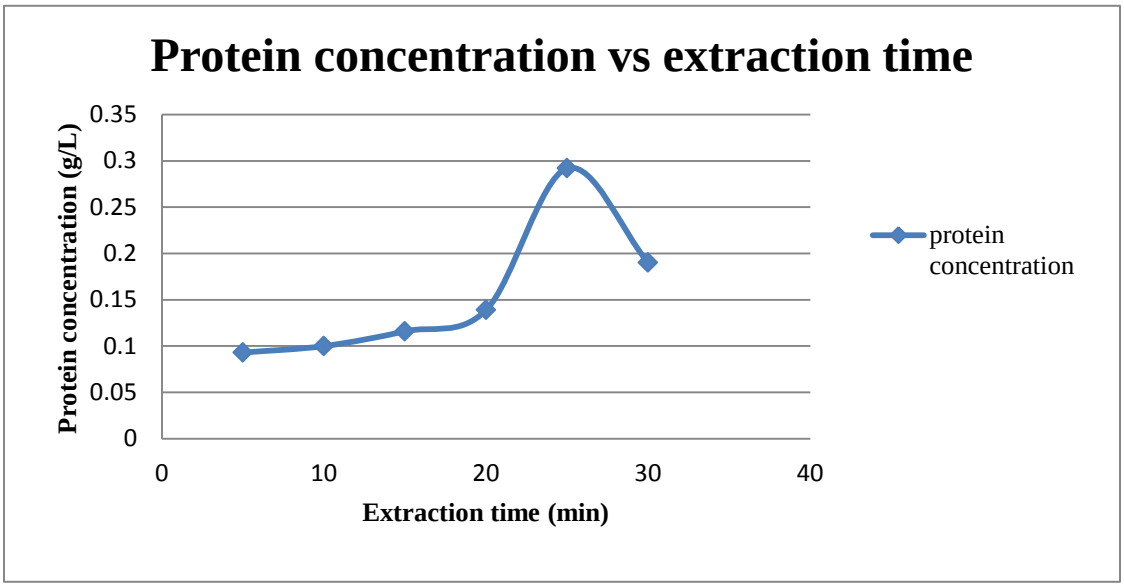

Figure 4-9: Effect of extraction time to protein concentration

4.3.2 Effect of NaOH concentration to protein concentration

Other parameter which also influence in the production of protein concentration is NaOH concentration. In order to study the effect of NaOH concentration to protein concentration, the extraction time used is 25 minutes while LSR used is 70:1 respectively. According to the Table 4.5, the protein concentration increased in the increased of NaOH concentration, however, beyond the optimum condition of NaOH concentration which is 100g/L, the protein concentration started to decreased in value. Obviously, it showed that protein was prone to degradation and denaturation. The denatured protein will form a three-dimensional solid-like network (or gel) at high concentration (Linqiang *et al*., 2008). In addition, from Table 4.5, it is observed that the protein concentration increases about 255% when using 100g/L of NaOH concentration and decreased about 5.16% when using 120g/L of NaOH concentration.

Table 4-5: Results of protein concentration (g/L) with NaOH concentration (g/L)

NaOH concentration (g/L)	Protein concentration (g/L)			
	Run 1	Run 2	Run 3	Average
40	0.320	0.333	0.343	0.332
60	0.349	0.353	0.351	0.351
80	0.699	0.741	0.834	0.758
100	1.100	1.210	1.233	1.181
120	1.098	1.101	1.161	1.120

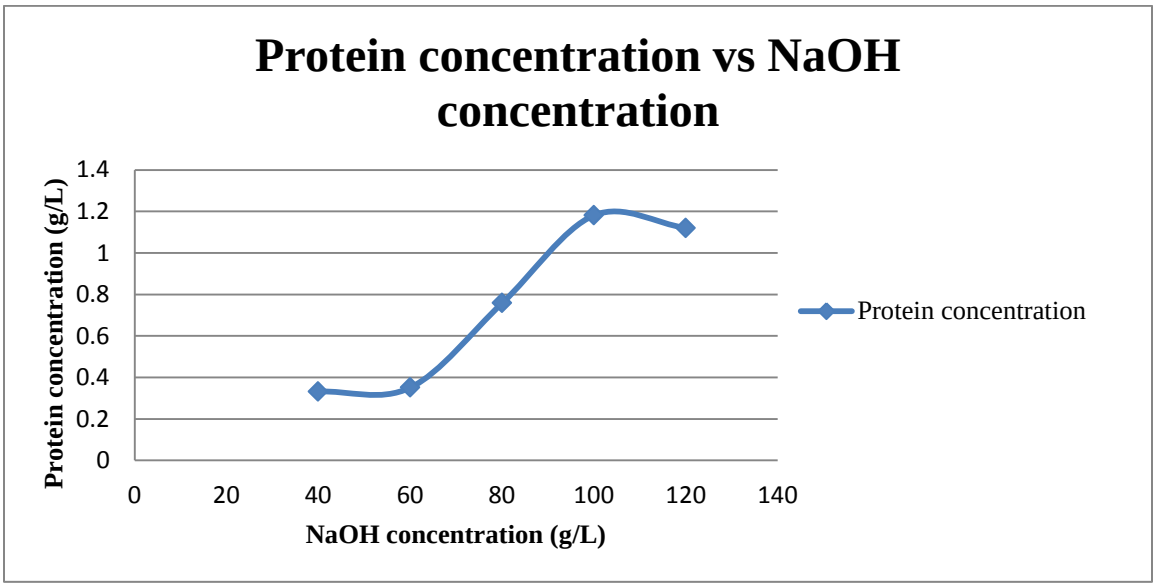

Figure 4-10: Effect of NaOH concentration to protein concentration

4.3.3 Effect of LSR to protein concentration

In order to study the effect of LSR on the protein concentration, 30:1- 70:1 of ratio were tested at 50 ºC of extraction temperature. 25 minutes of extraction time were used while varying NaOH concentration conditions are performed. From Figure 4.11, it showed that the highest protein concentration achieved was 1.260 g/L resulted from the highest curve plotted which is obtained from the curve of 30:1 LSR at 100g/L NaOH concentration. According to Stella *et al*., (2010), LSR is a ratio of the solvent volume per gram of raw material. In this study, it is clearly proved that the content of protein was higher in the 30:1 LSR compared to the 70:1 LSR that is when calculating the concentration of protein inside the sample (raw material) in the 30:1 and 70:1 resulted of 0.033 and 0.014 respectively. The optimum condition for EBN's protein extraction is identified as 30:1 LSR.

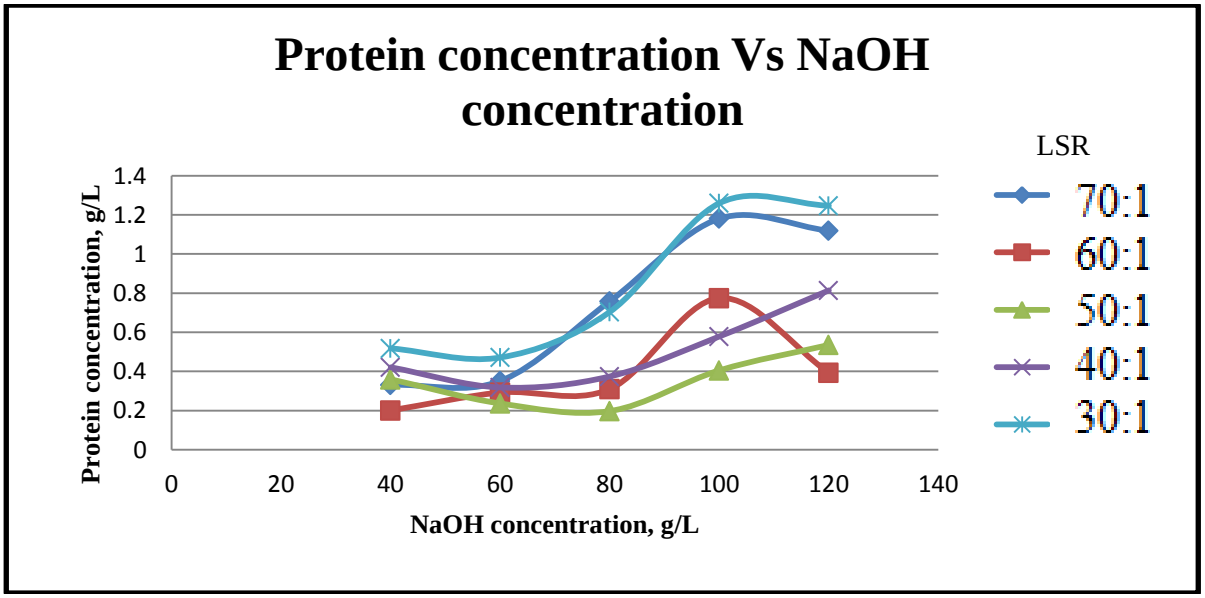

Figure 4-11: Effect LSR to protein concentration

4.3.4 Protein concentration versus NaOH concentration

At 50ºC of extraction temperature, LSR is ranging from 30:1 to 70:1 while extraction time is ranging from 5 minutes to 25 minutes and NaOH concentration is ranging from 40g/L to 120g/L and the result is shown in figure below. There were about five graph being plotted based on five different LSR which are 70:1, 60:1, 50:1, 40:1 and 30:1. According to each graph, the x-axis was NaOH concentration in ranging of 40g/L to 120g/L while y-axis showed the value of protein concentration based on different duration of extraction time.

Based on Figure 4.12 below, it showed that the highest protein concentration achieved was 1.221g/L. This protein concentration resulted from 10 minutes and 100g/L of NaOH concentration respectively. While the lowest protein concentration of 70:1 LSR was 0.008g/L for 20 minutes and 40g/L NaOH concentration. The trend of this graph was same as the protein concentration was increased from 40g/L to 100g/L but decline when the NaOH concentration reached to 120g/L for every minute except for 15 minutes. In 15 minutes, the protein concentration started to decline from 80g/L NaOH concentration to 100g/L but increased to 120g/L. This may resulted as the protein extracted from EBN did not suitable for 100g/L of 15 minutes duration of extraction as the protein concentration tended to declined immediately.

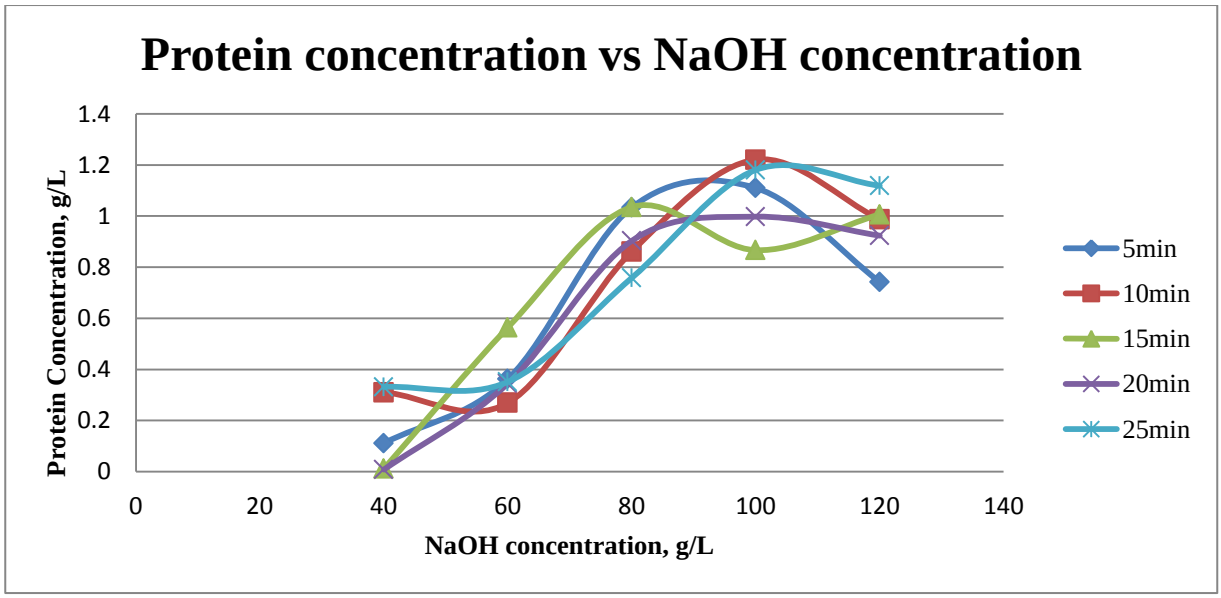

Figure 4-12: Graph of protein concentration versus NaOH concentration (Study on different time extraction and LSR 70:1)

According to Figure 4.13 below, it showed that the highest protein concentration achieved was 0.775g/L. This protein concentration resulted from 25 minutes and 100g/L of NaOH concentration respectively. When the LSR value was constant to 60:1, the lowest protein concentration was 0.057g/L for 10 minutes and 40g/L NaOH concentration. The trend of this graph was different from the graph before as the protein concentration was increased from 40g/L to 120g/L for 5 minutes to 20 minutes. Contrast to the trend of the graph plotted for the duration of extraction time of 25minutes. It showed that the trend of the protein concentration extract was same as LSR 70:1 graph plotted. The protein extract was increased from 40g/L to 100g/L but decline after that. Hence, the highest value of protein extract was taken from this trend of graph as the protein concentration was not increased to the unknown parameter.

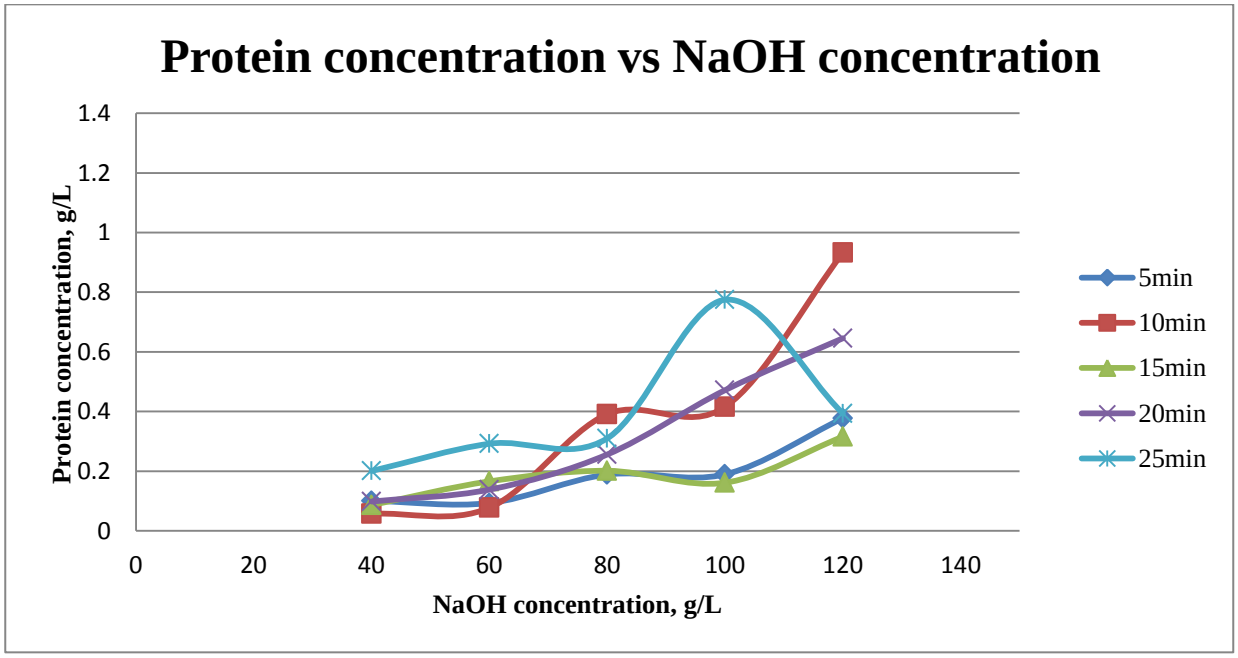

Figure 4-13: Graph of protein concentration versus NaOH concentration (Study on different time extraction and LSR 60:1)

Based on Figure 4.14 below, it showed that the highest protein concentration achieved was 0.567g/L. This protein concentration resulted from 10 minutes and 100g/L of NaOH concentration respectively. While the lowest protein concentration of 50:1 LSR was 0.048g/L for 10 minutes and 60g/L NaOH concentration. The trend of this graph was quite differed for each curve. The protein concentration was decreased for 40g/L to 80g/L NaOH concentration and started to increased when the NaOH concentration used was above 80g/L NaOH concentration for 15 minutes and 25 minutes extraction time. The best curve of this figure was for 10 minutes of extraction time in which the protein extract was increased from 40g/L to 100g/L of NaOH concentration and decreased when the concentration of NaOH is 120g/L. For 5 minutes extraction time of 50:1 LSR, the curve plotted was quite strange which is the curve was not consistent as the protein concentration was increased, and then decreased and increased afterwards. This was because the protein extracted for LSR 50:1 was not stabled enough if the extraction time used only for 5 minutes for different NaOH concentration.

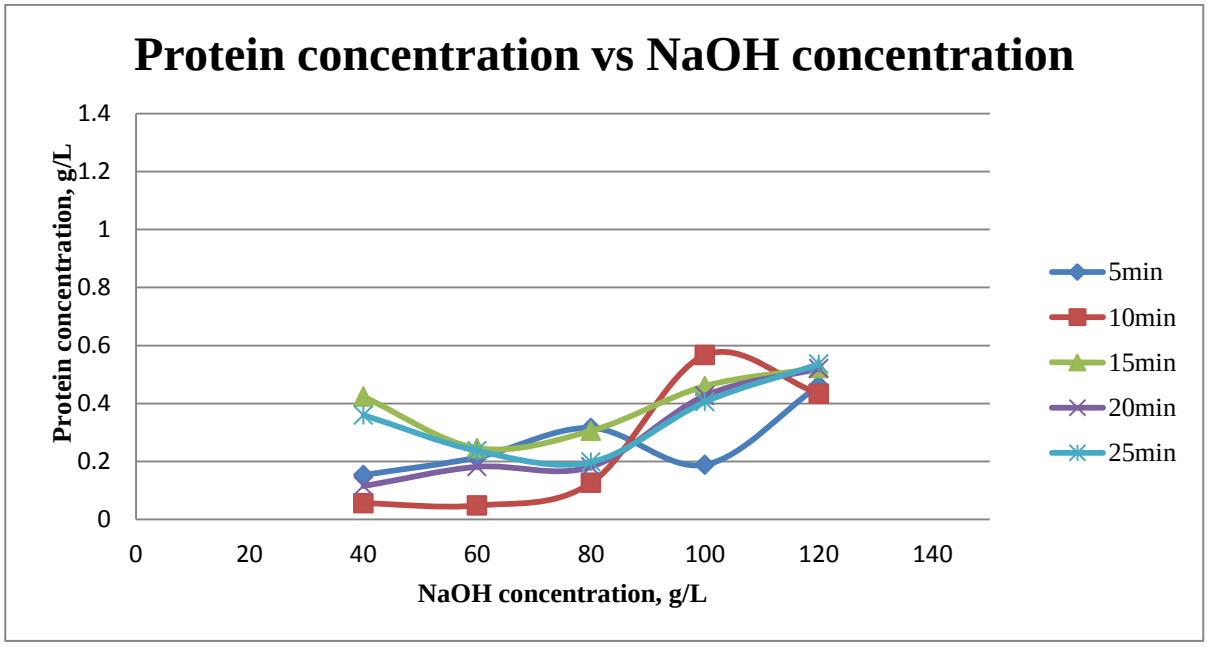

Figure 4-14: Graph of protein concentration versus NaOH concentration (Study on different time extraction and LSR 50:1)

According to Figure 4.15 below, the graph showed the protein concentration versus NaOH concentration for LSR 40:1. The highest protein concentration achieved was 1.112g/L resulted from 5 minutes and 120g/L of NaOH concentration. While the lowest protein concentration achieved was 0.318g/L for 25 minutes and 60 g/L NaOH concentration. The curve of four extraction time of 10, 15, 20 and 25 minutes showed the same trend as it increased from 40g/L to 120g/L of NaOH concentration. It was obviously differed to the curve plotted for extraction time of 5 minutes as it showed the best trend of curve. This was because, the protein concentration was increased from 40g/L to 80g/L NaOH concentration and decreased at 100g/L NaOH concentration but then, suddenly increased for the 120g/L NaOH concentration. For the accurate result, the data from the graph cannot be taken when the protein concentration was increased from 40g/L to 120g/L NaOH concentration as well as the graph showed that the protein extract can continuously to much higher NaOH concentration unstopped.

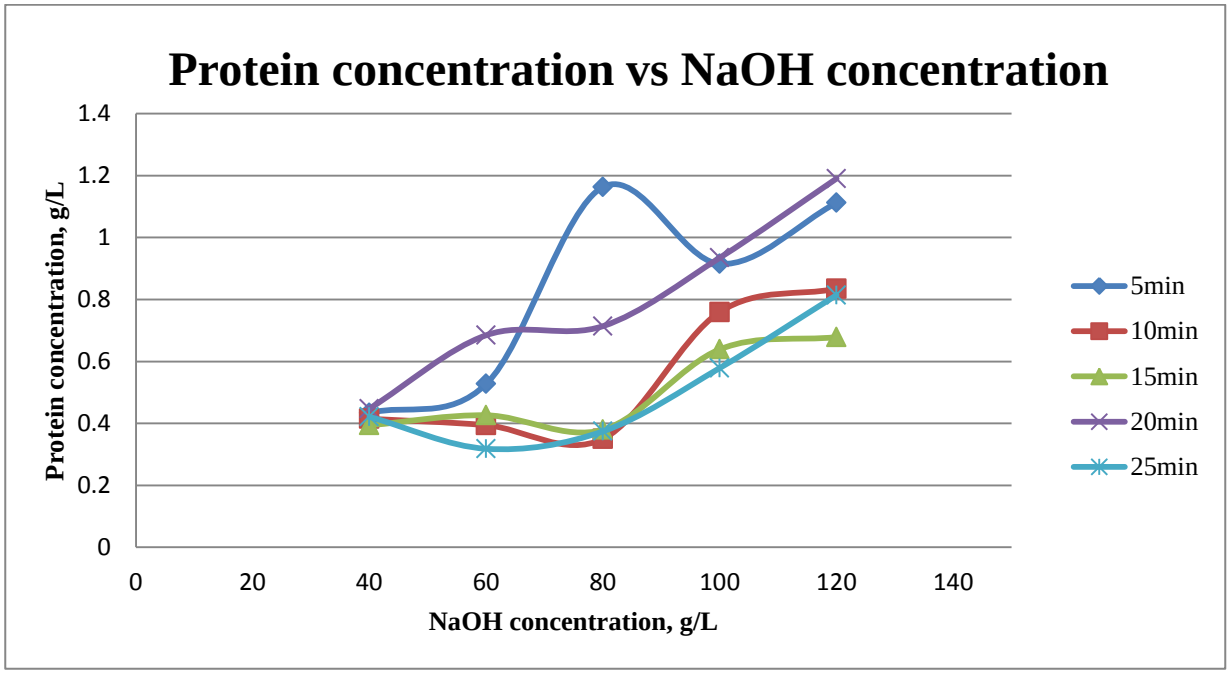

Figure 4-15: Graph of protein concentration versus NaOH concentration (Study on different time extraction and LSR 40:1)

The last graph plotted as Figure 4.16 below, it showed that the highest protein concentration achieved was 1.260g/L resulted from 25 minutes and 100g/L of NaOH concentration respectively while the lowest protein concentration was 0.372g/L resulted from 5 minutes and 60g/L NaOH concentration. Same to the graph plotted in the figure 4.3, there were four curves was in the same trend that was increased from 40g/L to 120g/L NaOH concentration. In between, the best curve was obtained from the curve of 25 minutes extraction time as the protein concentration decreased for 120g/L NaOH concentration. Hence, it is shown that the optimum condition for EBN's protein extraction is 30:1, 25minutes and 100g/L respectively for LSR, extraction time and

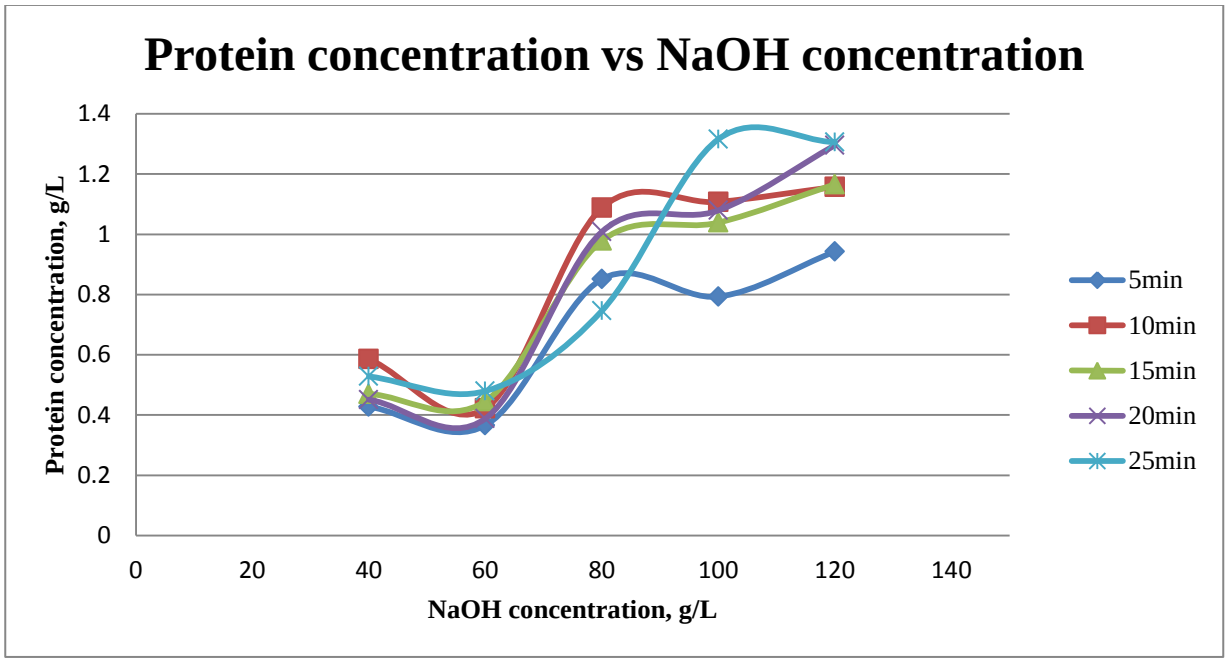

Figure 4-16: Graph of protein concentration versus NaOH concentration (Study on different time extraction and LSR 30:1)

4.3.5 Optimization of the protein concentration

In order to produce high concentration of protein, three factors of the alkaline hydrolysis were carried out in this study. The factors are extraction time, NaOH concentration and LSR. Apparently, the multiple regression equation for the protein concentration (Y) after extraction time (x_1) and NaOH concentration (x_2) and LSR (x_3) as the main variables was as follows:

$$Y: 8.561 + 0.061x_1 - 0.368x_2 + 0.700x_3 + 1.953E\text{-}03\ x_1^2 + 2.688E\text{-}03\ x_2^2 - 1.515E\text{-}03\ x_3^2 - 9.580\ E\text{-}04x_1\ x_2 - 3.315E\text{-}03x_1\ x_3 - 5.137E\text{-}03x_2\ x_3 \quad (1)$$

According to equation, the largest value of estimated regression coefficient for protein concentration was extraction time (x_1=0.061). Thus, it is worth to note that the extraction time was the prime factor which had the greatest effect on the protein concentration. This could be again due to the protein denaturation at higher extraction time beyond the optimum. Denaturation of protein involves the disruption of reactions of both secondary and tertiary structures. Since denaturation reactions are not strong enough to break the peptide bonds, the primary structure (sequence of amino acids) remains the same after a denaturation process. Denaturation disrupts the normal alpha-helix and beta sheets in a protein and uncoils it into a random shape.

The coefficient of determination (R^2) was found to be 0.9705 which corresponding to protein concentration. The value of R^2 is a measure of the total variation of the observed values of protein concentration about the mean explained by the fitted model, which is often described in percentage. This apparently indicates that 97.05% of the total variation in the protein concentration is explained by the fitted model (Equation 1). Based on Table 4.6, the differences between predicted R^2 and adjusted R^2 are less than 0.2, which implies that the predicted R^2 is in reasonable agreement with the adjusted R^2. In addition, the ratio of the adequate precision was greater than 4 which indicate adequate model discrimination. The analysis of variance also showed that there was a non-significant lack of fit that further indicates adequate model discrimination. Moreover, in this study, the statistical analysis of the model, was carried out using result was tabulated in Table 4.6.

The F-value is the ratio of mean square due to regression to the mean square of error. The F-value of the protein concentration is 32.91 which is greater than the tabulated $F_{7,10}$, the null hypothesis is rejected at the α level of significance and infers that the variation accounted for the model is significantly greater than the unexplained variation. In this case the null hypothesis is rejected at <0.0001 level of significance for protein concentration. This prob>F (<0.0001) indicated that the model terms were significant. Furthermore, according to Table 4.6, A was a significant model. The lack of fit F-value of 1.09 implied that the lack of fit was not significant.

Results from CCD clearly demonstrated that protein concentration increases when the extraction time increases from 5 minutes to 25 minutes. The optimum condition of protein were found at 25 minutes of extraction time, 100g/L NaOH concentration and 30:1 of LSR. Concisely, the extraction time was the most significant factor affecting the protein concentration (F-value = 161.65).

On the other hand, increase in NaOH concentration and LSR indicates insignificant effect to the degradation of protein and subsequently produced low yield of protein.

Table 4-6: Analysis of variance Table (partial sum of squares) for response surface Model

Source	Sum of Squares	DF	Mean Square	F value	Prob > F	
Model	19.73	9	2.19	32.91	< 0.0001	**significant**
A	0.55	1	0.55	8.19	0.0187	
B	0.27	1	0.27	4.11	0.0733	
C	0.062	1	0.062	0.94	0.3588	
A^2	0.059	1	0.059	0.89	0.3699	
B^2	14.38	1	14.38	215.92	<0.0001	
C^2	0.036	1	0.036	0.54	0.4828	
AB	0.073	1	0.073	1.10	0.3211	
AC	0.055	1	0.055	0.82	0.3874	
BC	2.11	1	2.11	31.69	0.0003	
Residual	0.60	9	0.067			
Lack of Fit	0.28	4	0.070	1.09	0.4499	**not significant**
Pure Error	0.32	5	0.064			
Cor Total	20.33	18				
Std. Dev.	0.26		**R-Squared**		0.9705	
Mean	1.36		**Adj R-Squared**		0.9410	
Adeq Precision	22.575		**Pred R-Squared**		0.8274	

Figure 4.17 below shows the contour plot of combined desirability profiler. Increase in extraction time results in increase in desirability profiler. Nevertheless, increase in NaOH concentration implies insignificantly effect to the desirability profiler in order to attain high concentration of protein. This scenario is again presumably related to the amount of protein effects by extraction time towards protein concentration.

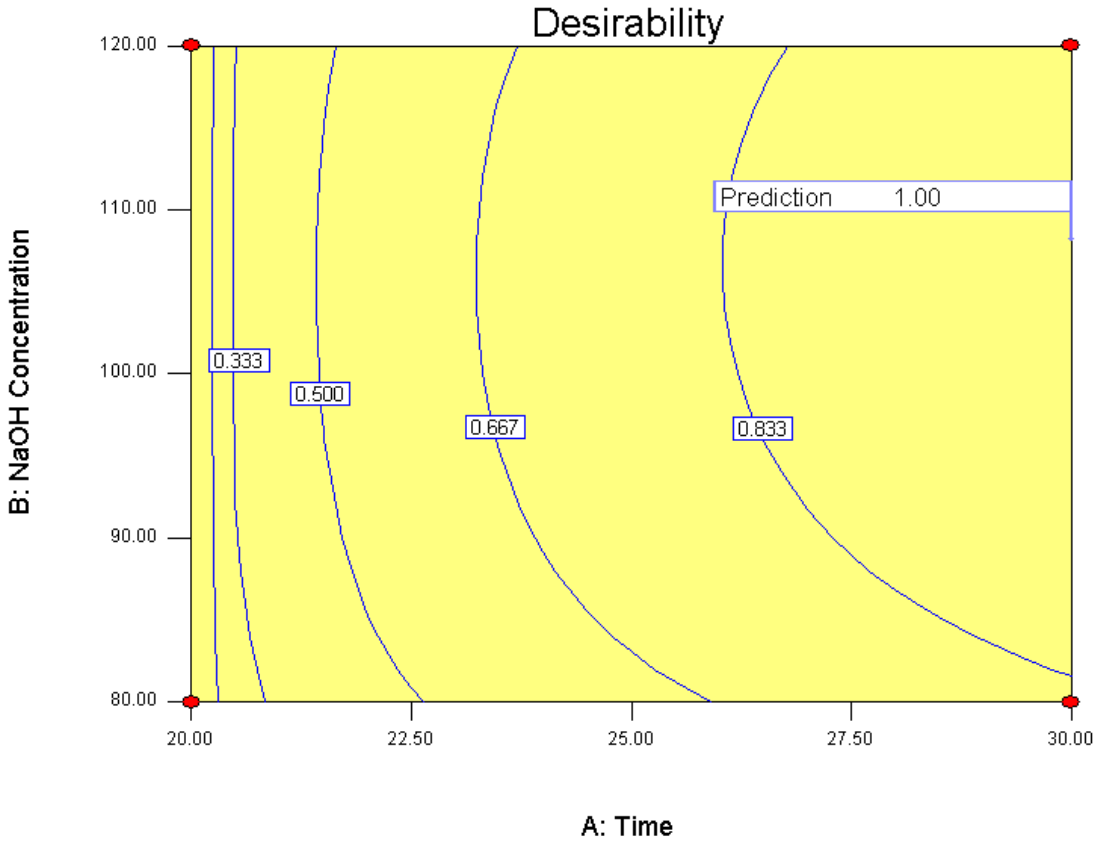

Figure 4-17: Effect of NaOH concentration and extraction time on protein concentration extracted from EBN

From the Figure 4.18 below, a normal plot was graphically illustrated to shows further diagnosed and comparison of significant effects and interactions of factors on response where the results of domination effects that are likely to represent the important and influential factors were found consistent with the ANOVA analysis results. In addition, the significance of interaction between factors on the response can be best considered using interaction analysis graph of Figure 4.19. From the Figure 4.18, concave response surface was found. A week effect on the response was observed for both the lowest levels of extraction time and NaOH concentration. The protein concentration increased with increasing the NaOH concentration from 80g/L to 100g/L within the studied range of extraction time. As predicted by the model, the max protein concentration of 1.235g/L was occurred when the NaOH concentration was 100g/L.

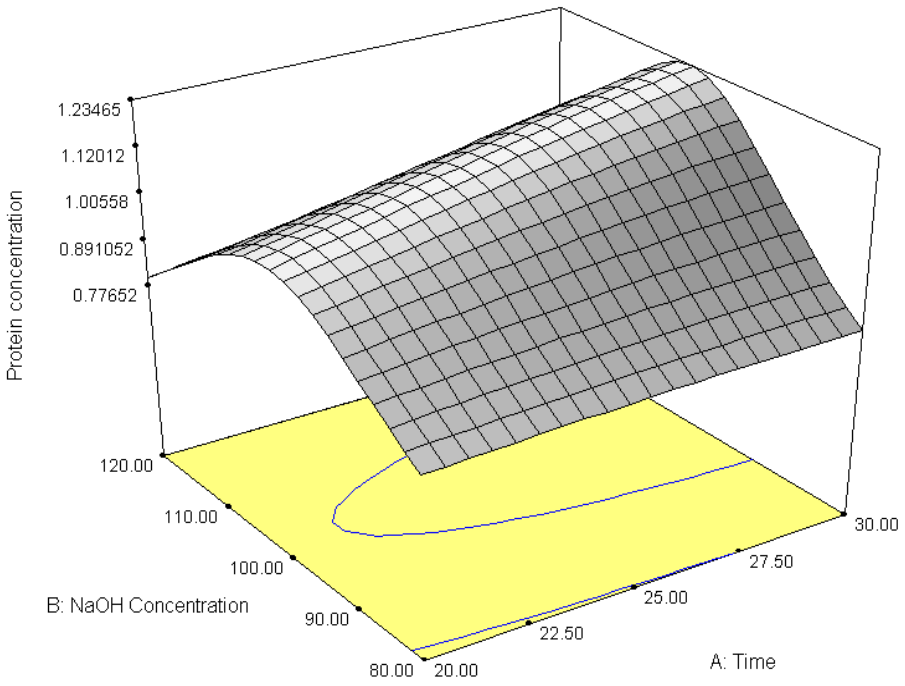

Figure 4-18: Surface plot for protein extract on EBN of factor A: B (Time; NaOH concentration)

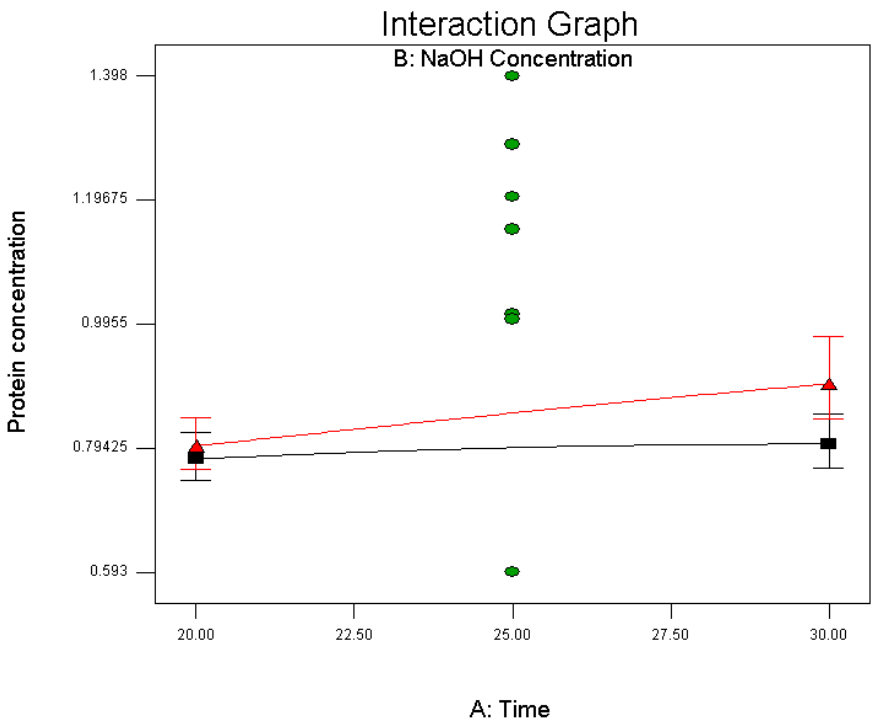

Figure 4-19: Interaction graph for the response of factor A:B (Time; NaOH concentration)

From the Figure 4.20 below, a normal plot was graphically illustrated to shows further diagnosed and comparison of significant effects and interactions of two factors which is (time and LSR) on response where the results of domination effects that are likely to represent the important and influential factors were found consistent with the ANOVA analysis results. In addition, the significance of interaction between these two factors on the response also was been best considered using interaction analysis graph of Figure 4.21. It was shown that the interaction effect between the extraction time and LSR depicts remarkable improvement in protein concentration as increasing of extraction time and LSR. The curve is shaped as shown in Figure 4.20 because of the limitation of the LSR range studied in this research.

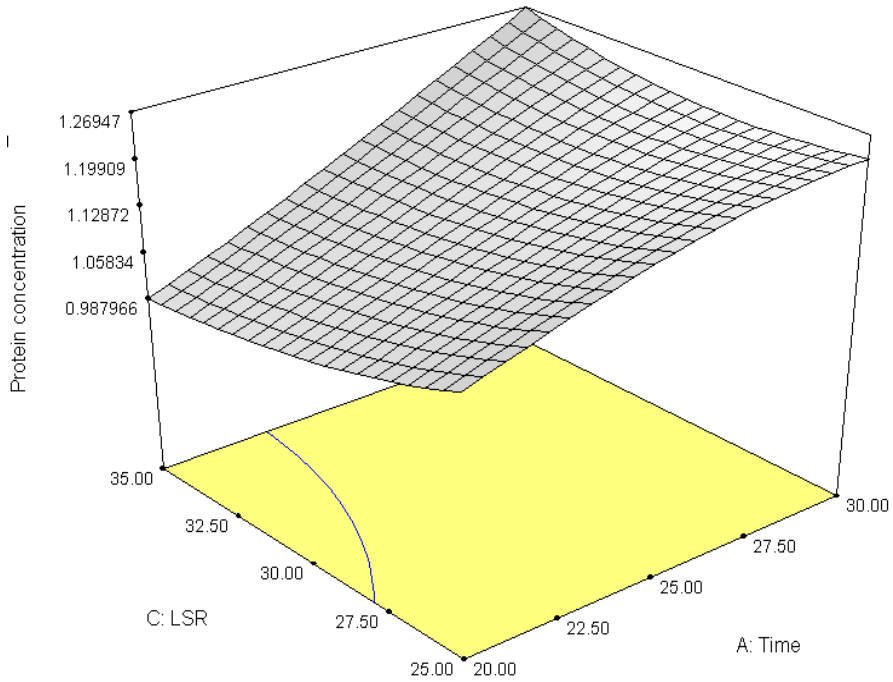

Figure 4-20: Surface plot for protein extract on EBN of factor A: C (time ; LSR)

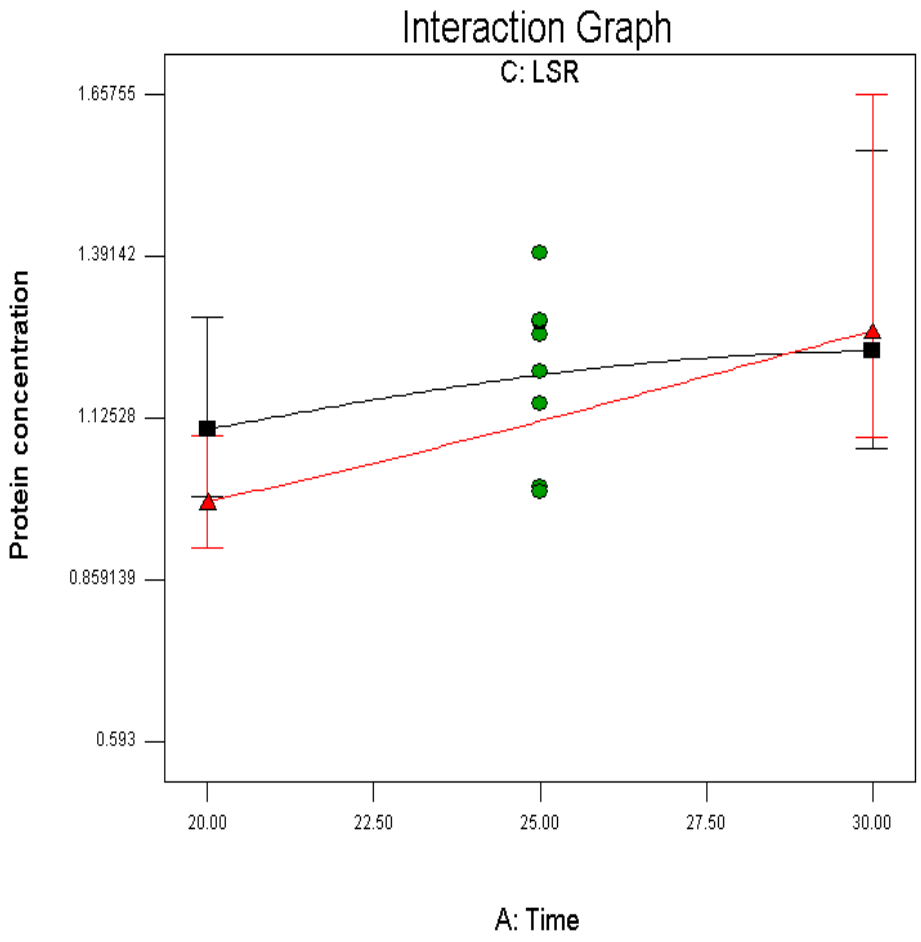

Figure 4-21: Interaction graph for the response of factor A: C (time; LSR)

The significant effects and interactions of two factors which are NaOH concentration and LSR on response were further diagnosed and compared and graphically illustrated in the normal plot as shown in Figure 2.2 below. Besides, the significance of interaction between these two factors on the response also was been best considered using interaction analysis graph as Figure 4.23. It was shown that the interaction effect between the NaOH concentration and LSR depicts remarkable improvement in protein concentration as increasing of LSR and NaOH concentration, which is however not beyond the optimum NaOH concentration at 100g/L.

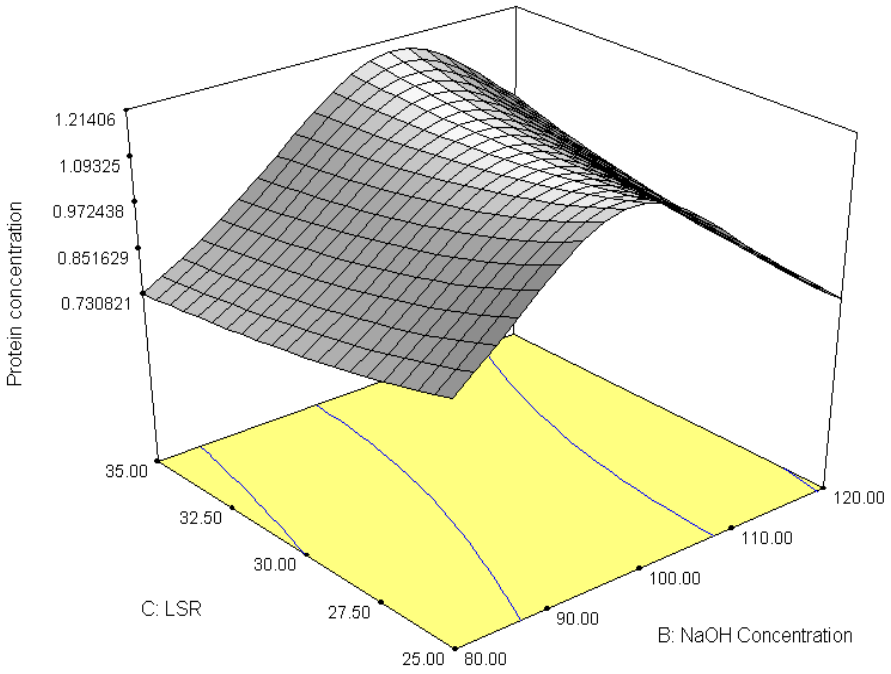

Figure 4-22: Surface plot for protein extract on EBN of factor B: C (NaOH concentration; LSR)

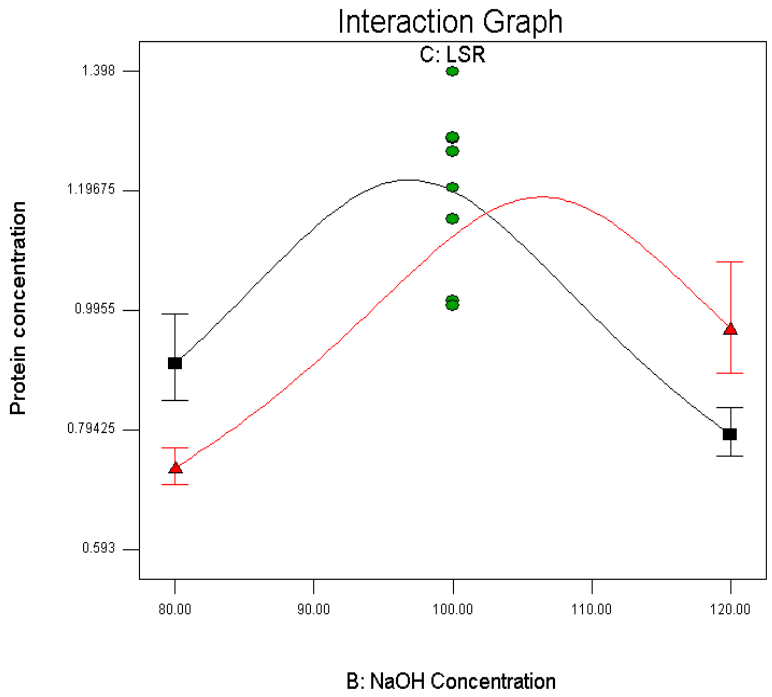

Figure 4-23: Interaction graph for the response of factor B:C (NaOH concentration; LSR)

It is observed from Figure 4.24, analyses on normal probability plot of residuals depicted nearly a straight line residual distribution in which denoting errors are evenly distributed and therefore support adequacy of the least-square fit. Somehow, the results illustrated in Figure 4.25, 4.26, and 4.27 revealed that the models proposed are distinctively adequate and reasonably free from any violation of the independence or constant variance assumption as the studentized residuals are equally tabulated within the red line of the x-axis.

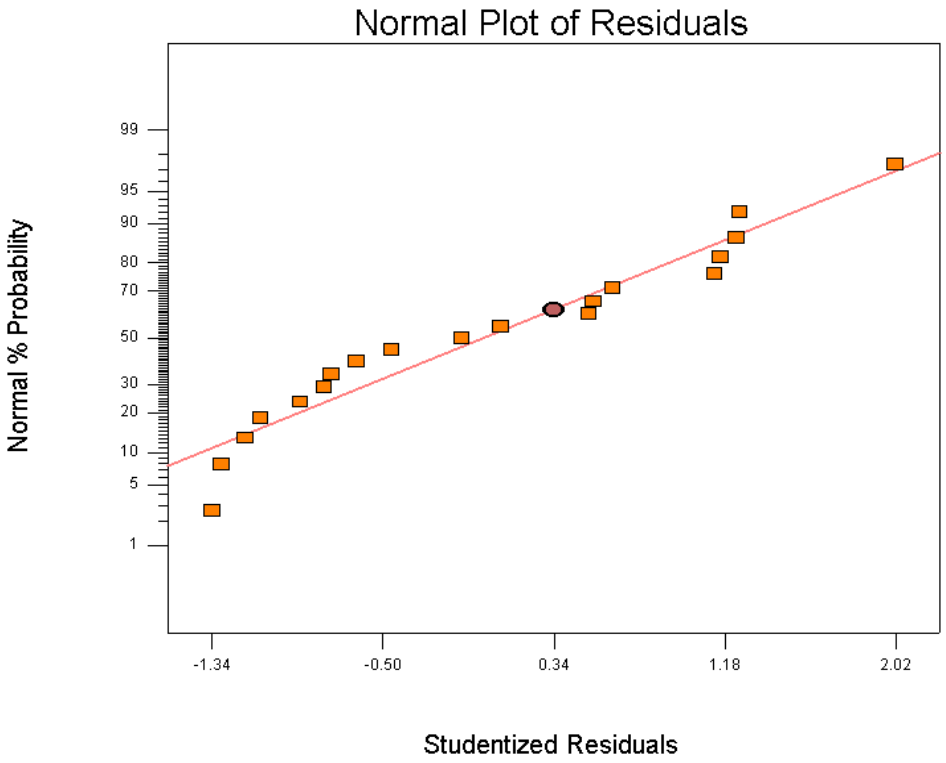

Figure 4-24: The Normal Plot of Residuals

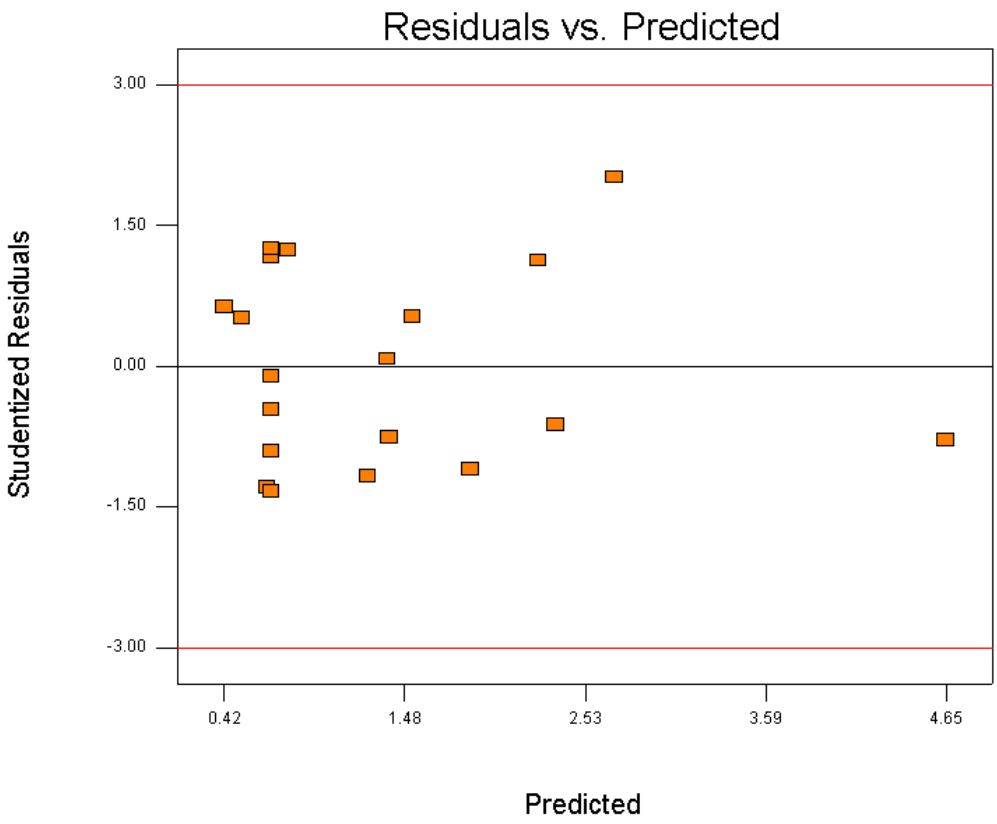

Figure 4-25: Plot of Residuals against predicted response

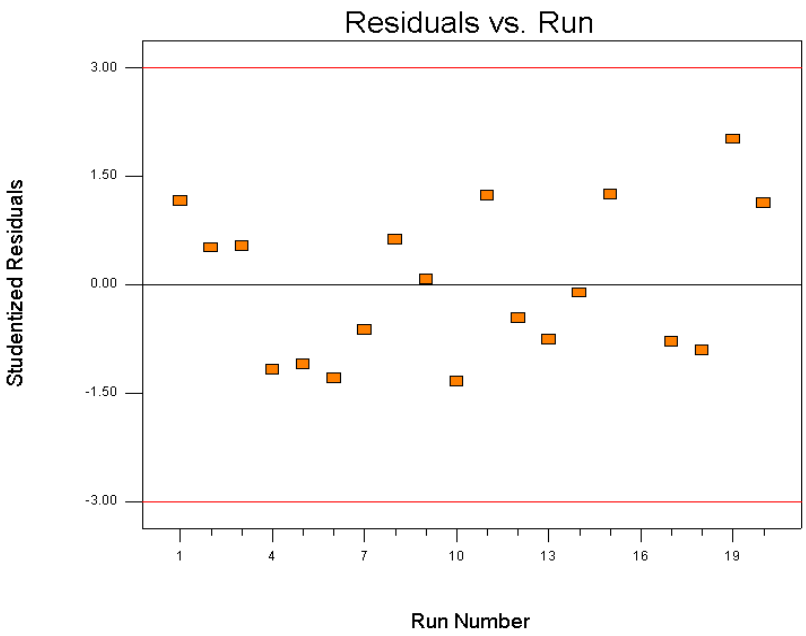

Figure 4-26: Plot of Residuals against run response

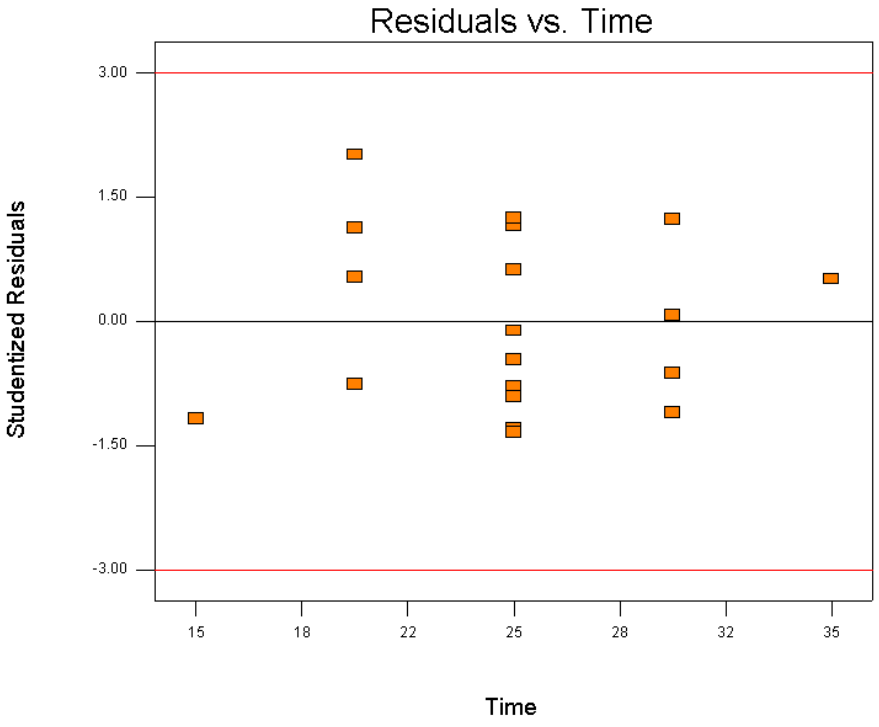

Figure 4-27: Plot of Residuals against time

In order to validate the adequacy of the model, a confirmation run was performed and resulted as Table 4.7. From the optimum experimental condition which was recommended by the software, the first condition of the confirmation run was taken. For other conditions in which for the last two confirmation experiments were among the preparation conditions that is applied previously. The comparison between the predicted values and the actual experimental values is made and the percentage error was calculated then. The calculated percentage error for protein concentration between the actual and predicted value was up to 4.000%

The model was successfully validated as the experimental values were found to be very close to the predicted values. Thus, the conclusion made is that the empirical model developed was reasonably accurate for the protein concentration as all actual values for the confirmation runs were in the high of prediction interval which is about 95%. Furthermore, this percent is in the range as there is an expectation for the value to fall into 95% of the time.

Table 4-7: Confirmation run

Time (minutes)	**NaOH concentration (g/L)**	**LSR (v:w)**	**Protein concentration (g/L)**		
			Actual (g/L)	Predict (g/L)	Error (%)
30	107.25	35	1.482	1.426	3.779
30	102.32	35	1.400	1.344	4.000
30	104.55	35	1.434	1.399	2.441
25	100.00	30	1.367	1.398	2.268
25	100.00	30	1.260	1.287	2.143

From Table 4.7, the confirmation run was revealed that the optimum condition of extraction time, NaOH concentration and LSR were 25 minutes, 100g/L and 30:1 respectively. This result is due to the lowest error obtained among others which is 2.143%. In the previous study which is the protein extraction from watermelon (Wani *et al*., 2008) revealed a significant increase in the protein concentration of extraction time 15 minutes which is lower than the present study; meanwhile, studies on the protein extraction of lupine (Lupinus termis L.) seeds (Khalil *et al.,*1990) are in agreement with the present study. The comparison and the differences of reported results may be due to the differences of the raw materials used, type of equipment used, procedure of the study/ experiment and other conditions used in the study.

5 CONCLUSIONS

The FTIR spectrums of the untreated and treated sample resulted in the different trend of spectrum. In the treated sample after pre-treatment process and alkaline hydrolysis, it is showed that N-H and C=O stretching peak is increased. Besides, it was confirmed that the material was correspond to amides as it consists of C=O stretch. While, from the analysis using the ICP-MS, amount of cadmium, arsenic and lead in the sample was 0.002mg/L, 0.00006mg/L and 0.262mg/L respectively after pre-treatment. It also showed that percentage decreasing of argentums, cadmium and magnesium is the highest compared to others metal which were 99%, 97.70% and 97.24% respectively. It can be concluded that EBN can be performed as an alternative source for protein. RSM technique proved to be a useful tool in establishing optimum conditions for extracting protein from EBN. From the experimental result, it showed that the optimum condition of protein concentration of 1.260 g/L were found at 25 minutes of extraction time, 100g/L NaOH concentration and 30:1 of LSR. From the ANOVA result, the significant factor was extraction time ($p=0.0187$) and was clearly showed that further increased in extraction time (25 minutes) resulted in decreased in protein concentration due to the degradation of amino acid structure at long duration of extraction time. It can be concluded that EBN can be performed as an alternative source for protein. Hence, according to the results above, the protein concentration of EBN were produced by extraction process is acceptable and excellent. Therefore this study is successful and objectives are achieved.

5.1 RECOMMENDATIONS

In this study, there are some recommendation was suggested for the further studies and maybe for application in laboratory or/and industry. This was to ensure the effectiveness on the protein extraction from EBN of *C.Fuciphaga* species. The recommendations are:

i) The temperature for the protein extracted was lower so the protein structure would not denature.

ii) Analysis of the sample must be made directly after the extraction process to ensure the protein extracted was not being denatured. This is because, if the sample is left in the chillers' for long duration of time, denaturation of protein occurred as there are many students will open and close the chillers frequently.

iii) The standard curve must be prepared in every preparation of the protein assay.

iv) Quantitative method such as High Performance Liquid Chromatography can be use in order to determine the compound inside the protein extracted.

6 APPENDIX

Table 6.1: BSA preparation

Volume distilled water, L	Mass of BSA, g	Final concentration, g/L
0.01	0	0
0.01	0.002	0.2
0.01	0.004	0.4
0.01	0.006	0.6
0.01	0.008	0.8
0.01	0.01	1

Table 6.2: The optical density on the different concentration of BSA

Concentration of BSA, g/L	Optical density (OD)		
	Run 1	Run 2	Average
0	0	0	0
0.2	0.324	0.328	0.326
0.4	0.521	0.509	0.515
0.6	0.640	0.701	0.6705
0.8	0.853	0.906	0.8795
1.0	1.002	0.962	0.982

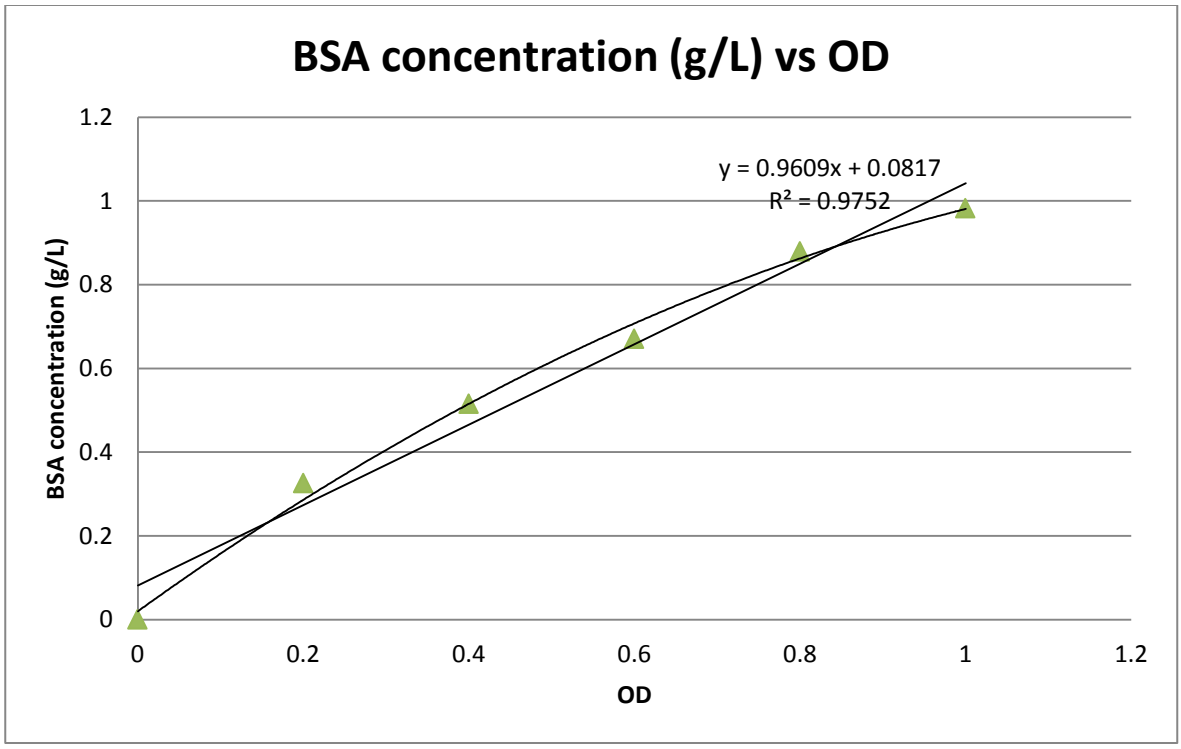

Figure 6.1 **:** BSA concentration (g/L) versus Optical density (OD)

Lowry's Method

Table 3: Reagents for Modified Lowry's Method

Reagent	**Preparation procedure**	**Notes**
A	Dissolve 20g of sodium carbonate and 4g of sodium hydroxide in 1L distilled water	Keep refrigerated
B	Dissolve 2.5g of copper sulfate ($CuSO_4.5H_2O$) and 5g of sodium citrate in 1 L distilled water	Wrap the bottle with aluminium foil to avoid discolorization and keep refrigerated
Lowry solution	Mix reagent A and B in 50:1 ratio	Prepare only when needed and keep refrigerated
Folin- Ciocalteu (stock 2.0 N)	Dilute the stock with distilled water in 1:1 ratio	Prepare only when needed

Infrared Spectroscopy

Table 4: Table B-1 IR Absorptions for Representative Functional Groups

Functional Group	Molecular Motion	Wavenumber (cm^{-1})
Alkenes	=CH stretch	3100-3010
	C=C stretch (isolated)	1690-1630
	C=C stretch (conjugated)	1640-1610
	C-H in-plane bend	1430-1290
	C-H bend (monosustituted)	~990 & ~910
Alcohol	O-H stretch	~3650 or 3400~3300
	C-O stretch	1260-1000
Aldehydes	C-H aldehyde stretch	~2850 & ~2750
	C=O stretch	~1725
Ketones	C=O stretch	~1715
	C-C stretch	1300-1100
Carboxylic acids	O-H stretch	3400-2400
	C=O stretch	1730-1700
	C-O stretch	1320-1210
	O-H bend	1440-1400
Amines	N-H stretch (1 per N-H bond)	3500-3300
	N-H bend	1640-1500
	C-N stretch (alkyl)	1200-1025
	C-N stretch (aryl)	1360-1250
	N-H bend (oop)	~800
Amides	N-H stretch	3500-3180
	C=O stretch	1680-1630
	N-H bend	1640-1550
	N-H bend (1^0)	1570-1515

Table 5: Preparation condition from RSM

Std	Run	Block	Factor 1	Factor 2	Factor 3	Response 1
			A : Time (min)	B : NaOH concentration (g/L)	C : LSR	Protein concentration (g/L)
15	1	Block 1	25.00	100.00	30.00	1.011
10	2	Block 1	35.00	100.00	30.00	1.197
7	3	Block 1	20.00	120.00	35.00	0.850
9	4	Block 1	15.00	100.00	30.00	0.970
4	5	Block 1	30.00	120.00	25.00	0.835
14	6	Block 1	25.00	100.00	40.00	1.284
6	7	Block 1	30.00	80.00	35.00	0.753
13	8	Block 1	25.00	100.00	20.00	1.263
2	9	Block 1	30.00	80.00	25.00	0.894
16	10	Block 1	25.00	100.00	30.00	1.398
8	11	Block 1	30.00	120.00	35.00	1.005
17	12	Block 1	25.00	100.00	30.00	1.202
1	13	Block 1	20.00	80.00	25.00	0.917
20	14	Block 1	25.00	100.00	30.00	1.149
19	15	Block 1	25.00	100.00	30.00	1.003
11	16	Block 1	25.00	60.00	30.00	0.724
12	17	Block 1	25.00	140.00	30.00	0.593
18	18	Block 1	25.00	100.00	30.00	1.287
5	19	Block 1	20.00	80.00	35.00	0.687
3	20	Block 1	20.00	120.00	35.00	0.736

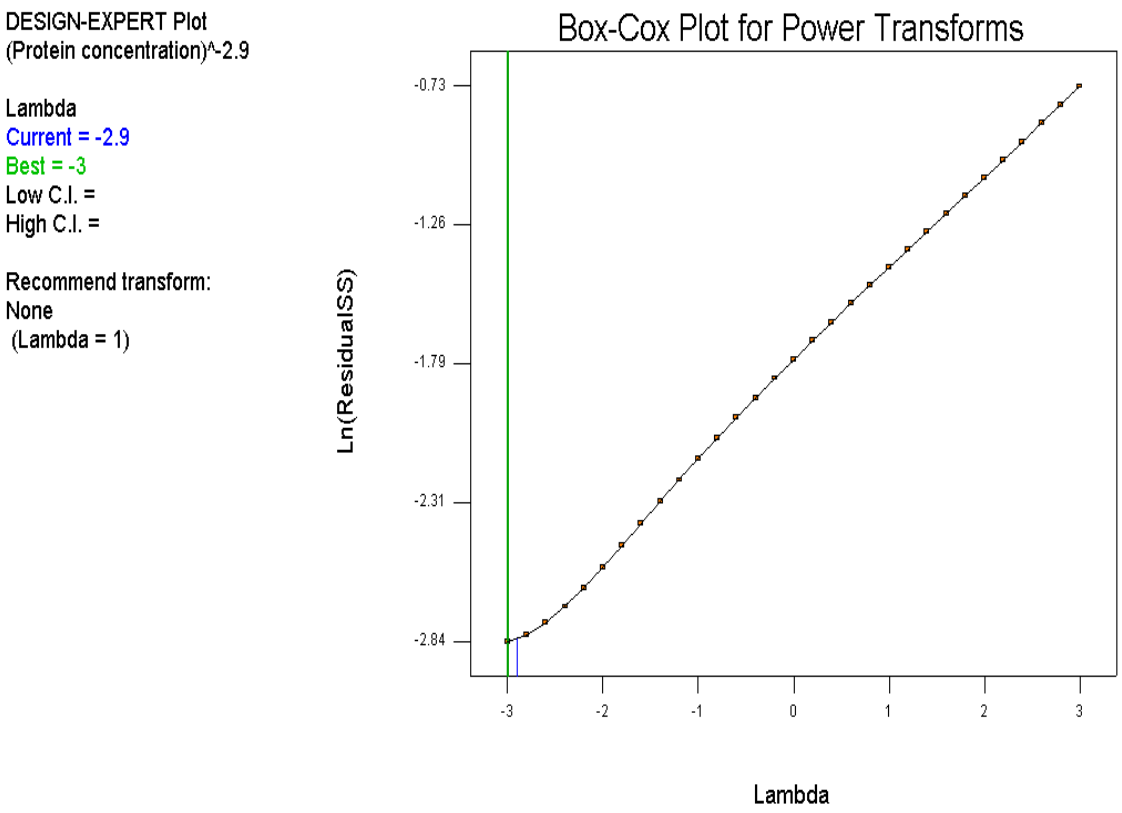

Figure 2: Box-Cox Plot

Response : conc

****WARNING: The Cubic Model is Aliased! *****

Sequential Model Sum of Squares

Source	Sum of square	DF	Mean square	F value	Prob > F	
Mean	35.38	1	35.38			
Linear	2.870	3	0.960	0.82	0.5020	
2FI	2.240	3	0.750	0.59	0.6342	
Quadratic	14.62	3	4.870	73.18	<0.0001	Suggested
Cubic	0.280	4	0.070	1.09	0.4499	Aliased
Residual	0.320	5	0.064			
Total	55.72	19	2.930			

"*Sequential Model Sum of Squares*": Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Lack of Fit Test

Source	Sum of square	DF	Mean Square	F Value	Prob > F	
Linear	17.14	10	1.71	2.681	0.0010	
2FI	14.90	7	2.13	33.30	0.0007	
Quadratic	0.280	4	0.070	1.09	0.4499	Suggested
Cubic	0.000	0				Aliased
Pure error	0.32	5	0.064			

"*Lack of Fit Tests"*: Want the selected model to have insignificant lack-of-fit

Model Summary Statistics

Source	Std Order	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	1.08	0.1411	-0.0306	-0.6865	34.29	
2FI	1.13	0.2513	-0.1231	-2.0861	62.76	
Quadratic	0.26	0.9705	0.9410	0.8274	3.51	Suggested
Cubic	0.25	0.9843	0.9434		+	Aliased

"*Model Summary Statistics"*: Focus on the model maximizing the "Adjusted R-Squared" and the "Predicted R-Squared".

Table 6 : Diagnostics Case Statistics

Standard order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	1.29	1.39	-0.10	0.737	-0.754	0.159	-0.735	13
2	1.38	1.37	0.01	0.737	0.078	0.002	0.074	9
3	2.43	2.25	0.18	0.629	1.13	0.217	1.15	20
4	1.69	1.86	-0.17	0.629	-1.1	0.205	-1.114	5
5	2.97	2.70	0.27	0.737	2.061	1.137	2.567	19
6	2.28	2.36	-0.083	0.737	-0.629	0.111	-0.606	7
7	1.60	1.52	0.084	0.629	0.534	0.048	0.512	3
8	0.99	0.79	0.19	0.629	1.236	0.259	1.279	11
9	1.09	1.26	-0.17	0.696	-1.175	0.317	-1.204	4
10	0.59	0.52	0.073	0.696	0.513	0.06	0.490	2
11	4.55	4.65	-0.094	0.786	-0.789	0.228	-0.771	17
13	0.51	0.42	0.090	0.696	0.629	0.091	0.607	8
14	0.48	0.67	-0.18	0.696	-1.292	0.383	-1.350	6
15	0.97	0.69	0.27	0.161	1.159	0.026	1.184	1
16	0.38	0.69	-0.32	0.161	-1.338	0.034	-1.409	10
17	0.59	0.69	-0.11	0.161	-0.458	0.004	-0.437	12
18	0.48	0.69	-0.21	0.161	-0.904	0.016	-0.894	18
19	0.99	0.69	0.30	0.161	1.254	0.030	1.302	15
20	0.67	0.69	-0.026	0.161	-0.111	0.000	-0.105	14

Proceed to Diagnostic Plots (the next icon in progression). Be sure to look at the:

1) Normal probability plot of the studentized residuals to check for normality of residuals
2) Studentized residuals versus predicted values to check for constant error.
3) Outlier t versus run order to look for outliers, ie., influential values.
4) Box-Cox plot for power transformations.

If all the model statistics and diagnostic plots are OK, finish up with the Model Graphs icon.

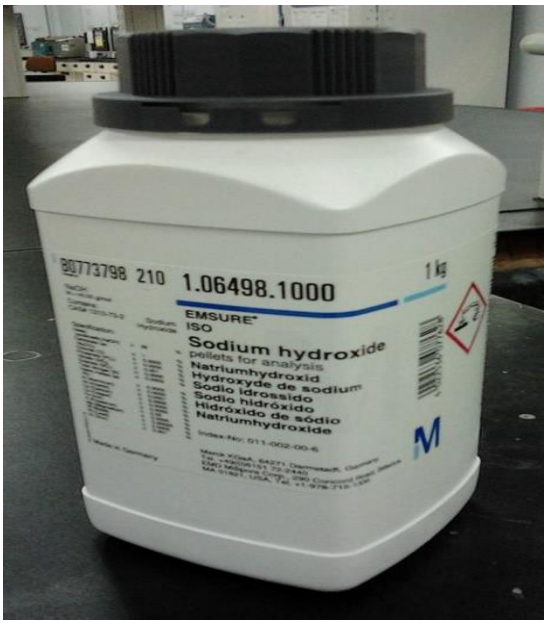

Figure 3: Sodium hydroxide (NaOH)

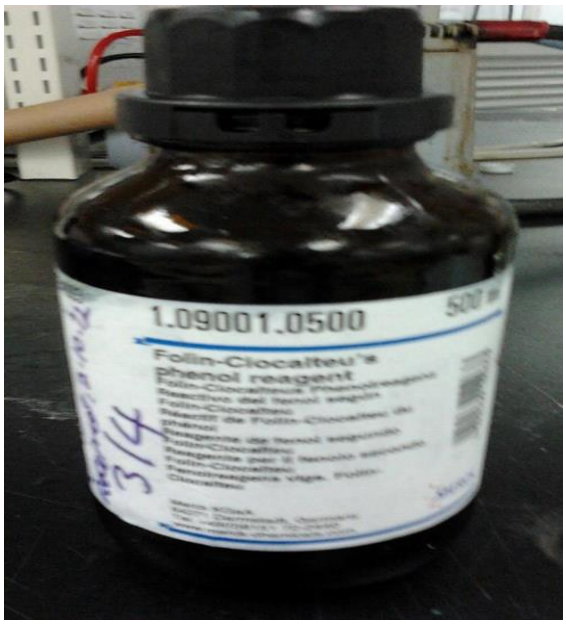

Figure 4: Folin-Ciocalteu's phenol reagent

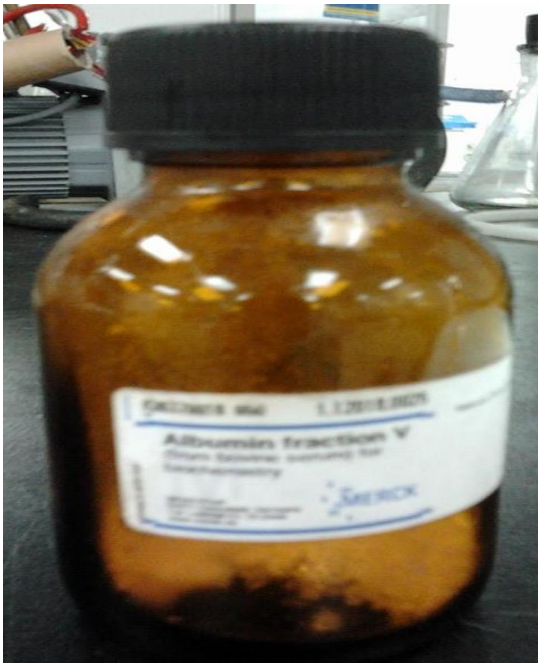

Figure 5: Albumin fraction V

Figure 6: Sample of NaOH dilution of different concentration

Figure 7: Dilution of NaOH

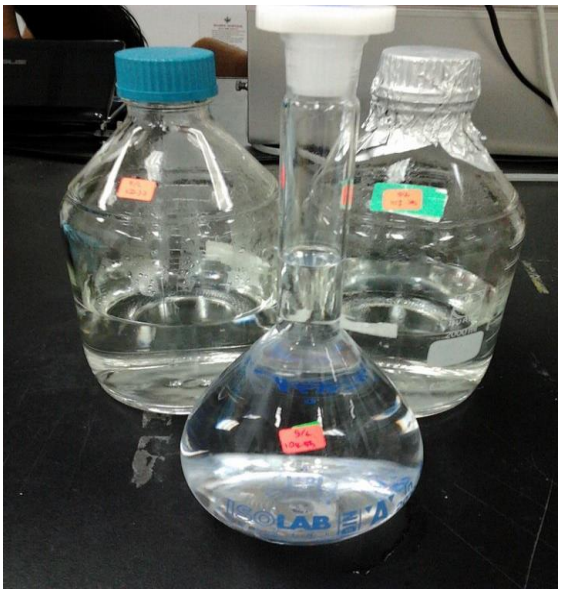

Figure 8: Dilution of NaOH for RSM

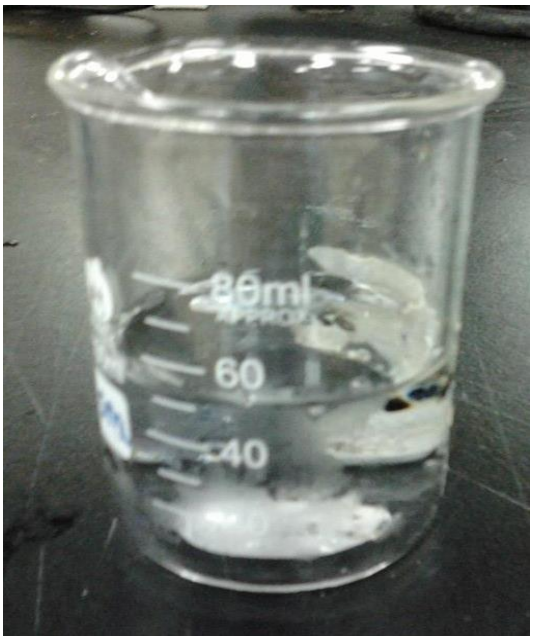

Figure 9: Lowry reagent

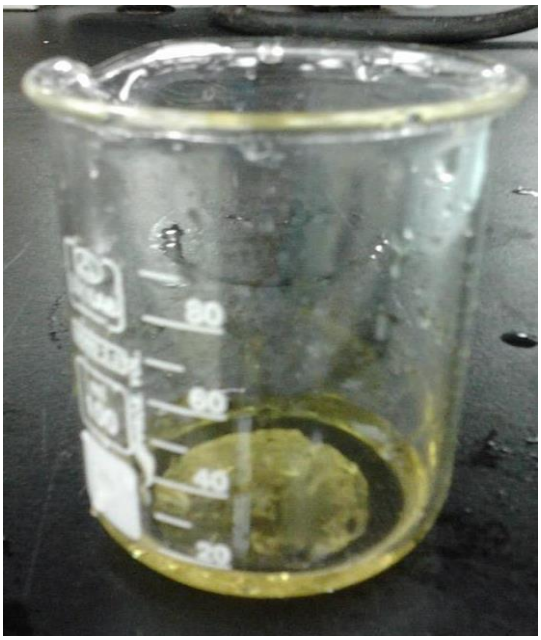

Figure 10: Folin-Ciocalteu's phenol reagent

Figure 11: Reagent A

Figure 12: Reagent B

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