CHARACTERIZATION AND PROCESS OPTIMIZATION OF *COLLOCALIA FUCIPHAGA* EXTRACT

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for the award of the degree of

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SUPERVISOR DECLARATION

"I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in terms of scope and quality for the award of the Bachelor in Chemical Engineering (Biotechnology)

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I hereby declare that the work in this thesis entitled "Characterization and Process Optimization of *Collocalia Fuciphaga*" is the results of my own research accept as cited in references. This thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

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ABSTRACT

The purpose of this study is to characterize and investigate the optimum condition of temperature and liquid solid ratio (LSR) in *Collocalia fuciphaga* extract. The formation of functional group in the *Collocalia fuciphaga* was confirmed by fourier transform infrared spectroscopy (FTIR) analysis of the untreated and treated sample while inductively coupled plasma mass spectrometry (ICP-MS) was used to determine the heavy metals contents inside the *Collocalia fuciphaga.* Water extraction method was employed as a function of temperature and LSR in order to identify their effects to the protein extract concentration from *Collocalia fuciphaga* and subsequently determine its optimum condition using response surface methodology (RSM). The FTIR spectrums of the untreated and treated sample resulted in the same trend of spectrum. This is because the functional group of the protein extract, O-H bond, N-H bond, C=O bond, and C-H bond, respectively did not change after the extraction process. From the analysis using the ICP-MS, it was clearly showed that concentration of aluminium, arsenic and lead in the *Collocalia fuciphaga* was 2.378 mg/L, 0.044mg/L and 0.125mg/L which is lower than the maximum concentration allowable of aluminium, arsenic and lead (7mg/L, 2mg/L and 3.402mg/L, respectively). The optimum condition of temperature and LSR were found to be 38^{O}C and 42:1 while the protein extract concentration was 0.3477g/L. Increase in temperature after this optimum value resulted in decrease in protein extract concentration due to the destruction of protein structure at high temperature.

ABSTRAK

Tujuan kajian ini adalah untuk mengenalpasti dan mengkaji keadaan optimum suhu dan nisbah cecair terhadap pepejal (LSR) bagi proses pengekstrakan protein daripada *Collocalia fuciphaga*. *Spektroskopi inframerah* transformasi *fourier* (FTIR) digunakan untuk menentukan kumpulan berfungsi sampel *Collocalia fuciphaga* yang tidak dirawat dan dirawat. Manakala *Spektrometer Jisim - Plasma Gandingan* Aruhan (ICP-MS) telah digunakan untuk menentukan kandungan logam berat di dalam *Collocalia fuciphaga.* Kaedah pengekstrakan menggunakan air telah digunakan dalam kajian ini. Faktor kesan suhu dan LSR terhadap kepekatan ekstrak protein daripada *Collocalia fuciphaga* telah dikaji dan seterusnya ditentukan keadaan optimum proses pengekstrakan ini dengan menggunakan kaedah tindak balas permukaan (RSM). Spektrum FTIR untuk sampel yang tidak dirawat dan dirawat menunujukkan bentuk graf yang sama. Ini adalah kerana ekstrak protein mempunyai kumpulan berfungsi O-H , N-H,C=O dan C-H tidak berubah selepas proses pengekstrakan. Daripada analisis menggunakan ICP-MS, ia jelas menunjukkan bahawa kandungan aluminium, arsenik dan plumbum dalam *Collocalia fuciphaga* adalah 2.378 mg/L, 0.044mg/L dan 0.125mg/L yang mana ianya kurang daripada kepekatan maksima yang dibenarkan iaitu 7mg/L, 2mg/L dan 3.402mg/L bagi kandungan aluminium, arsenik dan plumbum, masing-masing. Keadaan optima suhu dan LSR pada 38^{o}C dan 42:1 menghasilkan kepekatan protein ekstrak yang paling tinggi iaitu 0.3477g/L. Peningkatan suhu selepas nilai optima akan menyebabkan pengurangan kepekatan protein ekstrak kerana struktur protein akan terganggu pada suhu yang tinggi.

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

The word `protein' is defined as any of a group of complex organic compounds, consisting essentially of combinations of amino acids in peptide linkages, that contain carbon, hydrogen, oxygen, nitrogen, and usually, sulfur (Yada, 2004). Protein molecules tend to unfold and even become fully denatured under unfavoured conditions, such as a high temperature, an acidified condition, a high pressure or even excessive shear. Denatured protein molecules will aggregate and/or crosslink to form larger clusters and, at high concentrations, will form a three-dimensional solid-like network (or a gel). Therefore, proteins are regarded as one of the main classes of building blocks used in many semi-solid foods for conferring mechanical properties (Lianqing *et al*., 2008).

Proteins are also recognised as one of the main classes of surface-active agents in liquid foods for stabilising dispersed particles and fat droplets, due to the polarised distribution of hydrophobic and hydrophilic groups along the back bone. Protein molecules adsorb at the oil–water interface to lower the interfacial tension and, therefore, make such thermodynamically less favourable dispersed systems stable for an extended shelf life. The importance of protein application in foods can also be seen in many other aspects. For example, it was reported that, in combination with polysaccharides and starches, proteins could be applied as a meat alternative, as a fat replacer or a filler in manufacturing healthier foods. Proteins also have special uses as foaming agents or as functional ingredients for nutrient delivery in foods (Roger, 2001).

The diversity of the structural role of proteins in various food raw materials is illustrated by comparing protein structures in the muscle tissues of meat, fish and squid, the protein bodies of plant tissues such as cereals, legumes, oilseeds and shell (nut) fruits, and the casein micelle structure of bovine milk. Interactions of proteins with other components are exemplified in protein-starch interactions observed during dough processing and baking, protein-hydrocolloid interactions in dairy proteins in food processing products, protein-fat interactions in comminuted meat emulsions, mayonnaise and cheese, protein-water as well as protein-protein matrix interactions in fish surimi gels, yogurt and cheese (Yada, 2004).

According to Ebru *et al* (2010), the amount of protein was quantified using the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as the standard. The extraction method was assessed by the protein yield or the extraction rate, which was defined as the percentage ratio of the protein quantity extracted to the total amount of the protein in the tea leaf. A discontinuous SDS gel electrophoresis of 15% acrylamide was performed using a vertical mini-gel system of 0.75 mm thickness. The gel was prepared according to the method described by Laemmli (1970). Proteins of different molecular weights, b-lactoglobulin (18.4 kDa), lactate dehydrogenase (35.0 kDa), ovalbumin (45.0 kDa), bovine serum albumin (66.2 kDa) and galactosidase (116.0 kDa), were used as markers. The molecular weight of a tea protein was determined by comparing its migration distance with the standard markers. The amino acid analysis of tea protein was performed using a Hitachi 835-50 Amino Acid Analyser (Hitachi, Japan).

Besides that, other widely method use for the protein contain determination is The Lowry method. However, Pierce, 1996 (from the instruction manual) has developed a modified cupric sulphate-tartrate reagent that places two of the three reagents in the original established Lowry method with one stable reagent, thus, avoiding the necessity to prepare the fresh reagents daily. There is nearly a 100% correlation of the colour response curves with various proteins between the Pierce modified Lowry protein assay reagent and the original Lowry method.

Before protein assay were made, the Modified Lowry and Folin-Ciocalteu reagents must be prepared. The Modified Lowry reagent was prepared by adding the Reagent A (20 g Na_2CO_3 + 4 g NaOH dissolved in 1 liter distilled water) and Reagent B (2.5 g $CuSO_4.5H_2O$ + 5 g sodium citrate dissolved in 1 liter distilled water) in the proportion of 50:1. While the Folin-Ciocalteu reagent was prepared by diluting the supplied 2X reagent (2N) 1:1 with distilled water.

In this research, *Collocalia fuciphaga* is use as the raw material. *C. fuciphaga* species is popularly known as the White Nest or House Nest swiftlet. *C. fuciphaga* measures about 12 cm in its entire length and weighs about 15 to 18 gm. In recent years, hormone-like substances such as mitogen and avian epithelial growth factor have been found in *C. fuciphaga* (Jie *et al*., 2009).

The nests are cleaned by soaking them in water until the nest cement is softened and the tightly bound laminae partially loosen. Small feathers and fine plumage are then manually removed with tweezers with the cleaned strands subsequently being re-arranged and molded into chips of various shapes, air-dried, and packaged for sale around the world (Massimo, 2005). *C. fuciphaga* helps to stimulate cell division and growth and can enhance tissue growth and regeneration. They strengthen the body, moisturize the skin, maintain beauty, provide energy and enhance the metabolism of fat. The *C. fuciphaga* is adaptable for either sex or any age group (Jie *et al*., 2009).

It has been used for centuries whether as a tonic or a health food. However, its usage and benefits are mainly based on historical, anecdotal and observational reports. In Traditional Chinese Medicine (TCM), the nest is believe to offer good effect for treating consumptive diseases, curing tuberculosis, dry coughs, suppressing cough and phlegm-dyspnea (difficult breathing), alleviating asthma, hemoptysis (coughing blood), improving the voice, asthenia, stomach ulcer, relieving gastric troubles, and general weakness of bronchial ailments. It is also traditionally used to nourish the kidneys, lungs heart and stomach to aid renal functions, raise libido, strengthen the immune system, promote growth, enhance the immune system, improve concentration, increase energy and metabolism, and regulate circulation (Kong *et al*., 1987; Chan 2006).

Malaysia is currently the third largest producer of edible birds' nests (7% of gross supply value) in the world, behind Indonesia (60%) and Thailand (20%). A kilogram of unprocessed white edible birds' nests (around 90 to 120 nests) is able to fetch production level prices of RM$4500 to RM$6000 in 2006, with supply of white edible birds' nests being severely tight as compared to ever increasing levels of demand from consumer countries all over the world.

A kilogram of processed white edible birds' nests is able to fetch retail level prices of RM$15000 to RM$25000 in 2006 in Hong Kong and China. It has been hypothesized in their above publication that due to the following enduring qualitative reasons that the:

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

1.2 PROBLEM STATEMENTS

Taking care in a lifestyle is one of the important steps that need to concern in the daily life. Everyone has a different perspective on what a healthy lifestyle is, but it really comes down to practicing good health habits and giving up harmful ones.

With a healthy lifestyle, people can only get positive reinforcements out of it, such as feeling good; will have more energy, sleep better and be more relaxed, looking good; will have a nice toned body, strong muscles, bright eyes healthy hair and skin, and most important people will be happy and will have a better outlook on life.

Nowadays, there are some people that allergic with the protein extracted from the marine life and animal because it generally associated with high fat content and because of that, when consumed in large amounts, it attributed to high risks of disease, including high blood pressure and heart diseases. In an addition, there are many of the protein extracted are came from the porcine sources that cannot be used as a component of some foods, due to religious barriers.

Besides that, there is no research have been focus on the optimum value of temperature and liquid solid ratio of protein concentration from the *C.fuciphaga*.

1.3 OBJECTIVES

- The main objective of this research is to characterize and optimize protein extraction yield from *C.fuciphaga*.
  .
- In this research also have a few specific objectives. The specific objectives are:

    - To establish how temperature influence extraction protein yield
    - To establish how liquid solid ratio influence extraction protein yield
    - To optimize the extraction condition to produce higher extraction protein yield

1.4 SCOPES OF THE STUDY

- This research is focusing on the characterization of *C. fuciphaga* using the Fourier Transform Infrared Spectroscopy (FTIR) and inductively coupled plasma (ICP).
- Study the effect of two parameters, ratio of the C. *fuciphaga* with the distilled water (LSR) from 10:1 to 50:1 (v/w) and the temperature of the extraction from 25- 65^{o}C.

- Response surface methodology (RSM) is uses to determine the optimal condition of the extraction temperature and the ratio between distilled water and the *C.fuciphaga* for the protein extraction.

1.5 RATIONALE AND SIGNIFICANCE

Protein bioseperation, which refers to the recovery and purification of protein products from various biological feed streams, is considered to be one of the most important unit operations in the bioprocess industry. This is mainly because of the phenomenal development in the field of modern biotechnology and the increasing demand for more and more protein products to be purified in larger quantities (Daliya *et al*., 2007).

Previous studies by many researchers extract protein using pigeon pea (Ivone *et al*., 2000), germinant pumpkin seeds (Li *et* al., 2005), red pepper seed (*Capsicum frutescens*) (Ebru *et al*., 2010) and soybean (Rosenthal *et al*., 2001). There is no prior research that has been study about the optimum condition of extraction temperature and liquid solid ratio for the protein extracted from *C. fuciphaga*. Besides that, the sample is not from the marine life and animal source so it do not causes allergic to people and associated with high fat content. Therefore it is suitable for all ages including the pregnancy mother and the people that allergic with the protein extracted from the marine life. Besides that, this material is a halal product. So, it is suitable for Muslim and Vegetarian people.

CHAPTER 2

LITERATURE REVIEW

2.1 SWIFTLET FARMING INDUSTRIES IN MALAYSIA

The past 5 years ago, there is a new developments in the swiftlet farming industry and the industry is new in Malaysia as compared to other central and long-standing industries such as palm oil, oil and gas, timber, rubber and financial services. The swiftlet farming industry in Malaysia is a sudden becomes broader after the Asian Economic Crisis of 1997-1998. Many businesses, especially small to medium sized businesses, at that time experienced hard times and some of the business was close down. The places where the businesses reside were left clear due to the fact that no other businesses had sprung up to take their place as a result of the depressed economic environment at that time. Malaysia is currently the third largest producer of edible birds' nests (7% of gross supply value) in the world, behind Indonesia (60%) and Thailand (20%). (Hameed, 2007).

The swiftlet farming industry has a potential to bloom into a multi-million ringgit industry due to the industry's relatively profitable risk-return profile as well as a continuously growing request for edible birds' nests by the overseas countries. There is also a discernable world-wide trend pursued by international as well as home grown pharmaceutical and herbal products companies in using edible birds' nests as base materials for producing natural and organic health supplement products for local and overseas consumption. Currently, 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 (i.e. the white edible birds' nests swiftlets or the Aerodramus Fuciphagus species of swiftlets) that can only 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 (Hameed, 2007).

A continuous vocalization of swiftlet chirps and a mating sound are performed using speakers and audio systems installed every day 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. Almost 99% of all swiftlet farms in Malaysia are geared towards the production of white edible birds' nests (Hameed, 2007).

The swiftlet farming industry in Malaysia only started to gather momentum after the Asian Economic Crisis of 1997-1998. During that period, many businesses, especially small to medium sized businesses, experienced hard times and a great number of them closed down throughout the country. The premises that these businesses were located in were left empty due to the fact that no other businesses had sprung up to take their place as a result of the depressed economic environment at that time. Rather than leave their properties idle, quite a number of the landlords for these properties then had decided to convert their untenanted properties into swiftlet farms. At that time, there was only one research and development company specializing in the establishment of swiftlet farms in Malaysia and had almost single-handedly aided and helped grow the industry into becoming what it is today (Ibrahim *et al*., 2009).

Over the last 20 years, the swiftlet farming industry in the country basically grew through mostly private funding and operational initiatives and without any help whatsoever from the public sector. The major swiftlet farming areas are located mostly in secondary and tertiary townships where food source is in abundance 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 its essence, a swiftlet farm is a place in which edible white nests swiftlets mate, build their nests, raise their younglings and live in. Swiftlets had traditionally lived in caves. With their migration into the city and town centres through the years, these swiftlets will find places to live that are not dissimilar to that of their natural cave environment. Therefore, all swiftlet farmers have endeavored to design, construct and renovate their swiftlet farms in ways which will control the light intensities, humidity levels, air flow standards, pressure levels, safety perceptions, heat standards, odors and smells and swiftlet flight-paths in order to mimic swiftlet cave environments so as to encourage swiftlets to nest within the said farms.

Once a swiftlet farm has been completely constructed, swiftlet mating sounds and swiftlet chirps are played using audio systems through tweeters in order to ‘advertise’ to swiftlets flying above the new swiftlet farm that there is a new place for them to stay. These new swiftlets will then nest on wooden planks and lay eggs. Throughout the last 20 years or so, many technological advances, swiftlet farm design leaps as well as improvements in the behavioral characteristics of edible nests swiftlets have been made by the participants of the swiftlet farming industry in the areas of swiftlet farm design and construction, audio systems, mating and chirping sound identification and modulation, swiftlet flight-paths within farms and tweeter design (Hameed, 2007).

Many owners of swiftlet farms whose farms are located in close proximity to each other (within a 5km radius) are constantly trying to outdo their neighborly competition by implementing more and more scientifically researched and developed swiftlet farming products within their swiftlet farms in order to attract swiftlets from the surrounding competing farms into theirs. A stage has almost been reached whereby it is

now becoming more of a scientific endeavor of luring swiftlets to nest within a swiftlet farm as compared to the more unsuccessful and traditional ‘hit-or-miss’ method of swiftlet farming.

2.2 *COLLOCALIA FUCIPHAGA*

These birds (Figure 2.1) are found only in the Southeast Asian region. The nests are built almost exclusively by the 7–20 g male swiftlet over a period of approximately 35 days during breeding season. The salivary nest cement is the most important ingredient in the edible bird’s nest and is undoubtedly one of the most expensive food ingredients in the world. They construct their nests with a glutinous nest-cement produced by a pair of large, lobed salivary glands under the tongue. It is this nest-cement that constitutes the raw material of bird’s nest soup and renders the nest its commercial importance (Massimo, 2005).

Figure 2.1: Edible-nest Swiftlet

The nests are composed mainly of glycoprotein. The carbohydrate component consists of 9% sialic acid, 7.2% galactosamine, 5.3% glucosamine, 16.9% galactose,

and 0.7% fucose. The most abundant amino acids present are serine, threonine, aspartic acid, glutamic acid, proline, and valine. One study has shown the presence of a glycoprotein capable of promoting cell division, and another has demonstrated the presence of an epidermal growth factor–like protein (Denise *et al*., 2001).

Figure 2.2: *Collocalia Fuciphaga*

Harvesting of the edible bird's nest for human consumption is a painstaking and often times dangerous operation for local collectors. Most nests (Figure 2.2) are built hundreds of feet up on cave walls and require the use of temporary scaffolding made of locally collected bamboo or ironwood. After collection, the tedious process of cleaning approximately 10 nests takes a person approximately 8 h (Koon *et al.,* 2002).

Bird's nest is rich in protein. Protein is vital for tissue growth, maintenance and repair, muscle contraction and oxidation functions. Proteins are composed of 20 amino acids, 11 of which can be synthesized by human body (non-essential), and 9 have to be obtained through food (essential) (Table 2.2). Bird's nest contains 18 amino acids, including ALL 8 essential amino acids (Massimo, 2005). Practitioners of traditional Chinese medicine have consistently indicated that the consumption of birds' nest soup

is beneficial for the treatment of a variety of health problems. It is often administered to the elderly and very young alike who are recovering from various types of infections (Koon *et al.*, 2002).

Table 2.1: Nutritional Contents of Bird's Nest

Amino Acids in Bird's Nest (source: Massimo, 2005)	
Essential	**Non-Essential**
· Isoleucine	· Alanine
· Leucine	· Arginine
· Lysine	· Aspartic acid
· Methionine	· Cysteine
· Phenylalanine	· Glutamic Acid
· Threonine	· Glycine
· Tryptophan	· Histidine
· Valine	· Proline
	· Serine
	· Tyrosine
	· Asparagine

Due to the highly evaluated function both nutritiously (water-soluble protein, carbohydrate, iron, inorganic salt and fiber) and medically (anti-aging, anti-cancer, immunity-enhancing, etc). It was even referred as "Caviar of the East". Current scientific study confirmed that *C.fuciphaga* has haemagglutination inhibiting activity against the influenza virus and contained avian epidermal growth factor (Marcone, 2005; Lin et al., 2006). Nowadays with the help of modern commercialization and technology, *C.fuciphaga* was developed into various food products including drink, food additive, and even cosmetic ingredient. At present *C.fuciphaga* raw material mainly comes from Asian countries, such as China, Malaysia, Indonesia, Vietnam, Thailand, Philippine and most *C.fuciphaga* products were consumed in these areas as well as in North America. Trade scale of global market has been going up for decades. In order to improve the

quantity and quality of *C.fuciphaga* and to protect the natural environment of swiftlet residence, man-made bird house was built widely (Yajun *et al*., 2010).

It is considered as a great delicacy and effective medicine as well as beauty enhancer within the Chinese community throughout the world. The earliest history of recorded edible-bird nest trading can be traced back to the year 1589 (Yeap, 2002). Admiral Cheng Ho sailed to South East Asia and brought back edible-bird nests from Indonesia as a gift to the Ming Dynasty's Emperor which opened up the trade of this valuable nest. Some researchers have even stated that the trade can be traced back 1000 years ago during the Tang Dynasty (A.D. 618-907).

2.3 PROTEIN EXTRACTION

Proteins which have found applications in food and pharmaceutical industry may be produced as protein isolate or as protein concentrate. After many separation steps in aqueous extraction process, the bulk of proteins may be recovered as concentrate in the solid phase or as isolate in the aqueous phase depending on many parameters of the extraction medium(Tiezheng *et al*.,2010). It has been reported that the low extraction yields of aqueous processes can be overcome by using enzymes that hydrolyse the structural polysaccharides forming the cell wall of oilseeds, or that hydrolyse the proteins which form the cell and lipid body membranes(Rosenthal *et al*.,2001). Protein molecules tend to unfold and even become fully denatured under unfavoured conditions, such as a high temperature, an acidified condition, a high pressure or even excessive shear. Denatured protein molecules will aggregate and/or crosslink to form larger clusters and, at high concentrations, will form a three-dimensional solid-like network (or a gel). Therefore, proteins are regarded as one of the main classes of building blocks used in many semi-solid foods for conferring mechanical properties (Dickinson, 1997).

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main classes of building blocks used in many semi-solid foods for conferring mechanical properties. Proteins are also recognised as one of the main classes of surface-active agents in liquid foods for stabilising dispersed particles and fat droplets, due to the polarised distribution of hydrophobic and hydrophilic groups along the back bone. Protein molecules adsorb at the oil–water interface to lower the interfacial tension and, therefore, make such thermodynamically less favourable dispersed systems stable for an extended shelf life. The importance of protein application in foods can also be seen in many other aspects. For example, it was reported that, in combination with polysaccharides and starches, proteins could be applied as a meat alternative, as a fat replacer or a filler in manufacturing healthier food (Lianqing *et al*., 2008)

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Huge efforts have been made in extracting proteins from various sources for food applications. So far, proteins from two major sources (milk proteins and soy protein) are most widely used in the food industry, either as a general nutrients supply or as functional ingredients.Milk proteins (e.g., whey proteins or caseins) are probably the most commonly used proteins in all major types of food products. As from non-animal source, soy protein becomes increasingly used in food products because of its health benefits and characteristic physico-chemical properties (Endres, 2001). Proteins from other sources have also been studied for their functionality and potential food applications. Examples include corn protein (Myers *et al*., 1994), wheat protein (Hettiarachch *et al*., 1996), rice protein (Morita *et al*., 1996), seaweed protein (Fleurence, 1999), protein from yeast (Ganeva *et al*., 1999), fish protein (Afonso *et al*.,

2004), etc. However, the applications of these proteins were still very limited, either due to the limited source of supply or the non-satisfactory functionality.

Proteins also have special uses as foaming agents or as functional ingredients for nutrient delivery in foods (Chen *et al*., 2004). The ongoing development in protein extraction, purification and identification methods has significantly advanced our ability to address an increasing number of biological questions using proteomic approaches. Two-dimensional gel electrophoresis (2- DE) is one of the most widely used techniques for resolving complex protein extracts. The extraction of comprehensive protein populations from plants is particularly challenging due to the metabolic and structural characteristics of plant tissues, including the plant cell wall matrix. With the exception of mature and dormant structures, i.e., seeds and pollen, most plant cells have relatively low protein content, and are also rich in proteases and oxidative enzymes (Inder *et al*., 2009). Protein extraction from oat bran will increase the concentration of B-glucan or soluble fibre and in turn the value of oat by-product. The alkali method is reported to be the most commonly used procedure for protein extraction from oat flour (Xiao *et al*., 2008).

2.4 TYPES OF PROTEIN EXTRACTION

There are two types extraction used in the extraction of protein. There are the extraction using chemicals and enzymes.

2.4.1 Chemicals

The use of chemical has been widely recognised as a feasible method for protein extraction from plant sources. However, its effectiveness depends highly on the extraction conditions such as, agent concentration, extraction temperature, extraction time, volume–weight ratio between the extraction solvent and the raw material. Table 2.2 show the examples of chemicals used in the protein extraction.

2.4.2 Enzymes

Nowadays, some of researchers use enzymes in the extraction of protein Different enzymes may have different capabilities in extracting proteins because of their characteristic nature, as have been observed by many other researchers. In Table 2.3, it has been showed the examples of enzymes used in the protein extraction.

Table 2.2 Examples of chemicals used in the protein extraction

Chemical	Experimental	Reference
• Sodium hydroxide	• Extraction optimization of pigeon pea protein • Extraction optimization of germinant pumpkin seeds protein • Effect of Temperature, Alkali Concentration, Mixing Time and Meal/Solvent Ratio on the Extraction of Watermelon Seed Proteins—a Response Surface Approach	• Ivone *et al*.,2000 • Li *et* al., 2005 • Ali et al., 2008
• Deionized water	• Protein extraction optimization of red pepper seed (*Capsicum frutescens*)	• Ebru *et al*., 2010
• TCA–acetone	• Plant protein extraction methods with mass spectrometry for proteome analysis	• Inder *et al*., 2009
• Ethanol	• Extraction of protein from distiller's grain	• Drew J. C *et al*., 2008

Table 2.3 Examples of enzymes used in the protein extraction

Enzyme	Experimental	Reference
• Viscozyme L	• Extraction of oat bran protein	• Xiao *et al*., 2008
• Cellulase, hemicellulase, pectinase, protease	• Enzymatic extraction of oil and protein from soybean	• Rosenthal *et al*.,2001
• neutrase, alcalase, protamex and flavourzyme.	• Extraction of tea protein	• Lianqinq *et al*., 2008
• mascerase	• Protein extraction during production of varietally derived apple juices	• Beveridge *et al*., 1997
• Protease, amylase	• Protein Extraction from Heat-Stabilized Defatted Rice Bran. 1. Physical Processing and Enzyme Treatments	• Tang et al., 2002

• Alcalase, Nutrase, Protamex, Complex Cellulase	• Aqueous enzymatic extraction of peanut oil and protein hydrolysates	• Li hua *et al*., 2010

2.5 INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP-MS)

The inductively coupled plasma-mass spectrometer ICP-MS., consisting of an inductively coupled plasma ion source, a quadrupole mass analyzer, and a single electron multiplier, was developed and commercialized in the early 1980s and has since between widely adopted for trace element analysis. In principle, these instruments are also capable of isotopic analysis, but have not been widely used for such because the achievable precision is considerably poorer than thermal ionization mass spectrometry (TIMS) The poor precision results from the relatively poor peak shape and abundance sensitivity of quadrupole mass analyzers, small count rates, and the inherent instability of the plasma ion source. The limitations of the mass analyzer were overcome in the late 1980s by replacing the quadrupole with a magnetic sector mass analyzer (White *et al*., 2000).

According Eggins *et al* (1998), inductively coupled plasma mass spectrometry (ICP-MS) is an analytical technique which has undergone rapid development and seen application in diverse fields. As the argon ICP is an atmospheric pressure ion source, laser ablation sampling must also be carried out in an atmospheric pressure ambient gas in order to ensure contamination-free transport of the ablation products to the ICP. Under these conditions, expansion of the laser induced plasma above the ablation site is constrained by the ambient gas to a volume of only a few cubic millimeters. The dissipation of this plasma promotes redeposition of ablated material onto the sample substrate following the laser light pulse. This, together with other possible transport losses and condensation onto the sidewalls as the plasma plume exits the ablation hole, reduces the ablation yield and may influence measured element concentrations.

The replacement of the single detector with an array of Faraday detectors and the addition of an electrostatic energy filter aimed at focusing ions of different initial energy overcame the final obstacles to high precision isotopic analysis required by geochemists. In the last several years, multi-collector inductively coupled plasma-mass spectrometry. The instrument has also shown potential for elements conventionally

analyzed by TIMS. For example, Luais et al. (1997) showed that MC-ICP-MS could achieve precision comparable to TIMS in isotopic analysis of Nd.

Inductively coupled plasma mass spectrometry (ICP-MS) (Figure 2.4) is a type of mass spectrometry that is highly sensitive and capable of the determination of a range of metals and several non-metals at concentrations below one part in 10^{12} (part per trillion). It is based on coupling together inductively coupled plasma as a method of producing ions (ionization) with a mass spectrometer as a method of separating and detecting the ions. In trace elemental analysis, the method has advantages of high speed, precision and sensitivity compared to atomic absorption techniques. Analysis of lower concentrations at the same time is more prone to disruption by trace contaminants in labware and reagents used. Specific analytes suffer from interferences exclusive to ICP-MS technique. Verification of analysis results requires additional effort.

The variety of applications exceeds that of ICP-OES and includes isotopic speciation. Due to possible applications in nuclear technologies, ICP-MS hardware is a subject for special exporting regulations.

Figure 2.3: Inductively coupled plasma mass spectrometry (ICP-MS)

2.6 HEAVY METALS

Increasing industrialization has been accompanied throughout the world by the extraction and distribution of mineral substances from their natural deposits. Many of these have undergone chemical changes through technical processes and finally pass, finely dispersed and in solutions, by way of effluent, sewage, dumps and dust, into the water, the earth and the air and thus into the food chain. These include metals and thus also the heavy metals relevant for this document. Together with essential nutrients, plants and animals also take up small amounts of contaminant heavy metal compounds and can concentrate them (Kumar *et al*., 1995).

As certain heavy metals such as Lead, Cadmium, Mercury, Arsenic (Arsenic is usually regarded as a hazardous heavy metal even though it is actually a semi-metal) have been recognized to be potentially toxic within specific limiting values, a considerable potential hazard exists for human. Those metals are described as "heavy metals" which, in their standard state, have a specific gravity (density) of more than about 5 g/cm3. Some of them, such as copper, nickel, chromium and iron, for example, are essential in very low concentrations for the survival of all forms of life. These are

described as essential trace elements. Only when they are present in greater quantities, can these, like the heavy metals lead, cadmium which are already toxic in very low concentrations, cause metabolic anomalies. Here, the boundary between the essential and the toxic effect is somewhat problematic (Kumar *et al.*, 1995).

Essentially, the heavy metals have only become a focus of public interest since analytical techniques have made it possible to detect them even in very small traces. The relatively reckless handling of heavy metals and their compounds in former times can partly be explained by the fact that their effects were unknown. Today, analytical detection is possible down to a thousand of a mg/kg for certain matrixes. This has made it possible for toxicologists, in animal experiments, to follow up the effects of individual substances down to the smallest concentrations.

Heavy metals are chemical elements with a specific gravity that is at least 5 times the specific gravity of water. The specific gravity of water is 1 at 4°C (39°F). Simply stated, specific gravity is a measure of density of a given amount of a solid substance when it is compared to an equal amount of water. Some well-known toxic metallic elements with a specific gravity that is 5 or more times that of water are arsenic, 5.7; cadmium, 8.65; iron, 7.9; lead, 11.34; and mercury, 13.546 (Lide 1999).

Susan *et al* (1998) stated that heavy metal contamination exists in aqueous waste streams of many industries, such as metal plating facilities, mining operations, and tanneries. The soils surrounding many military bases are also contaminated and pose a risk of metals groundwater and surface water contamination. Some metals associated with these activities are cadmium (Cd), chromium (Cr), lead (Pb), and mercury (Hg). Heavy metals are not biodegradable and tend to accumulate in living organisms, causing various diseases and disorders.

Lead has been mined since ancient times and has been processed in many ways, e.g. for water pipes, containers and, as acetate, even for sweetening wine ("lead sugar"). World production amounts to millions of tons and is used in the manufacture of accumulators, solders, pigments, cables and anti-rust agents (red lead/lead oxide) and, a considerable amount still, into anti-knock petrol. The main sources of lead pollution in

the environment are: Industrial production processes and their emissions, road traffic with leaded petrol, the smoke and dust emissions of coal and gas-fired power stations, the laying of lead sheets by roofers as well as the use of paints and anti-rust agents. Problems for foodstuffs were caused for a long time, and are still caused today on occasion, by the soldered seams of cans and the soldered closures of condensed milk cans, the metal caps of wine bottles and, still, by lead pipes in drinking water systems (Susan *et al*.,1998).

Lead can trigger both acute and chronic symptoms of poisoning. Acute intoxications only occur through the consumption of relatively large single doses of soluble lead salts. Chronic intoxications can arise through the regular consumption of foodstuffs only slightly contaminated with lead. Lead is a typical cumulative poison. The danger of chronic intoxications is the greater problem. Basically, as a result of their comparatively high affinity for proteins, the lead ions consumed bond with the haemoglobin (red blood pigment) and the plasma protein of the blood. This leads to inhibition of the synthesis of red blood cells and thus of the vital transport of oxygen. If the bonding capacity here is exceeded, lead passes into the bone-marrow, liver and kidneys.

The routes of human exposure to aluminum are considered first. Drinking water and other dietary sources are examined in some detailed, and occupational exposures are also reviewed. Studies of aluminum absorption and mass balance are then summarized, with emphasis on the bioavailability of aluminum in the diet, drinking water, and auxiliary sources. Reported aluminum concentrations in body fluids and tissues, including blood, serum, plasma, urine, bone, and brain are critically evaluated to indicate which values in the literature can be considered with confidence for making comparisons between different groups of individuals. The toxicokinetics of aluminum excretion are also considered. In reviewing the evidence for the effects of aluminum on human health, bone disease and dialysis encephalopathy (DE) are examined in some depth, as well as the recent literature on occupational exposures. The epidemiological evidence with respect to aluminum in drinking water and dementia is formally treated as a subproject of the study and thus constitutes a research overview (Oxman and Guyatt 1988). A strict statistical meta-analysis was not justified by the quality of the

epidemiologic studies. Further, the aluminum-related epidemiological evidence is discussed in the context of the current status of the etiology of AD (Niebeor *et al*., 1995).

Arsenic is ubiquitous in the biosphere and occurs naturally in both organic and inorganic forms. While arsenic can be found to a small extent in the elemental form, the most important inorganic arsenic compounds are arsenic trioxide, sodium arsenite, arsenic trichloride (i.e. trivalent forms), and arsenic pentoxide, arsenic acid and arsenites, such as, lead and calcium arsenates (i.e. pentavalent forms). Common organic arsenic compounds are arsanilic acid, methylarsonic acid, dimethylarsinic acid (cacodylic acid), and arsenobetaine (AB). This latter compound (AB) is considered to be the most predominant organoarsenical in marine animals. Other, organoarsenicals including, arsenocholine, dimethyloxyarsylethanol, trimethylarsonium lactate, arsenic containing sugars and phospholipids have also been found in fish (Lau *et al.,* 1987; Friberg *et al.,* 1986). Although arsenic compounds were commonly used in the past as drugs, their main use today is as pesticides, veterinary drugs and in industrial applications, such as, the manufacture of integrated circuits and the production of alloys (Friberg *et al.,* 1986).

2.7 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTI-R)

Fourier Transform InfraRed (FTI-R) was preferred method of infrared spectroscopy. In infrared spectroscopy, IR radiation is passed through a sample (Figure 2.5). Some of the infrared radiation is passed through (transmitted) and some of it is absorbed by the sample. The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. Like a fingerprint no two unique molecular structures produce the same infrared spectrum. This makes infrared spectroscopy useful for several types of analysis (Mariey *et al*., 2001).

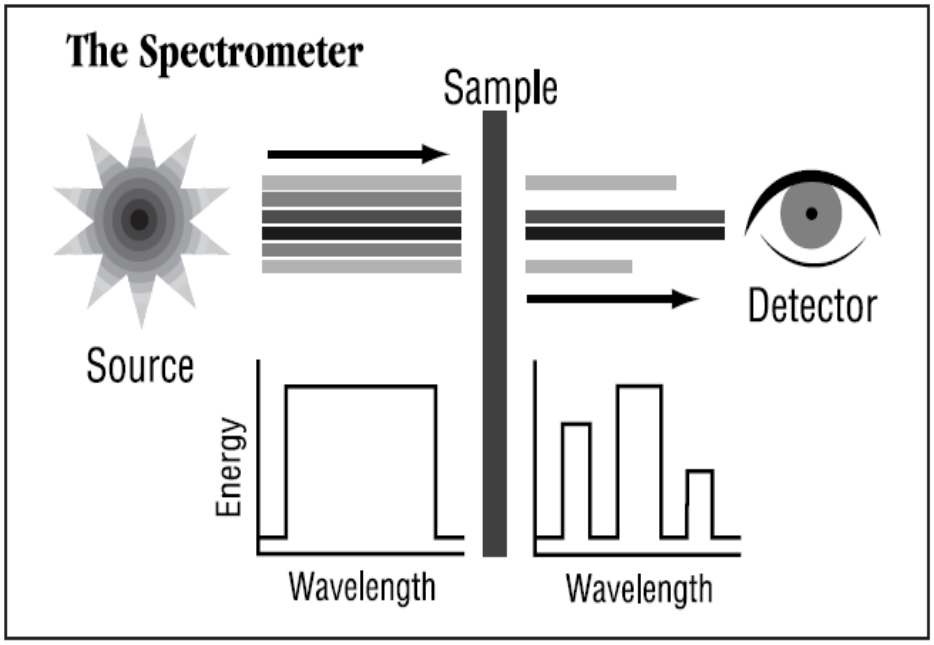

Figure 2.4: Mechanism for FTI-R

FTI-R can be used to identify unknown materials, determine the quality or consistency of a sampleand to determine the amount of components in a mixture. Infrared spectroscopy has been a workhorse technique for materials analysis in the laboratory for over seventy years. An infrared spectrum represents a fingerprint of a sample with absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. Therefore, infrared spectroscopy can result in a positive identification (qualitative analysis) of every different kind of material. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present. With modern software algorithms, infrared is an excellent tool for quantitative analysis (Salimi *et al.*, 2003).

The original infrared instruments were of the dispersive type. These instruments separated the individual frequencies of energy emitted from the infrared source. This was accomplished by the use of a prism or grating. An infrared prism works exactly the same as a visible prism which separates visible light into its colors (frequencies). A grating is a more modern dispersive element which better separates the frequencies of

infrared energy. The detector measures the amount of energy at each frequency which has passed through the sample. This results in a spectrum which is a plot of intensity vs. frequency (Mariey *et al*., 2001).

Fourier Transform Infrared (FT-IR) spectrometry was developed in order to overcome the limitations encountered with dispersive instruments. The main difficulty was the slow scanning process. A method for measuring all of the infrared frequencies simultaneously, rather than individually, was needed. A solution was developed which employed a very simple optical device called an interferometer. The interferometer produces a unique type of signal which has all of the infrared frequencies "encoded" into it. The signal can be measured very quickly, usually on the order of one second or so. Thus, the time element per sample is reduced to a matter of a few seconds rather than several minutes.

Most interferometers employ a beamsplitter which takes the incoming infrared beam and divides it into two optical beams. One beam reflects off of a flat mirror which is fixed in place. The other beam reflects off of a flat mirror which is on a mechanism which allows this mirror to move a very short distance (typically a few millimeters) away from the beamsplitter. The two beams reflect off of their respective mirrors and are recombined when they meet back at the beamsplitter. Because the path that one beam travels is a fixed length and the other is constantly changing as its mirror moves, the signal which exits the interferometer is the result of these two beams "interfering" with each other. The resulting signal is called an interferogram which has the unique property that every data point (a function of the moving mirror position) which makes up the signal has information about every infrared frequency which comes from the source (Signolle *et al*., 2001).

This means that as the interferogram is measured; all frequencies are being measured simultaneously. Thus, the use of the interferometer results in extremely fast measurements. Because the analyst requires a frequency spectrum (a plot of the intensity at each individual frequency) in order to make identification, the measured interferogram signal cannot be interpreted directly. A means of "decoding" the individual frequencies is required. This can be accomplished via a well-known

mathematical technique called the Fourier transformation. This transformation is performed by the computer which then presents the user with the desired spectral information for analysis (Figure 2.6).

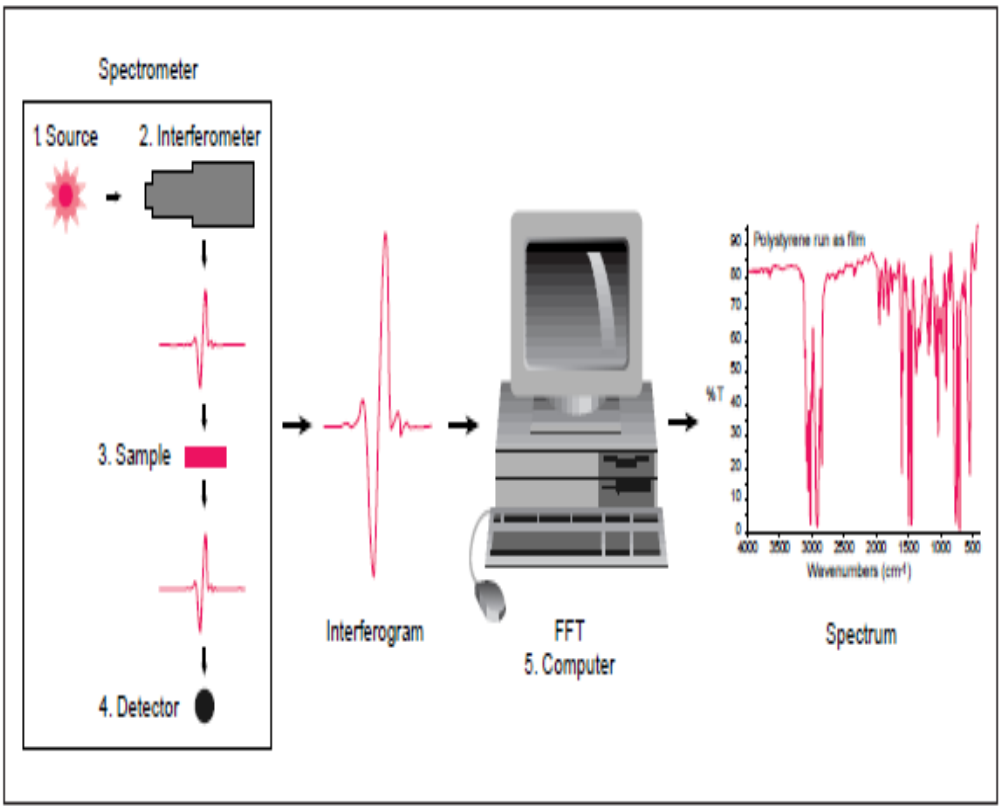

Figure 2.5: Result analyse by the computer

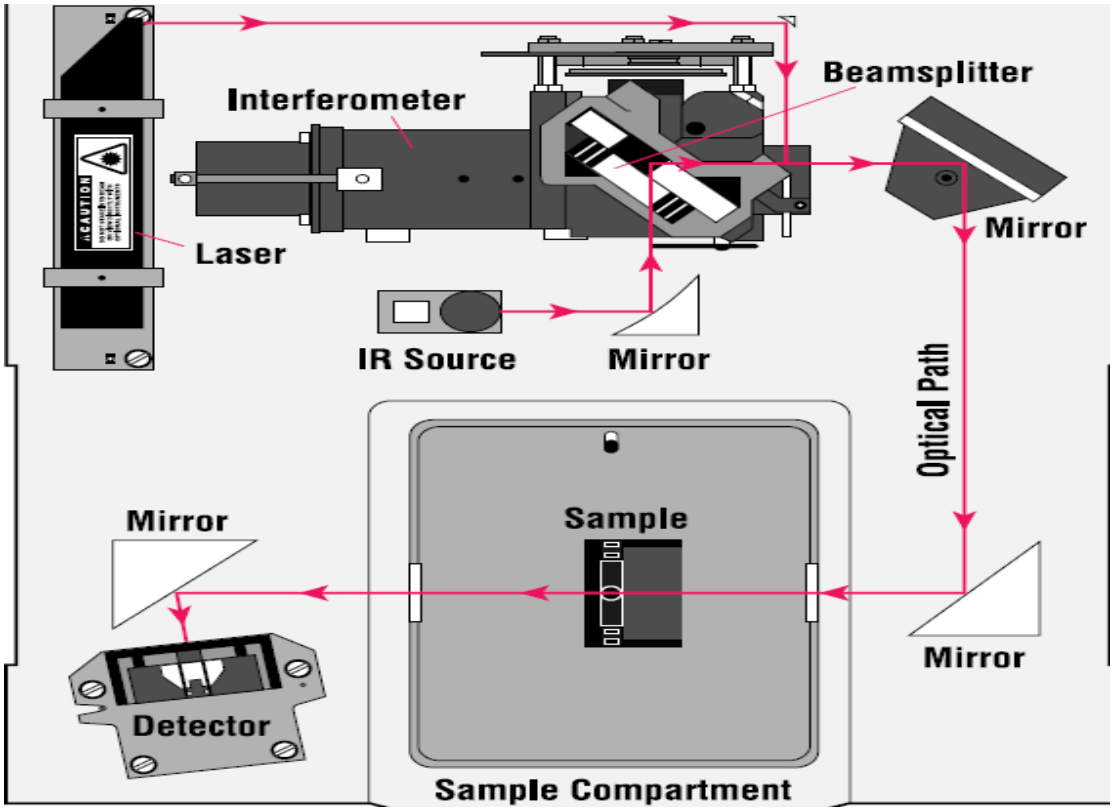

Figure 2.6: A Simple Spectrometer Layout

These advantages, along with several others, make measurements made by FT-IR extremely accurate and reproducible. Thus, it a very reliable technique for positive identification of virtually any sample. The sensitivity benefits enable identification of even the smallest of contaminants. This makes FT-IR an invaluable tool for quality control or quality assurance applications whether it is batch-to-batch comparisons to quality standards or analysis of an unknown contaminant. In addition, the sensitivity and accuracy of FT-IR detectors, along with a wide variety of software algorithms, have dramatically increased the practical use of infrared for quantitative analysis. Quantitative methods can be easily developed and calibrated and can be incorporated into simple procedures for routine analysis (Signolle *et al*., 2001).

Thus, the Fourier Transform Infrared (FT-IR) technique has brought significant practical advantages to infrared spectroscopy. It has made possible the development of many new sampling techniques which were designed to tackle challenging problems

which were impossible by older technology. It has made the use of infrared analysis virtually limitless.

According to Harberhauer *et al* (1998), FTIR is most useful for identifying chemicals that are either organic or inorganic. It can be utilized to quantitate some components of an unknown mixture. It can be applied to the analysis of solids, liquids, and gasses. The term Fourier Transform Infrared Spectroscopy (FTIR) refers to a fairly recent development in the manner in which the data is collected and converted from an interference pattern to a spectrum. Today's FTIR instruments are computerized which makes them faster and more sensitive than the older dispersive instruments. FTIR can be used to identify chemicals from spills, paints, polymers, coatings, drugs, and contaminants. FTIR is perhaps the most powerful tool for identifying types of chemical bonds (functional groups). Figure 2.8 is show an example for Infrared Spectrum of Silicon (Polydimethylsiloxane). The wavelength of light absorbed is characteristic of the chemical bond as can be seen in this annotated spectrum.

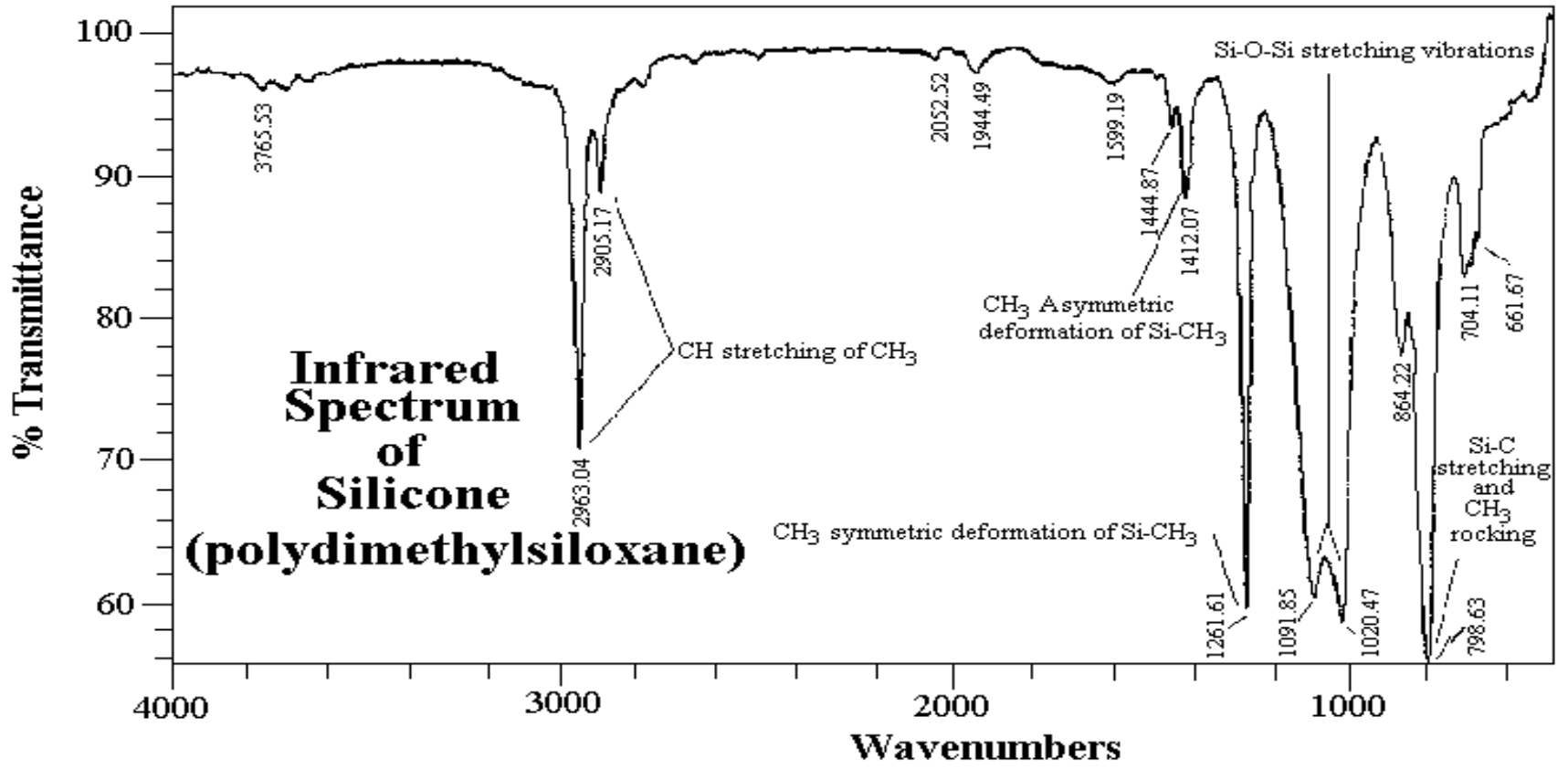

Figure 2.7: Infrared Spectrum of Silicon (Polydimethylsiloxane)

2.8 PROTEIN CONTENT DETERMINATION

According to Ebru *et al* (2010), the amount of protein was quantified using the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as the standard. The extraction method was assessed by the protein yield or the extraction rate, which was defined as the percentage ratio of the protein quantity extracted to the total amount of the protein in the tea leaf. A discontinuous SDS gel electrophoresis of 15% acrylamide was performed using a vertical mini-gel system of 0.75 mm thickness. The gel was prepared according to the method described by Laemmli (1970). Proteins of different molecular weights, b-lactoglobulin (18.4 kDa), lactate dehydrogenase (35.0 kDa), ovalbumin (45.0 kDa), bovine serum albumin (66.2 kDa) and galactosidase (116.0 kDa), were used as markers. The molecular weight of a tea protein was determined by comparing its migration distance with the standard markers. The amino acid analysis of tea protein was performed using a Hitachi 835-50 Amino Acid Analyser (Hitachi, Japan).

Besides that, other widely method use for the protein contain determination is The Lowry method. However, Pierce, 1996 (from the instruction manual) has developed a modified cupric sulphate-tartrate reagent that places two of the three reagents in the original established Lowry method with one stable reagent, thus, avoiding the necessity to prepare the fresh reagents daily. There is nearly a 100% correlation of the colour response curves with various proteins between the Pierce modified Lowry protein assay reagent and the original Lowry method.

Before protein assay were made, the Modified Lowry and Folin-Ciocalteu reagents must be prepared. The Modified Lowry reagent was prepared by adding the Reagent A (20 g Na_2CO_3 + 4 g NaOH dissolved in 1 liter distilled water) and Reagent B (2.5 g $CuSO_4.5H_2O$ + 5 g sodium citrate dissolved in 1 liter distilled water) in the proportion of 50:1. While the Folin-Ciocalteu reagent was prepared by diluting the supplied 2X reagent (2N) 1:1 with distilled water.

Lowry's method (1951) is applied to estimate the protein content in the extracted alkaline and acidic solution by using spectrophotometer. A reaction mixture

containing 0.7 mL Modified Lowry reagent and 0.5 mL sample was incubated at room temperature for 20 minutes. Distilled water was used as a blank. At the end of the incubation period, 0.1 mL of 1 N Folin-Ciocalteu reagent, which was bought from Merck Darmstadt Germany was added into the reaction mixture and left at room temperature for 30 minutes. The absorbance of the sample was measured at 750 nm. A standard curve was prepared by plotting the average blank corrected 750 nm reading for each BSA (bovine serum albumin) standard, which was purchased from Merck Darmstadt Germany versus its concentration in μg/mL. The protein concentration was determined for each sample using the standard curve.

2.9 RESPONSE SURFACE METHODOLOGY

According to (Li Q et al, 2005), response surface methodology (RSM) was employed to study the effect of liquid:solid ratio, NaCl concentration and reaction time on the production of protein from germinant pumpkin seeds. Furthermore, (Li .Q *et al*, 2005) also state that response surface methodology (RSM) is an effective tool for optimizing the process. 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.

Response surface experiments attempt to identify the response that can be thought of as a surface over the explanatory variables experimental space. It usually uses an experimental design such as central-composite experimental design (CCED) 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).

Other than that, response surface methodology is also a useful technique to investigation about several input variables which influence the performance and quality characteristics of the product or process under investigation. The technique provides mathematical and statistical procedures to study relationships between one or more

responses (dependent variables) and a number of factors (independent variables) (Diniz *et al*., 1996).

RSM has been used to optimize hydrocolloid extraction (Bostan *et al*., 2010; Cui *et al*., 1994; Furuta *et al*., 1998; Koocheki *et al*., 2008; Li *et al*., 2007; Razavi *et al*., 2009; Triveni *et al*., 2001; Wu *et al*., 2007) and to investigate the effect of the different extraction conditions on the gum obtained from different sources (e.g. Balke *et al*., 2000; Milani *et al*., 2007; Wang *et al*., 2006). Hydrocolloids extraction is influenced by a number of variables, such as temperature, water to seed ratio, pH, salts concentration, nature of the solvent, extraction time etc (Wu et al., 2007).

Response surface methodology (RSM) has also become a very popular and successful method for the optimisation of enzymatic hydrolysis processes with different substrates and proteases. Generally, RSM defines the effect of the independent process parameters, alone or in combinations, and generates a mathematical model that describes the overall process (Bas & Boyaci, 2007).

RSM enables information to be obtained at less cost and in a shorter time (Myers *et al*., 1995). According to (Ali *et al.,* 2008), RSM is a statistical technique that helps us in getting information with less cost and short time. This technique relates input and output parameters. Its use leads to rapid and efficient development of new/improved products or processes.

Ivone *et al* (2000) stated that the optimisation of maximum pigeon pea protein extraction was investigated using RSM, with three variables: NaCl concentration, liquid:solid ratio and pH. RSM is a statistical- mathematical method that is uses quantitative data in an experimental design to determine and simultaneously solve, multivariate equations and to optimise processes or products while (Ali *et al*., 2006) have stated in their journal that response surface methodology is one of a statistical technique that have been used to design the experiments that yield with the relevant information in the shortest time with the least cost. The basic principle of RSM is to relate the product properties with the regression equations that describe inter-relations between input parameters and product properties. Its use leads to the rapid and efficient

development of new and improved products and processes. In the present study, RSM was employed to standardise the parameters (temperature, alkali concentration, extraction time and solvent/meal ratio) for maximum protein extraction from watermelon seed meal.

Table 2.4 show the summary for the researches that have been focus on the optimization using response surface methodology.

Table 2.4: Summary table for Researches that involve Response Surface Methodology

TITLE	FINDING		REFERENCE
	PURPOSE	**VARIABLES**	
Response surface methodology for extraction optimization of pigeon pea protein	To optimisation of maximum pigeon pea protein extraction	• NaCl concentration • pH • liquid solid ratio (LSR)	Ivone *et al*., 2000
Application of response surface methodology for extraction optimization of germinant pumpkin seeds protein	To optimize and study the effect of the variables for the production of protein from germinant pumpkin seeds.	• Liquid solid ratio (LSR) • NaCl concentration • reaction time	Li *et al*., 2005
Extraction optimization of watermelon seed protein using response surface methodology	To maximize protein yield and to predict optimum conditions for maximum solubility of proteins from watermelon seed meal.	• pH • meal/solvent ratio	Ali *et al*., 2008

		• alkali concentration	
Response surface methodology for protein extraction optimization of red pepper seed (*Capsicum frutescens*)	To optimize the protein extraction process from red pepper seeds for maximum yield.	• Temperature • pH • extraction time • solvent/meal ratio	Ebru *et al.*, 2010
Response surface optimization of enzymatic hydrolysis of *Cistus ladanifer* and *Cytisus striatus* for bioethanol production	The enzymatic hydrolysis of rock-rose and broom was studied employing preliminary tests and experimental design as a statistical problem solving approach, as the Plackett–Burman method and a response surface methodology (RSM) of central composite Rotatable design (CCRD).	• pH • incubation time • temperature • cellulases concentration • polymer(PEG) concentration	Susana *et al.*, 2009
Optimization of Viscozyme L-	To study the effects of pretreatment	• Viscozyme L	Xiao *et al.*, 2008

assisted extraction of oat bran protein using response surface methodology	variables on protein extraction from oat bran.	concentration • pH • incubation time • temperature	
Combined effect of operational variables and enzyme activity on aqueous enzymatic extraction of oil and protein from soybean	To establish how process variables influence extraction yield especially when cell-wall degrading enzymes (mainly protease and cellulase) are added to aqueous extraction media.	• enzyme concentration • time of hydrolysis • particle size • liquid;solid ratio (LSR)	Rosenthal *et al*., 2001
Extraction optimization of a hydrocolloid extract from cress seed (*Lepidium sativum*) using response surface methodology	To give maximum yield of mucilage, viscosity and minimum protein content and to predict optimum conditions for extraction of the mucilage from seeds.	• temperature • pH • extraction time • liquid;solid ratio	Hojjat *et al*., 2010

		(LSR)	
Response surface methodology for optimisation of protein concentrate preparation from cowpea	to apply RSM to study the effect of protein extraction parameters on protein content, protein yield; reactive lysine and zinc content from cowpea, and to determine the optimum preparation conditions.	• pH • NaCl concentration	Martin *et al.*,2008

CHAPTER 3

METHODOLOGY

3.1 MATERIALS

3.1.1 *Collocalia fuciphaga*

Collocalia fuciphaga was choosing as the raw material for this research. The sample is composed mainly of glycoprotein. The carbohydrate component consists of 9% sialic acid, 7.2% galactosamine, 5.3% glucosamine, 16.9% galactose, and 0.7% fucose. The most abundant amino acids present are serine, threonine, aspartic acid, glutamic acid, proline, and valine. One study has shown the presence of a glycoprotein capable of promoting cell division, and another has demonstrated the presence of an epidermal growth factor–like protein (Denise *et al*., 2001). This sample was purchased from Pekan, Pahang.

3.2 METHODS

3.2.1 Characterization with Fourier Transform Infrared Spectrocopy (FTIR)

FTIR (Figure 3.1) was used to separate the individual frequencies of energy emitted from the infrared source. This was accomplished by the use of a prism or grating. An infrared prism works exactly the same as a visible prism which separates visible light into its colors (frequencies). A grating is a more modern dispersive element which better separates the frequencies of infrared energy. The detector measures the amount of energy at each frequency which has passed through the sample. This results in a spectrum which is a plot of intensity vs. frequency (Mariey *et al*., 2001).

Figure 3.1: Fourier Transform Infrared Spectrocopy (FTIR)

3.2.2 Prepare the standard protein curve

A different dilution of Bovine Serum Albumin (BSA) solutions was prepared (Table 3.1) by mixing the BSA with water in the test tube. The final volume in each of the test tubes is 5 ml. The BSA range is 0 to 1.0 g/L. The dilution was prepared by the following recipe at the table shown below:

Table 3.1: Dilution from the BSA solution (1.0 g/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	0.01	1.0

Before protein assay were made, the Modified Lowry and Folin-Ciocalteu reagents was prepared. The Modified Lowry reagent was prepared by adding the Reagent A (20 g Na_2CO_3 + 4 g NaOH dissolved in 1 liter distilled water) and Reagent B (2.5 g $CuSO_4.5H_2O$ + 5 g sodium citrate dissolved in 1 liter distilled water) in the proportion of 50:1. While the Folin-Ciocalteu reagent was prepared by diluting the supplied 2X reagent (2N) 1:1 with distilled water.

From these different dilutions, 0.2 ml protein solution was pipette out (Figure 3.1) to different test tubes and 1.0ml of the Modified Lowry reagent was added into the test tubes.

Figure 3.2: The BSA Solution was pipette out to the Test Tube

Figure 3.2 showed that the solutions were mix well. After that, the solutions were incubated at room temperature for 10 mins. At the end of the incubation period, 0.1 mL of 1 N Folin-Ciocalteu reagent (Figure 3.3), which was bought from Merck Darmstadt Germany was added into the reaction mixture and left at room temperature for 30 minutes.

Figure 3.3: The solution was mix well using the Vortex

Figure 3.4: The Folin-reagent was added to the solutions

After 30 minutes, the sample was analyzed using the UV-vis spectrophotometer in order to determine the concentration of protein in each of the sample. Distilled water was used as a blank. Zero the colorimeter with blank and take the optical density (measure the absorbance) at 743 nm. The absorbance of the sample was measured at 743 nm. A standard curve was prepared by plotting the average blank corrected 743 nm reading for each BSA (bovine serum albumin) standard, which was purchased from Merck Darmstadt Germany versus its concentration in g/L. The protein concentration of the unknown sample was determined for each sample using the standard curve.

3.2.3 Pre-treatment of *Colllocalia Fuciphaga*

The nests (Figure 3.4) were cleaned by soaking them in water until the nest cement is softened and the tightly bound laminae partially loosen. Then the sample was filtered out and the filtrate was removed. The small feathers and line plumage are then manually removed with tweezers and toothpicks (Figure3.5 & 3.6). The purpose is to make the nests as if it is first constructed by the swiftlet without impurity. The cleaned bird's nests are freeze-dried/dried using a fan. Throughout the process, no additives, bleaching agent and chemicals are adulterated into the bird nests (Massimo, 2005).

Figure 3.5: The nests

Figure 3.6: The Small Feathers Was Removed From Sample

Figure 3.7: Sample was being treated using the tweezer

3.2.4 Protein Extraction

There are two parameters that has been control in this research. The parameters are the ratio between the distilled water with the *C.fuciphaga* (LSR) and the extraction temperature. In this research, the one-factor-at-a-time method (or OFAT) was applied. The range of the ratio is between 1:10 – 1:50 and the extraction temperature is in the range 25 ^{O}C-65 ^{O}C (Table 3.2). The extraction is occurring inside the Erlenmeyer flask, of capacity 250ml and the flasks shaken in orbital shaker incubator (Figure 3.8) at 180 rpm for 1 hour (Rosenthal *et al*., 2001).

Table 3.2: Data for the one factor at a Time (OFAT)

LSR (w/v)	Temperature (^{o}c)
1:10	25
1:20	35
1:30	45
1:40	55
1:50	65

Figure 3.8: Samples inside the incubator shaker

3.2.5 Solid-liquid separation

Following extraction, the suspension was centrifuged under 3000 rpm for 15 min at 20°C in a Sorvall RC5C centrifuge (Figure 3.9). The precipitate will be removed, and the supernatant will be taken for moisture content determination (Rosenthal, *et al*., 2001).

Figure 3.9: Sorvall RC5C centrifuge

3.2.6 Determination concentration of protein

The sample was analysing using the UV-vis Spectrophotometer (Figure 3.10). The supernatant were pipette out to semi micro disposable cuvettes (Figure 3.11) and the concentration of the protein is measure by using the spectrophotometer (Lowry *et al*., 1951). The graph standard protein versus temperature will be plotted. After that, the new range of temperature and ratio between the distilled water and *C.fuciphaga* is taken from the graph.

Figure 3.10: UV-vis spectrophotometer

Figure 3.11: Samples That Need To Analyze

3.2.7 Response Surface Methodology

RSM will be used to find the effect of independent variables on the protein content (%). A face-centered central composite design was used with extraction temperatures and water to *C. fuciphaga* ratio. All experiments were conducted at three replicates. RSM was applied to the experimental data using a commercial statistical package: Design-Expert version 6.0.4. (Hojjat *et al*., 2010).

3.2.8 `Flow Diagram

Figure 3.12 was show the overall flow diagram of the methodology for this study.

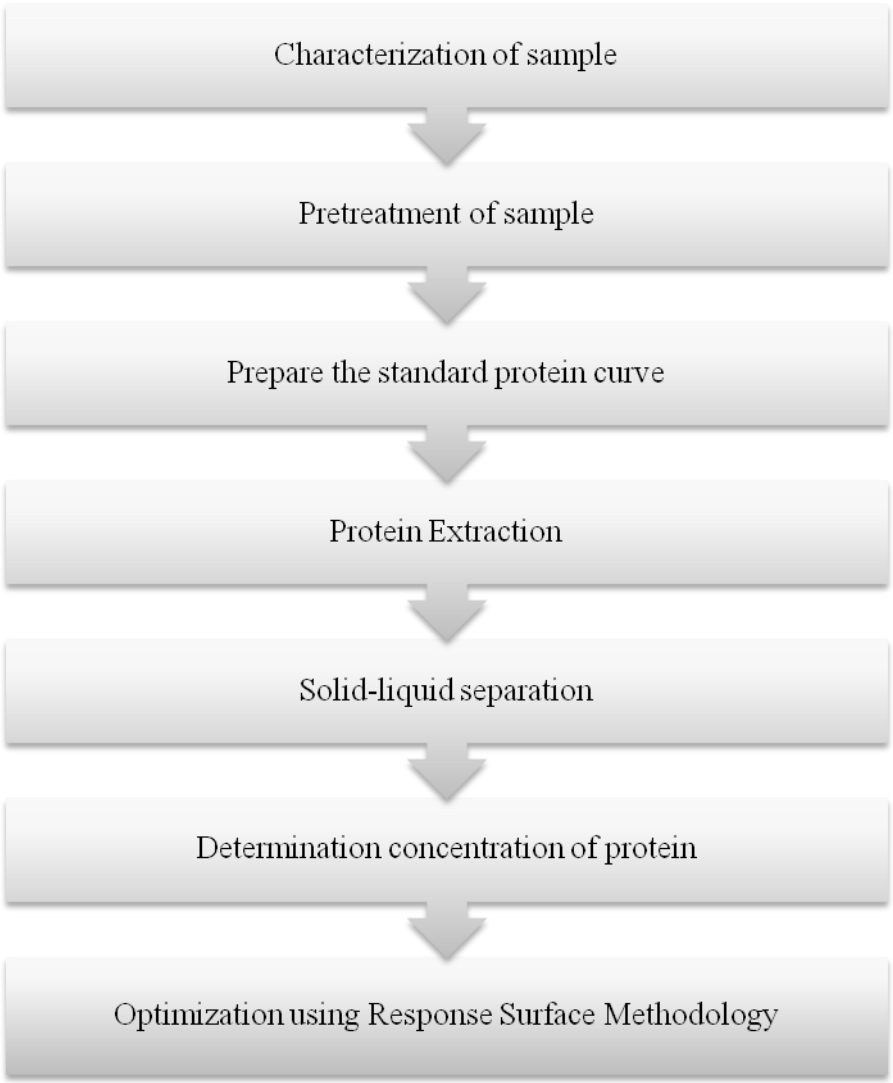

Figure 3.12: Flow diagram of the methodology

CHAPTER 4

RESULT 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 transforms infrared spectroscopy (FTIR)

The formation of functional group in the *Collocalia fuciphaga* was confirmed by FTIR spectroscopic analysis of the untreated and treated sample as shown in Figure 4.1. The FTIR spectrums of the untreated and treated sample were come out with the same trend of spectrum. This is due to the all the samples are come from same functional group. In the untreated sample, it was clearly shows that the absorption bands in the region of 3549 cm–1, 3450 cm–1, 1650 cm–1 and 1420 cm-1due to O-H stretching vibration, N-H stretching vibration, C=O stretching vibration, and C-H stretching vibration, respectively. These absorption bands are due to hydroxyl group in the samples and amide bonds in the samples due to the peptide bond of the protein (Anchouri *et al*., 2011). The four-atom functional group -C (=O) NH- is called a peptide link. The peptide bonds in proteins are metastable, meaning that in the presence of water they will break spontaneously.

Then, the absorption band at treated sample show at O-H which shifted towards 3500 cm–1 and at C-H the absorbance shifted towards into 1380cm–1 respectively. It

can be seen that, the amide peak C=O at 1650 cm–1 was slightly shifted towards 11645 cm–1 and the N-H peak at 3450cm-1 was slightly shifted towards 3400cm-1 in the spectra of treated sample because the amide bonds and peptide link in the sample was break due to the extraction process (Ahmad *et al.*, 2011). All the difference happens due to the treatment onto the sample.

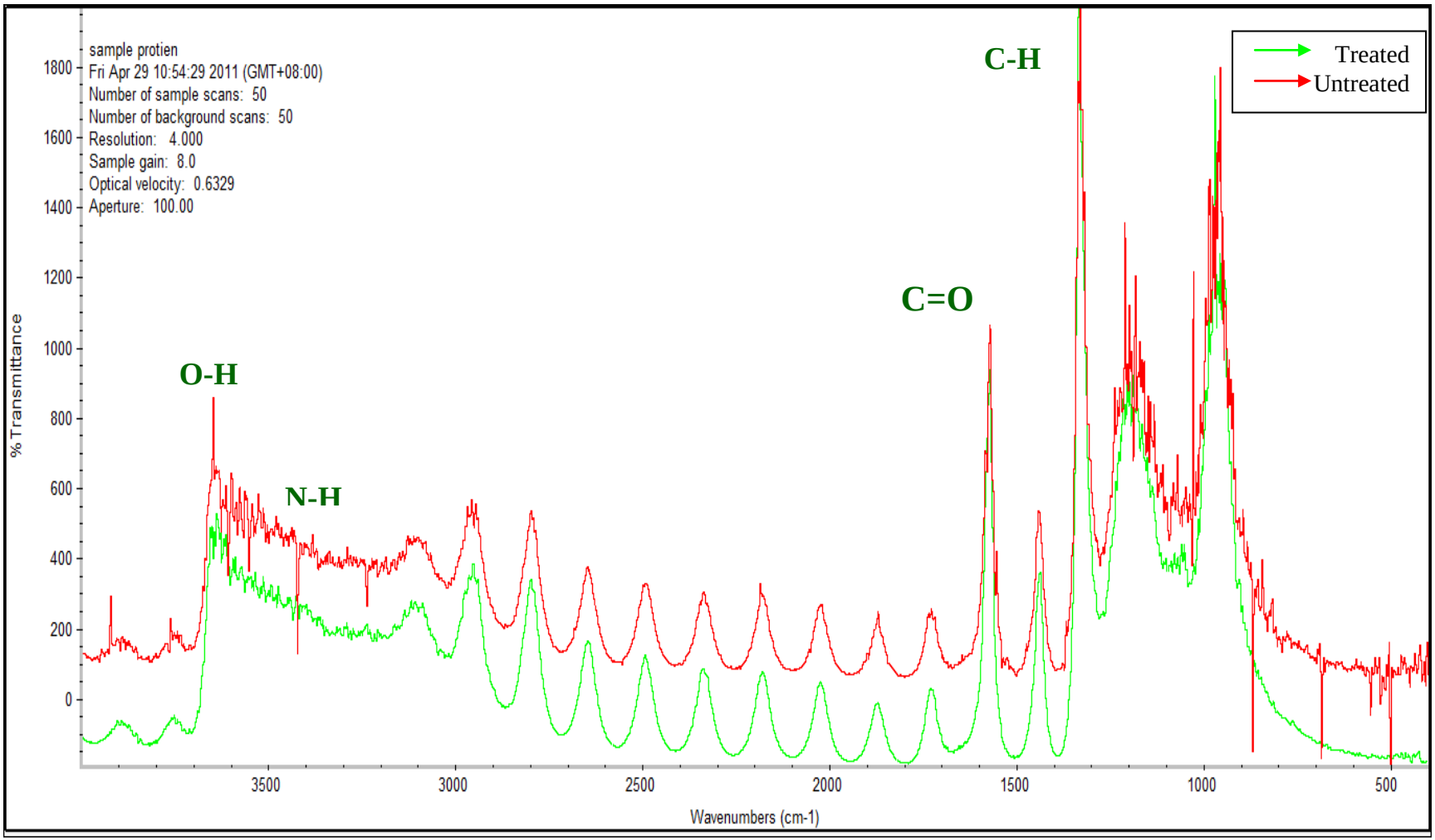

Figure 4.1: Result from FTIR analysis for treated and untreated sample

4.2.2 Inductively Coupled Plasma (ICP)

From Table 4.1, aluminium concentration in the sample was 2.378 mg/L. Aluminium is the third most abundant element (after oxygen and silicon), and the most abundant metal, in the Earth's crust. An average person has a daily intake of 7mg/L (Lide 1999) of aluminium. In very high doses, aluminium can cause neurotoxicity, and is associated with altered function of the blood-brain barrier.

In any event, if there is any toxicity of aluminium, it must be via a very specific mechanism, since total human exposure to the element in the form of naturally occurring clay in soil and dust is enormously large over a lifetime. Scientific consensus does not yet exist about whether aluminium exposure could directly increase the risk of Alzheimer's disease. Therefore, we can conclude that this sample does not have a high concentration of aluminium that can give a health effect if we consume it into our body. Therefore, aluminium concentration contain inside the samples was save for us to consume into our body (Niebeor *et al*., 1995).

Arsenic is a naturally occurring chemical element found throughout our environment and its living systems. Arsenic can enter groundwater through erosion and weathering of soils, minerals, and ores. Arsenic compounds are used in the manufacture of a variety of products and may enter our environment directly from industrial effluents and indirectly from atmospheric deposition. From the table shown above, the value for arsenic concentration in this sample was 0.044 mg/L. With this concentration of arsenic; it was not give a negative health effect to our body because it will give an effect if we consumed it above a level of 2 mg/L (Lau *et al.,* 1987).

Arsenic exists in different chemical forms, which can be classified into two groups: organic arsenic and inorganic arsenic. Inorganic arsenic is considered to be the most toxic to human health, while organic arsenic is considered to be non-toxic. Both organic and inorganic forms of arsenic can be found in food. While the levels of each depend on the type of food, inorganic arsenic is not usually found at high levels. Higher levels of arsenic are generally found in fish and shellfish, but in the organic form, which is not of concern to human health (Lau *et al.,* 1987).

According to Friberg *et al* (1986), long-term exposure (over many years or decades) to high levels of inorganic arsenic is known to contribute to the risk of human cancer and can affect the gastrointestinal tract, kidneys, liver, lungs and epidermis. Short term exposure (days/weeks) to very high levels of inorganic arsenic can also cause various health effects including skin effects, nausea, diarrhea, vomiting and numbness in hands and feet.

Lead is the most significant of all the heavy metals because it is both very toxic and very common. It is a poisonous metal that can damage nervous connections (especially in young children) and cause blood and brain disorders. From the table above, the value of the lead concentration inside the sample was 0.125 mg/L. the maximum value of lead concentration that we can consume daily is 3.402 mg/L. Therefore, this sample is safe and not brings a negative effect to our health.

Susan *et al* (1998) have stated in their study that long-term exposure to lead or its salts (especially soluble salts or the strong oxidant PbO_2) can cause nephropathy, and colic-like abdominal pains. The effects of lead are the same whether it enters the body through breathing or swallowing. Lead can affect almost every organ and system in the body. The main target for lead toxicity is the nervous system, both in adults and children. Long-term exposure of adults can result in decreased performance in some tests that measure functions of the nervous system. It may also cause weakness in fingers, wrists, or ankles. Lead exposure also causes small increases in blood pressure, particularly in middle-aged and older people and can cause anemia. Exposure to high lead levels can severely damage the brain and kidneys in adults or children and ultimately cause death. In pregnant women, high levels of exposure to lead may cause miscarriage.

Table 4.1: Result of concentration f heavy metals contain in the sample

NO	PARAMETER	RESULTS	UNIT
1	ALUMINIUM(Al)	2.378	mg/L
2	ARSENIC (As)	0.044	mg/L
3	LEAD(Pb)	0.125	mg/L

4.3 ONE FACTOR AT TIME

4.3.1 Temperature

Extraction temperature is one of the parameters that influence in the production of protein concentration. In this study, temperature ranging from 25 to 65 ^{o}C was used for protein extraction from *C.fuciphaga*. The effect of extraction temperature on the protein concentration is shown in Fig.4.2. In Fig 4.2, a low extraction temperature (below 35^{o}C), attributes to larger amount of protein concentration produced. This phenomenon was due to the protein denaturation due to high temperature. Denaturation occurs because the bonding interactions responsible for the secondary structure (hydrogen bonds to amides) and tertiary structure are disrupted. In tertiary structure there are four types of bonding interactions between "side chains" including: hydrogen bonding, salt bridges, disulfide bonds, and non-polar hydrophobic interactions which may be disrupted. Therefore, protein molecules aggregate and the solubility of protein decrease due to the exposure and expansion of hydrophobic groups, thus the yield of protein concentration decrease.

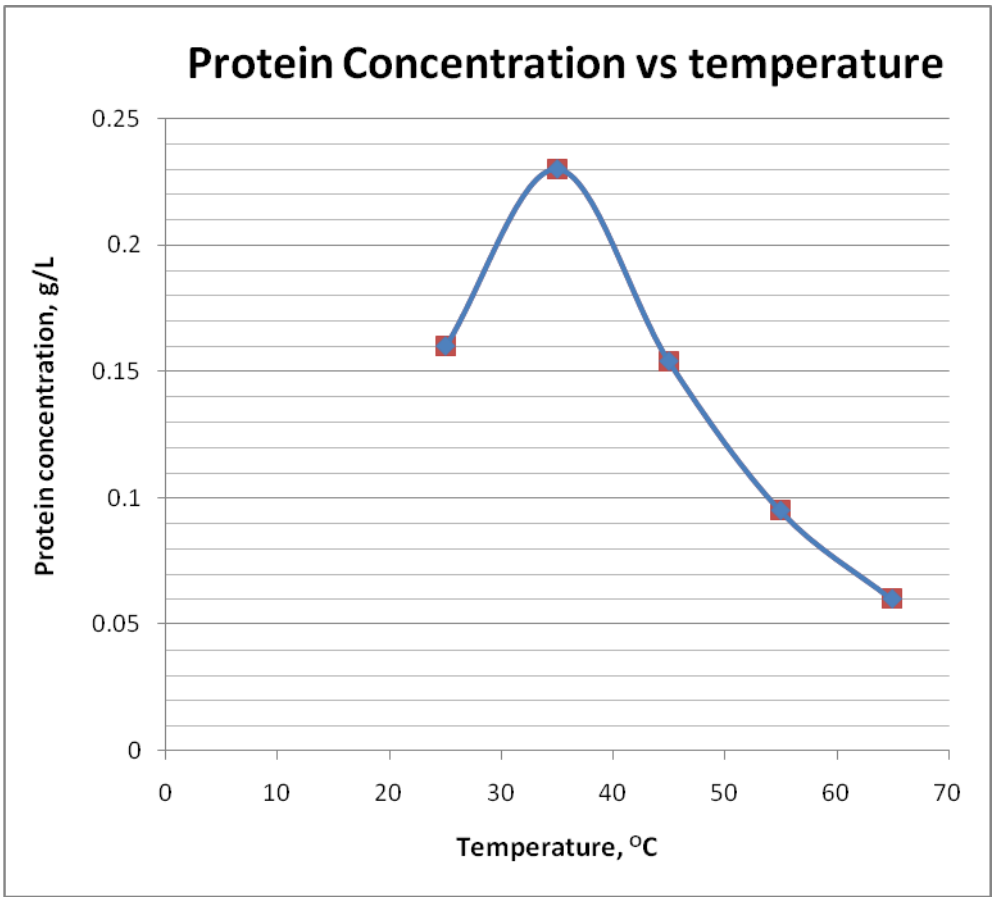

Figure 4.2: Graph of protein concentration versus temperature

4.3.2 Liquid Solid ratio (LSR)

In order to study the effect of liquid; solid ratio (v/w) on the protein concentration, 10:1-50:1 of ratio were tested at 35 ^{o}C of extraction temperature and the result are shown in Fig 4.3. In this figure, the protein concentration for the 10:1-40:1 of the liquid: solid ratio increase rapidly after an hour of extraction time. This is due to the concentrated of protein inside the samples. At the 50:1 ratio, the protein concentration was decrease rapidly and this is due to the larger quantity of water in the samples. Therefore, the protein concentration was lower.

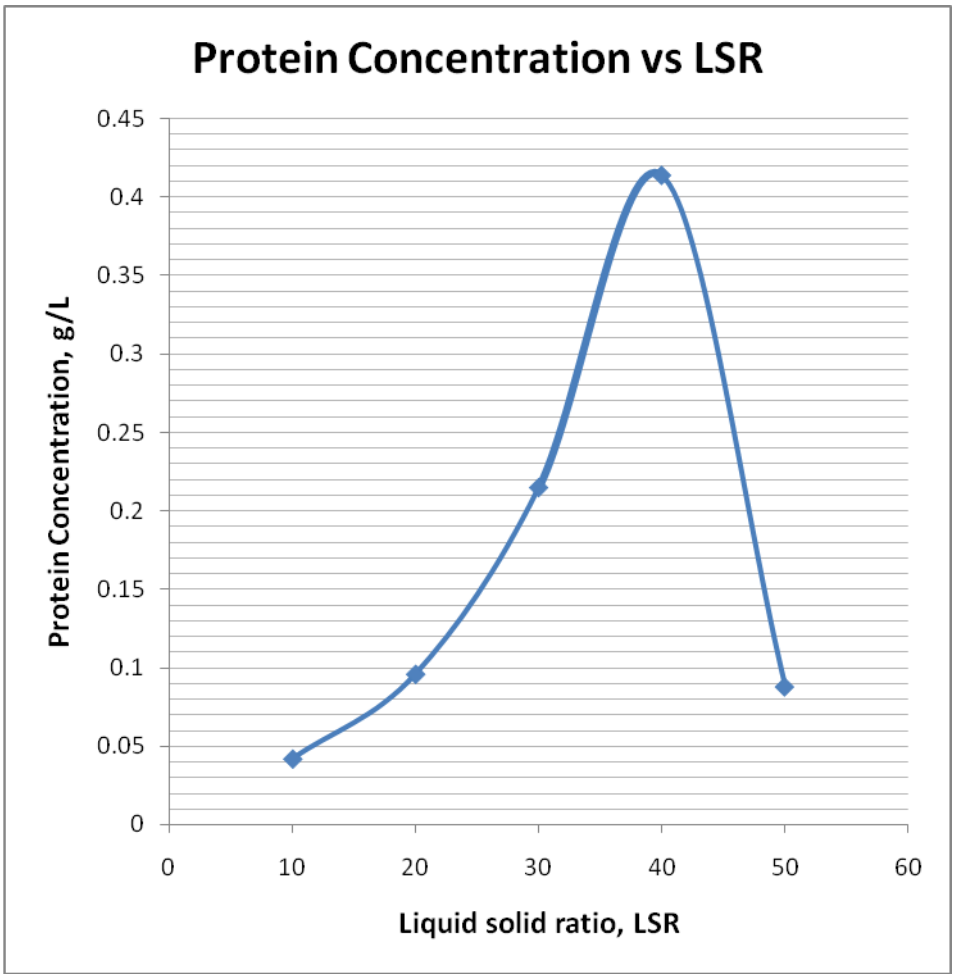

Figure 4.3: Graph of protein concentration versus liquid: solid ratio

4.4 OPTIMIZATION USING RESPONSE SURFACE METHODOLOGY (RSM)

In order to produce high concentration of protein concentration, two factors were carried out in this study. The factors are extraction temperature and liquid; solid ratio. Apparently, the multiple regression equation for the protein concentration (Y) after the extraction temperature (x_1) and liquid; solid ratio (x_2) as the main variables was as follows:

$$Y= +0.35 - 0.060x_1 + 4.583E\text{-}03x_2 - 4.642E\text{-}03x_1^2 - 1.642E\text{-}03x_2^2 +3.250E\text{-}03\ x_1x_2 \quad (1)$$

According to Eq. (1), the largest value of estimated regression coefficient for protein concentration was the extraction temperature (x_1 = -0.06). Thus, it is worth noting that the extraction temperature was the prime factor, which had the greatest effect on the protein concentration. This could be again due to the protein denaturation at higher temperature. Denaturation of proteins involves the disruption and possible destruction of both the 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 0.8380 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 83.8% of the total variation in the protein concentration is explained by the fitted model [Eq. (1)]. Earlier studies have reported R^2 ranging from 71.00%- 95.20% (Mizubuti *et al*., 2000; Sogi *et al*., 2003; Shahidi, 1996) for flaxseed, pigeon pea and tomato seed. Based on Table 4.2, 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 indicates adequate model discrimination. The analysis of variance also showed that there was a non-significant lack of fit that further validates the model. Moreover, in this study, the statistical analysis of variance was tabulated in Table 4.2.

The F-value is the ratio of mean square due to the regression to the mean square error. The F-value of the protein concentration is 7.24, which is greater that the tabulated $F_{7,10}$, the null hypothesis is rejected at the a level of significance, and infers that the variation accounted for by 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(<0.001) indicated that the model terms were significant. Furthermore according to Table 4.2, A was a significant model. The lack of fit F-value of 0.35 implied that the lack of fit was not significant.

Results from CCD clearly demonstrated that protein concentration increases when the temperature increase from 32 to 41^{o}C and the liquid: solid ratio from 39:1 to 45:1. As the matter of fact, 38^{o}C of extraction temperature and 42:1 of liquid; solid ratio resulted in highest protein concentration. The extraction temperature was the most significant factor affecting the protein concentration (F-value= 35.56).

On the other hand, increase in liquid;solid ratio indicates insignificant effect to the degradation of protein and subsequently produced low yield of protein. However, this finding was found to contrast with the results obtained by Ivone *et al.,* 2000 who reported that the liquid;solid ratio was found to be significant to the protein concentration yield.

Table 4.2: Analysis of Variance Table (partial sum of squares) for response surface Model

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	0.044	5	8.774E-03	7.24	0.0109	**significant**
A	*0.043*	1	*0.043*	*35.56*	*0.0006*	
B	*2.521E-04*	1	*2.521E-04*	*0.21*	*0.6621*	
A^2	*4.938E-04*	1	*4.938E-04*	*0.41*	*0.5435*	
B^2	*6.180E-05*	1	*6.180E-05*	*0.051*	*0.8278*	
AB	*4.225E-05*	1	*4.225E-05*	0.035	0.8572	
Residual	*8.480E-03*	7	*1.211E-03*			
Lack of Fit	*1.744E-03*	*3*	*5.814E-04*	*0.35*	*0.7956*	**not significant**
Pure Error	*6.736E-03*	*4*	*1.684E-03*			
Cor Total	*0.052*	*12*				
Std. Dev.	0.035		**R-Squared**		0.8380	
Mean	0.34		**Adj R-Squared**		0.7223	
Adeq Precision	10.136		**Pred R-Squared**		0.5493	

The confirmation run was performed in order to validate the adequancy of the model and the result is tablulated in Table 4.3. the first condition of the confirmation run was taken from the optimum experimental condition which was recommendation by the software, while the other conditions for the last three confirmation experiments were among the preparation conditions which were applied previously. The predict values and the actual experimental values were compared and the percentage error were calculated. The precentage eror between the actual and predicted value for protein concentration was up to 3.653%.

In other words, the experimental values were found to be very close to the predicted values and thus, the model was succesfully validated. Therefore, it can be concluded that the empirical model developed was reasonably accurate for the protein concentration as all actual values for the confirmation runs were within the 95% prediction interval. The 95% prediction interval is the range in which there is an expectation for any value to fall into 95% of the time.

Table 4.3 : Confirmation Run

Temperature (^oC)	**LSR (v:w)**	**Protein Concentration (g/L)**		
		Actual (g/L)	Predict (g/L)	Error (%)
38	42:1	0.354	0.348	1.695
41	39:1	0.219	0.211	3.653
35	45:1	0.332	0.321	3.313
32	42:1	0.243	0.238	2.058

Variation in temperature and LSR revealed that maximum protein extraction was obtained when the temperature was 38^{o}C and when LSR was 42:1 (v/w), respectively (Figure 4.4 and Figure 4.5). previous studies on extraction of melon seed kernel protein isolates (Khalil, 1998), revealed a significant increase in the protein yield up to 30:1 ratio which is lower than the present study; however, studies on the protein extraction of flaxseed and deoiled tomato seed meal are in agreement with the present study (Oomach *et al*., 1994; Sogi *et al*., 2003). The difference in the reported may b due to differences in the raw materials used, type of equipments used and other conditions used in the study.

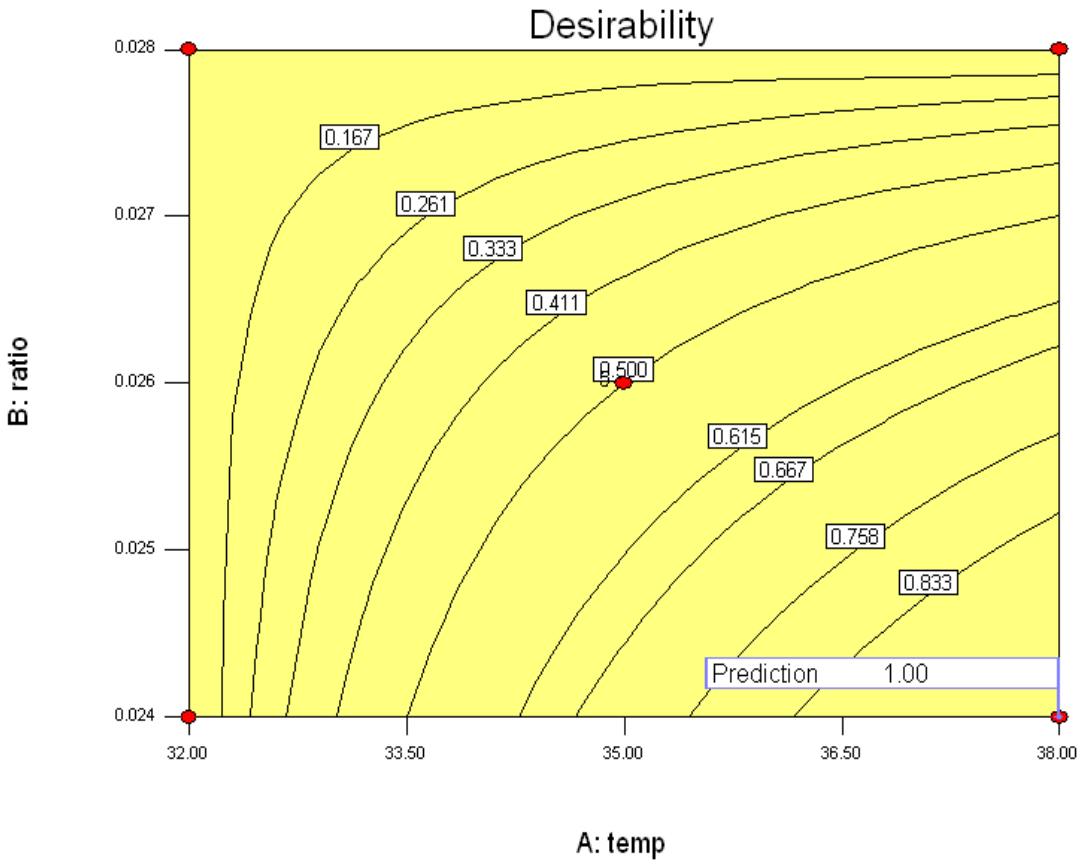

Figure 4.4: Effect of temperature and LSR on protein yield extracted from *Collocalia fuciphaga*

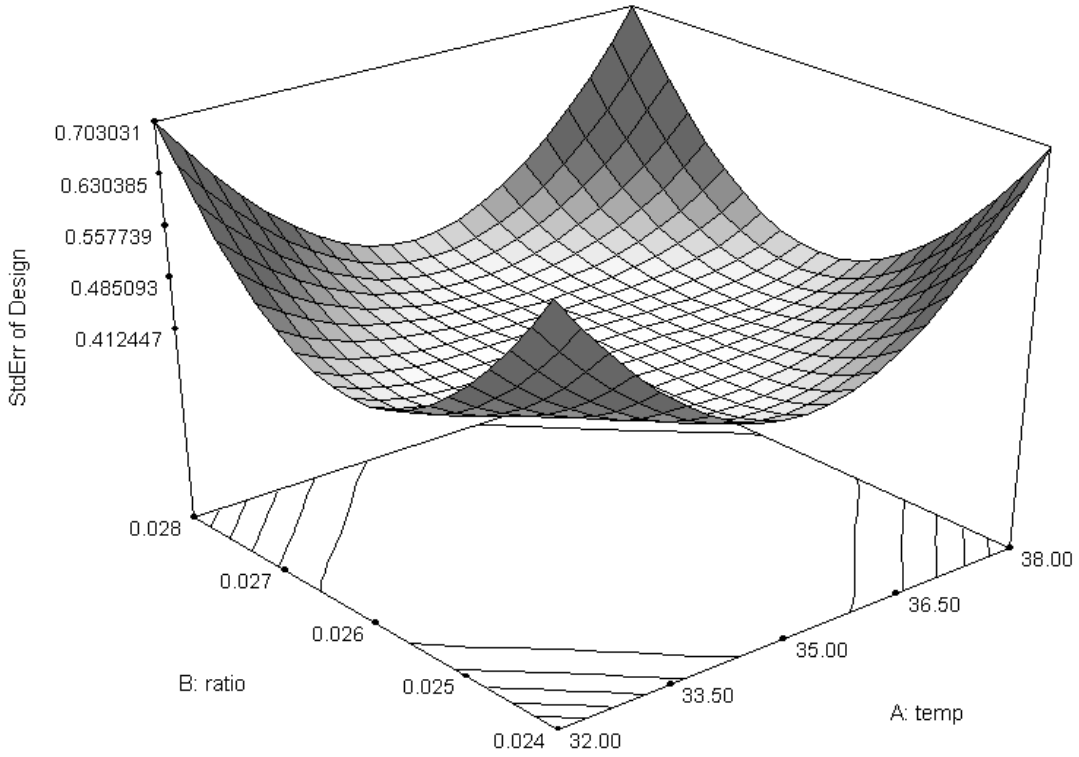

Figure 4.5: Surface plot for protein yield of *Collocalia fuciphaga*

Significances of effects and interactions of factors on response were further diagnosed and compared, and graphically illustrated in the normal plot (Figure 4.6), where results of dominating effects that are likely to represent the important and influential factors were found consistent with the ANOVA analysis results. Moreover the significance of interactions between factors on the response can be best considered using interaction analysis graph of Figure 4.7. It has been observed that the interaction effect between temperature and LSR depicts remarkable improvement in protein concentration as the decreasing of temperature and LSR.

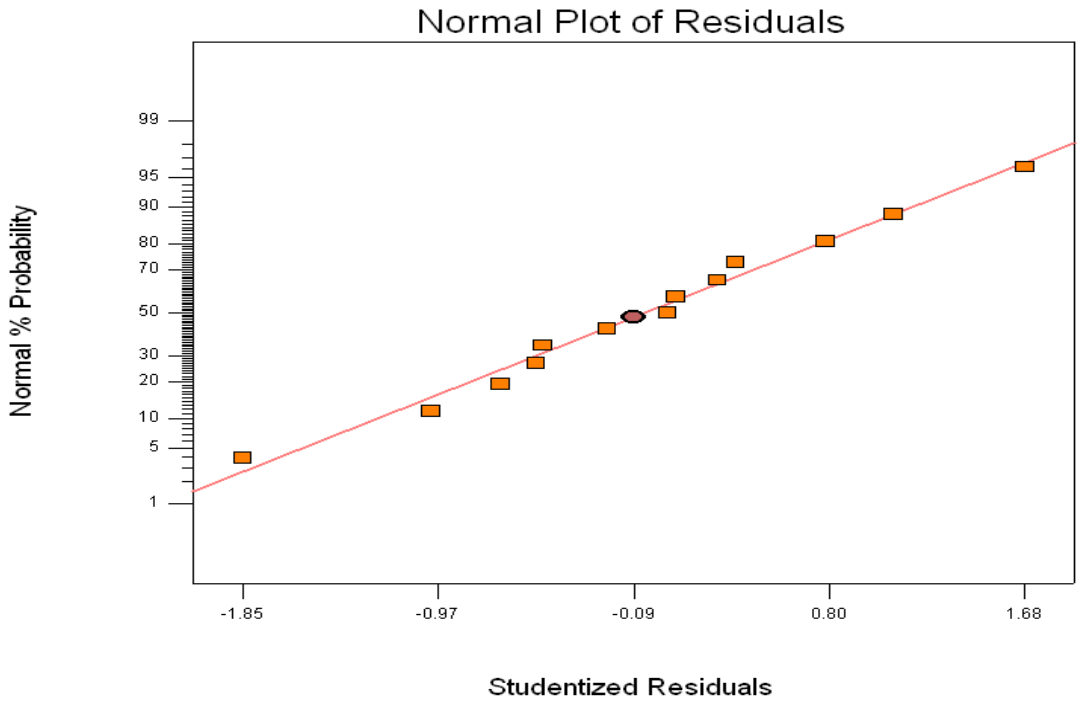

Figure 4.6: The Normal Plot of Residuals

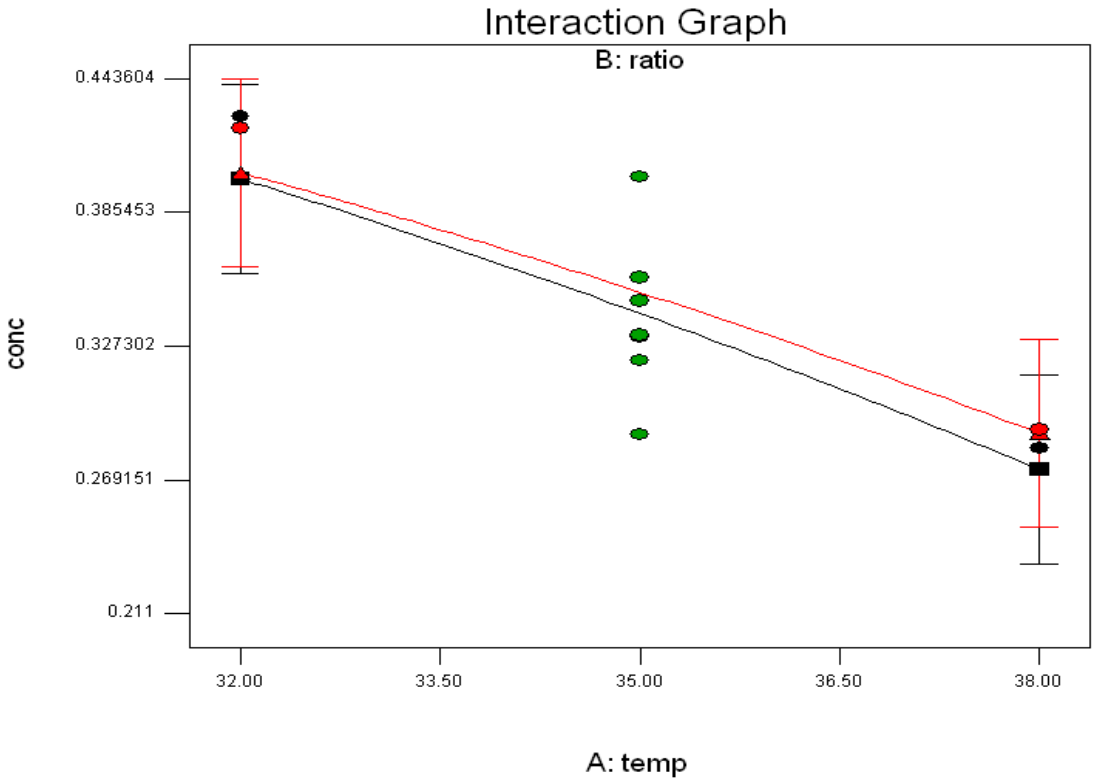

Figure 4.7: Interaction graph for the response

Analyses on normal probability plot of residuals (Figure 4.6) depicted nearly a straight line residuals distribution, which denoting errors are evenly distributed and therefore support adequacy of the least-square fit, while results illustrated in Figure 4.8, 4.9 and 4.10 revealed that the models proposed are distinctively adequate and reasonably free from any violation of the independence or constant variance assumption, as studentized residuals are equally tabulated within the red line of the x-axis.

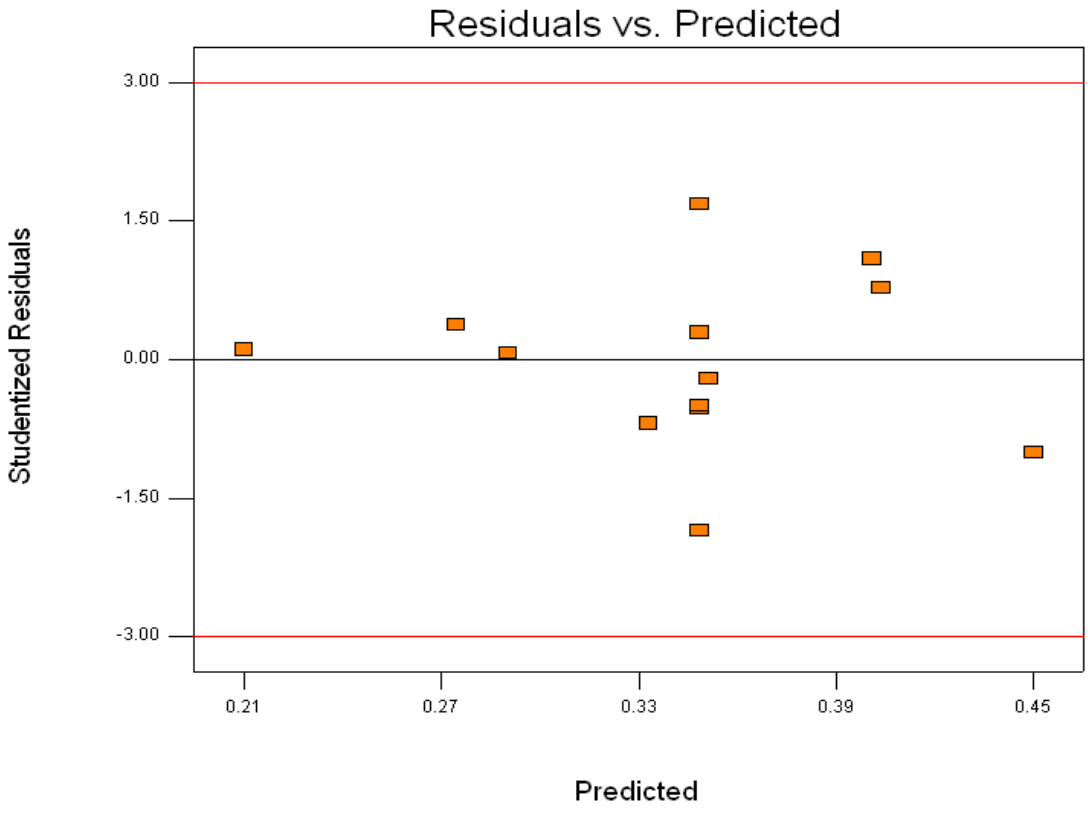

Figure 4.8: Plot of Residuals against predicted response

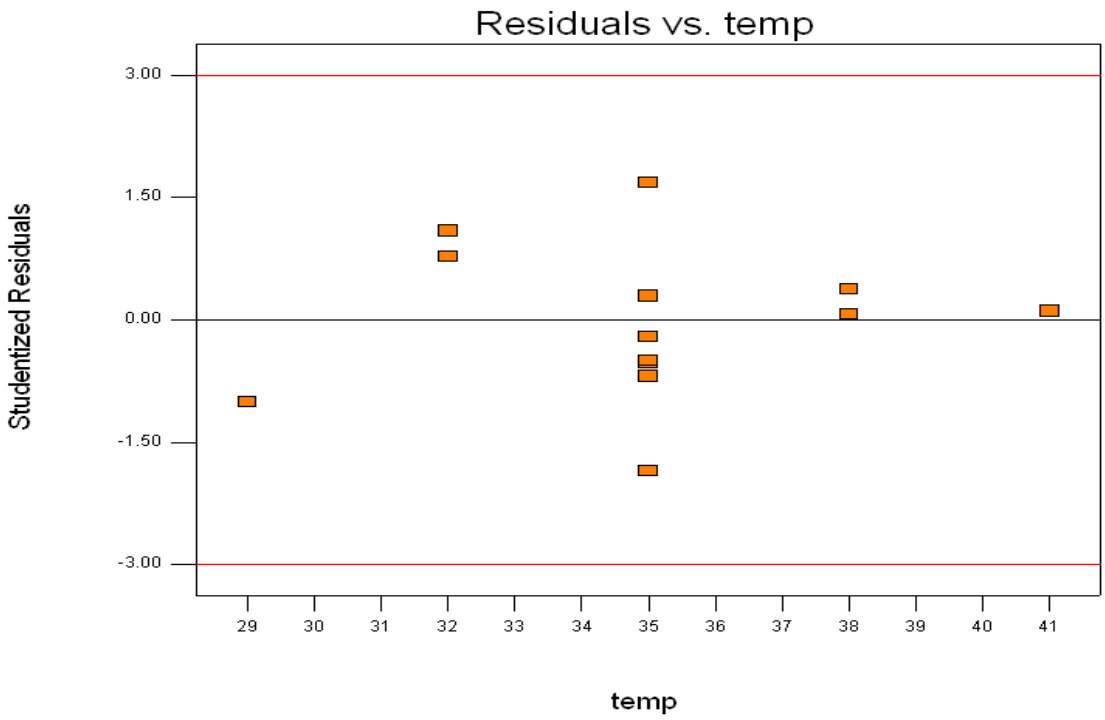

Figure 4.9: Plot of Residuals against temperature

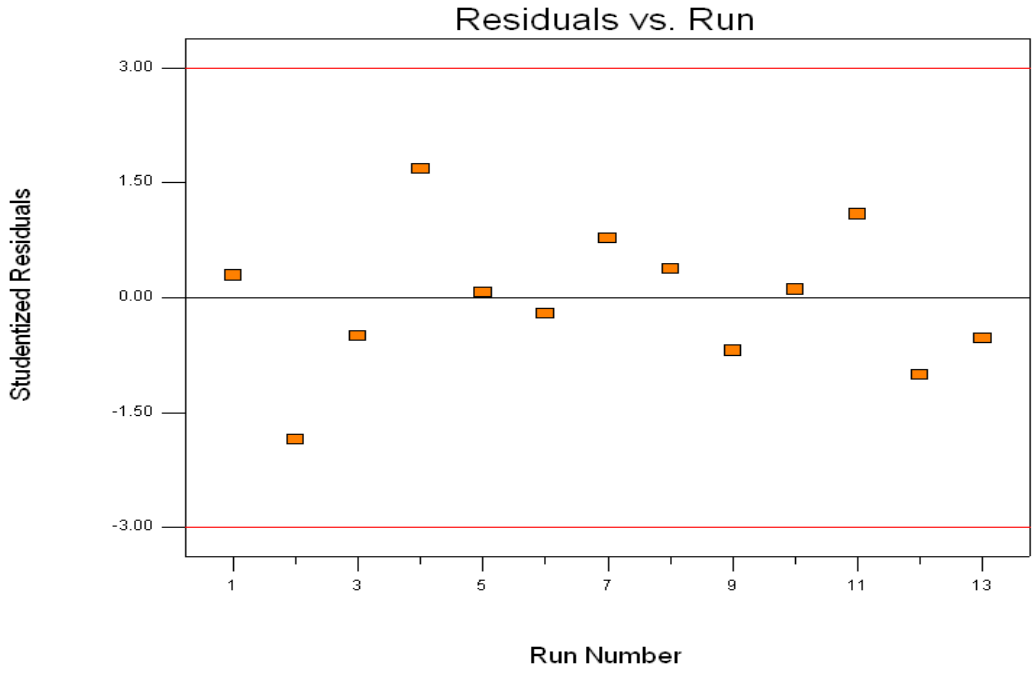

Figure 4.10: Plot of Residuals against run response

CHAPTER 5

CONCLUSION

In conclusion, from the FTI-R analysis, it show that there are O-H bond, N-H bond, C=O bond, and C-H bond in the untreated and treated sample while in the ICP-MS analysis, amount of aluminium, arsenic and lead in the sample was 2.378 mg/L, 0.044 mg/L and 0.125mg/L, respectively. Response surface methodology technique proved to be a useful tool in establishing optimum conditions for extracting protein from *Collocalia fuciphaga*. The optimum protein extraction yield will be produced at temperature of 38^{o}*C and liquid:solid ratio at 42:1 (v/w).* Based on the results above, the protein concentration of *Collocalia Fuciphaga* were produced by extraction process is acceptable and excellent. Therefore this study is successful and objective is achieved.

5.1 RECOMMENDATIONS

For the further studies and maybe for application in laboratory or industry, some recommendations are required to make sure this study more effectively in order to extract protein from *Collocalia fuciphaga:*

i. The extraction time for the protein must be longer that an hour. Therefore, the protein can be fully extracted from the sample.
ii. The temperature for the protein extracted was lower so the protein structure would not denature.
iii. The standard curve must be prepared in every preparation of the protein assay.
iv. High Performance Liquid Chromatography can be use as the quantitative method to determine the compound inside the protein extracted.

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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: Data collected for Standard Curve Preparation

concentration of BSA, g/L	OD			
	Run 1	Run II	Run III	Average
0	0	0	0	0
0.2	0.306	0.308	0.313	0.309
0.4	0.518	0.516	0.521	0.518
0.6	0.685	0.697	0.706	0.696
0.8	0.794	0.875	0.926	0.865
1	0.965	0.989	1.001	0.985

Table 6.3: Second data Collected for Standard Curve Preparation

concentration of BSA, g/L	OD			
	Run 1	**Run II**	**Run III**	**Average**
0	0	0	0	0
0.2	0.336	0.315	0.324	0.325
0.4	0.531	0.507	0.519	0.519
0.6	0.712	0.695	0.639	0.682
0.8	0.857	0.853	0.915	0.875
1	0.957	0.895	1.090	0.984

Table 6.4: Third Data collecte for Standard Curve preparation

concentration of BSA, g/L	OD			
	Run 1	**Run II**	**Run III**	**Average**
0	0	0	0	0
0.2	0.306	0.328	0.299	0.311
0.4	0.557	0.497	0.527	0.527
0.6	0.703	0.695	0.639	0.679
0.8	0.857	0.853	0.915	0.875
1	0.957	0.895	1.090	0.984

Table 6.5: Preparation condition from RSM

Std	Run	Block	Factor 1	Factor 2	Response 1
			A:temp	B:ratio	Conc g/L
12	1	Block 1	35.00	0.026	0.357
10	2	Block 1	35.00	0.026	0.289
11	3	Block 1	35.00	0.026	0.332
13	4	Block 1	35.00	0.026	0.401
4	5	Block 1	38.00	0.028	0.291
8	6	Block 1	35.00	0.030	0.347
3	7	Block 1	32.00	0.028	0.422
2	8	Block 1	38.00	0.024	0.283
7	9	Block 1	35.00	0.022	0.321
6	10	Block 1	41.00	0.026	0.211
1	11	Block 1	32.00	0.024	0.427
5	12	Block 1	29.00	0.026	0.433
9	13	Block 1	35.00	0.026	0.331

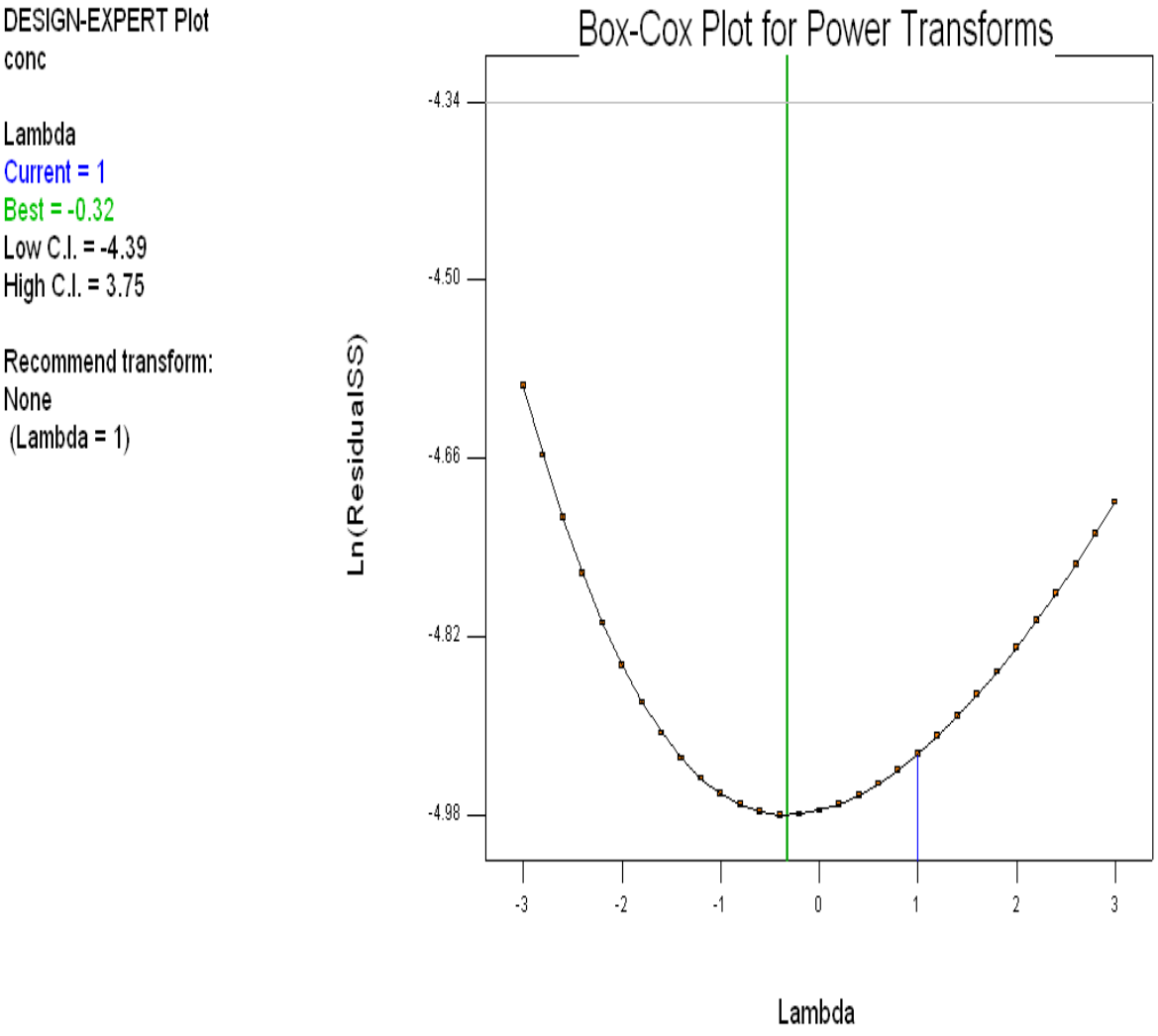


Figure 6.1: Box- Cox Plot

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

Sequential Model Sum of Squares

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Mean	1.52	1	1.52			
Linear	0.043	2	0.022	24.02	0.0002	Suggested
2FI	4.225E-005	1	4.225E-005	0.042	0.8415	
Quadratic	4.964E-004	2	2.482E-004	0.20	0.8195	
Cubic	5.563E-004	2	2.782E-004	0.18	0.8440	Aliased
Residual	7.924E-003	5	1.585E-003			
Total	1.57	13	0.12			

"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 Tests

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Linear	2.283E-003	6	3.805E-004	0.23	0.9475	Suggested
2FI	2.241E-003	5	4.481E-004	0.27	0.9105	
Quadratic	1.744E-003	3	5.814E-004	0.35	0.7956	
Cubic	1.188E-003	1	1.188E-003	0.71	0.4483	Aliased
Pure Error	6.736E-003	4	1.684E-003			

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

Model Summary Statistics

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	0.030	0.8277	0.7933	0.7490	0.013	Suggested
2FI	0.032	0.8285	0.7714	0.7037	0.016	
Quadratic	0.035	0.8380	0.7223	0.5493	0.024	
Cubic	0.040	0.8486	0.6367	-1.8243	0.15	Aliased

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

Table 6.6: Diagnostics Case Statistics

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	0.43	0.40	0.027	0.494	1.090	0.193	1.107	11
2	0.28	0.27	9.310E-03	0.494	0.376	0.023	0.352	8
3	0.42	0.40	0.019	0.494	0.780	0.099	0.756	7
4	0.29	0.29	1.644E-03	0.494	0.066	0.001	0.061	5
5	0.43	0.45	-0.016	0.790	-1.003	0.632	-1.003	12
6	0.21	0.21	1.678E-03	0.790	0.105	0.007	0.098	10
7	0.32	0.33	-0.011	0.790	-0.689	0.298	-0.661	9
8	0.35	0.35	-3.322E-03	0.790	-0.208	0.027	-0.194	6
9	0.33	0.35	-0.017	0.172	-0.528	0.010	-0.499	13
10	0.29	0.35	-0.059	0.172	-1.855	0.119	-2.408	2
11	0.33	0.35	-0.016	0.172	-0.497	0.009	-0.468	3
12	0.36	0.35	9.276E-03	0.172	0.293	0.003	0.273	1
13	0.40	0.35	0.053	0.172	1.683	0.098	2.019	4

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.

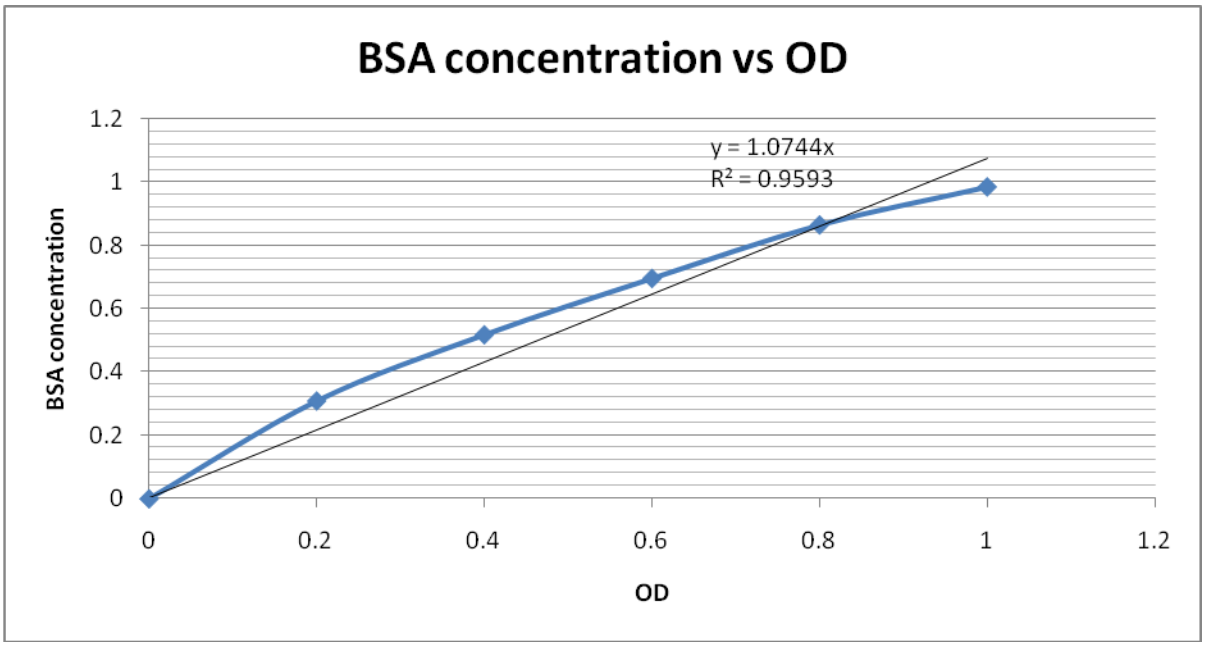
2) Studentized residuals versus predicted values to check for constant error.

3) Outlier t versus run order to look for outliers, i.e., influential values.

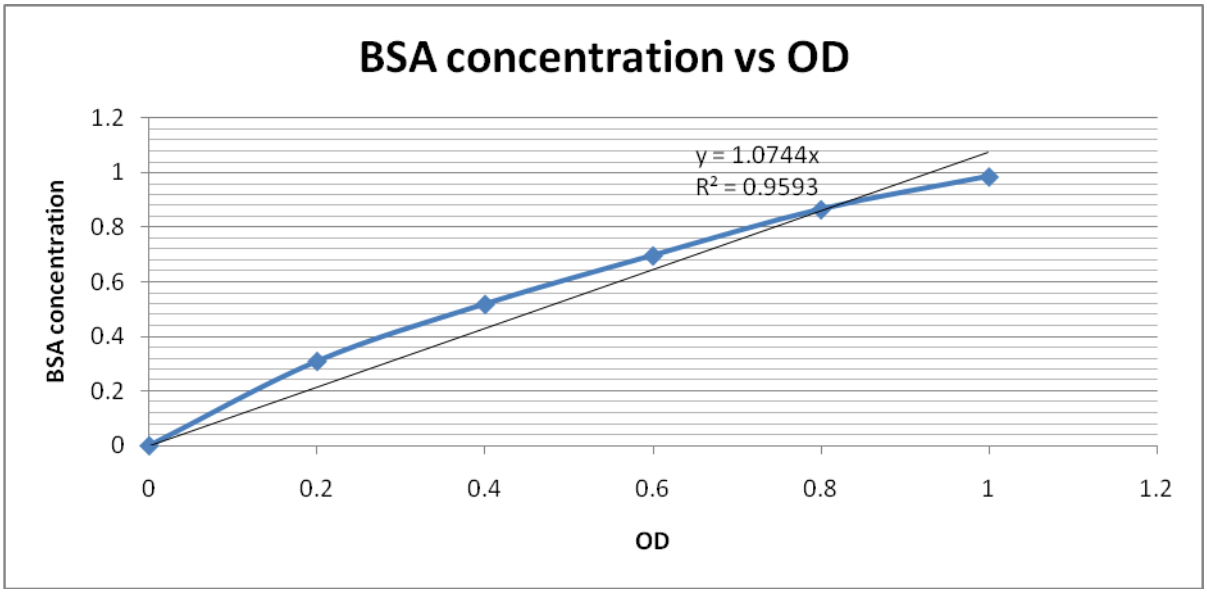
4) Box-Cox plot for power transformations.

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

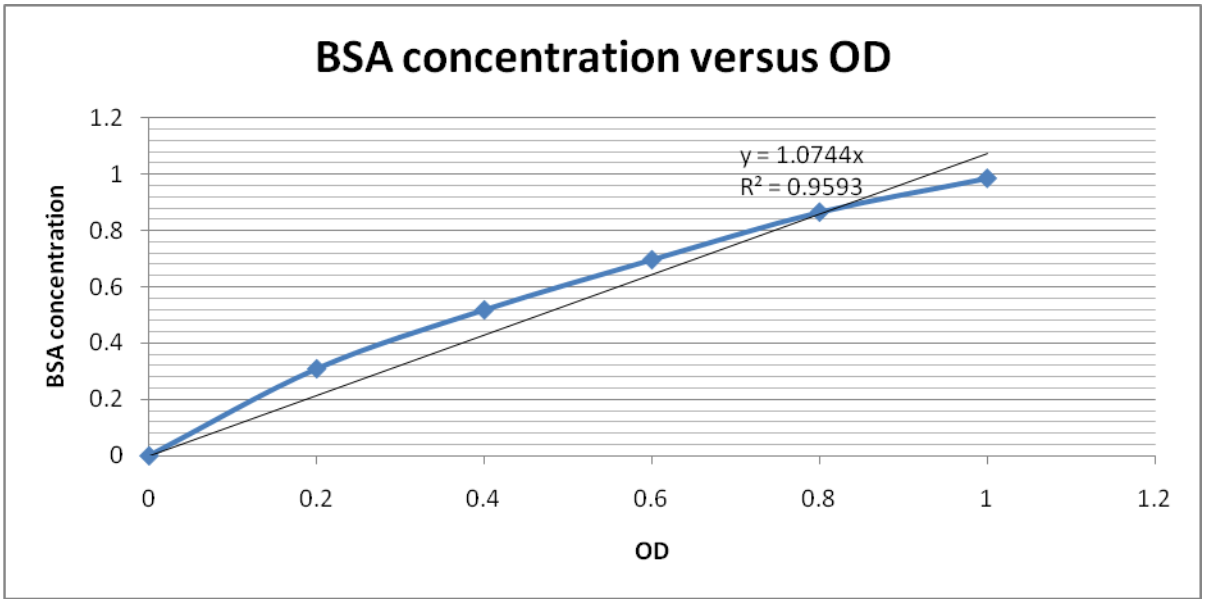
28 September 2011



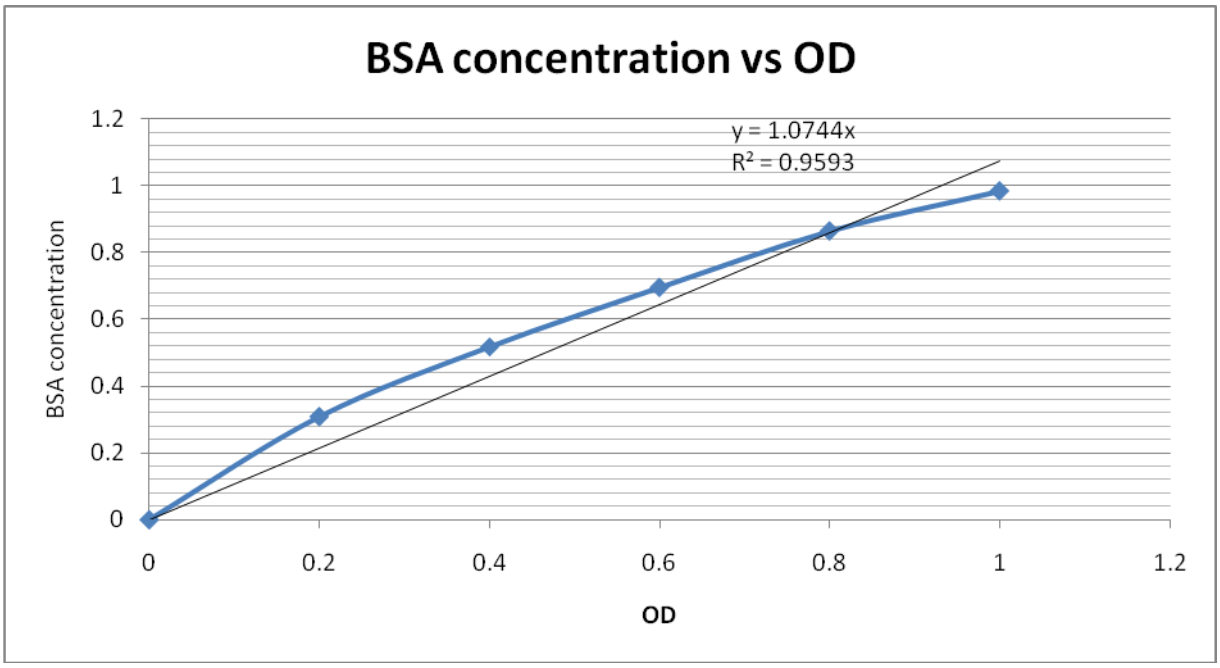
5 October 2011



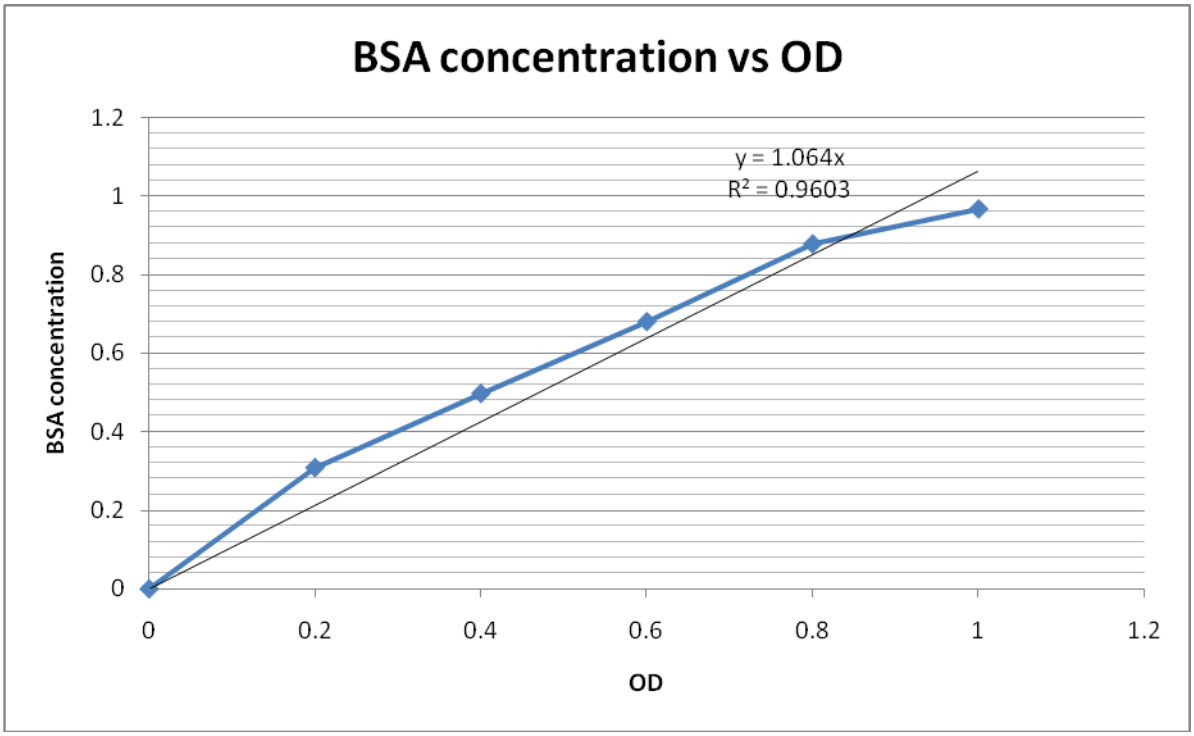
6 Oktober 2011



14 Oktober 2011



18 November 2011



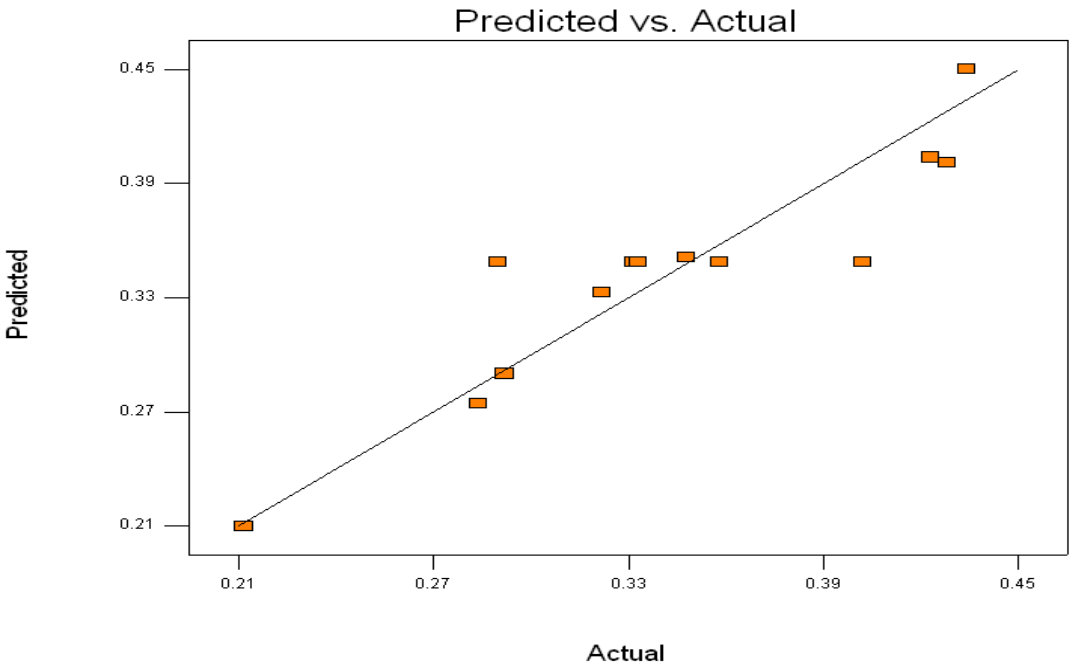


Figure 6.2: Predicted vs Actual response

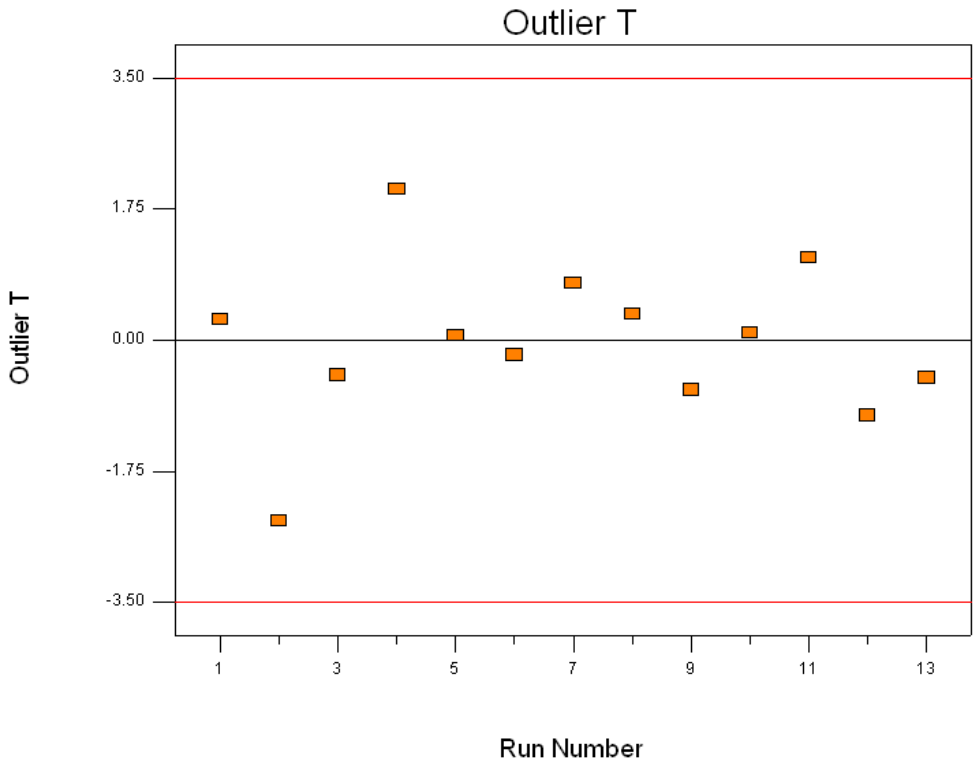


Figure 6.3: Outlier T Plot

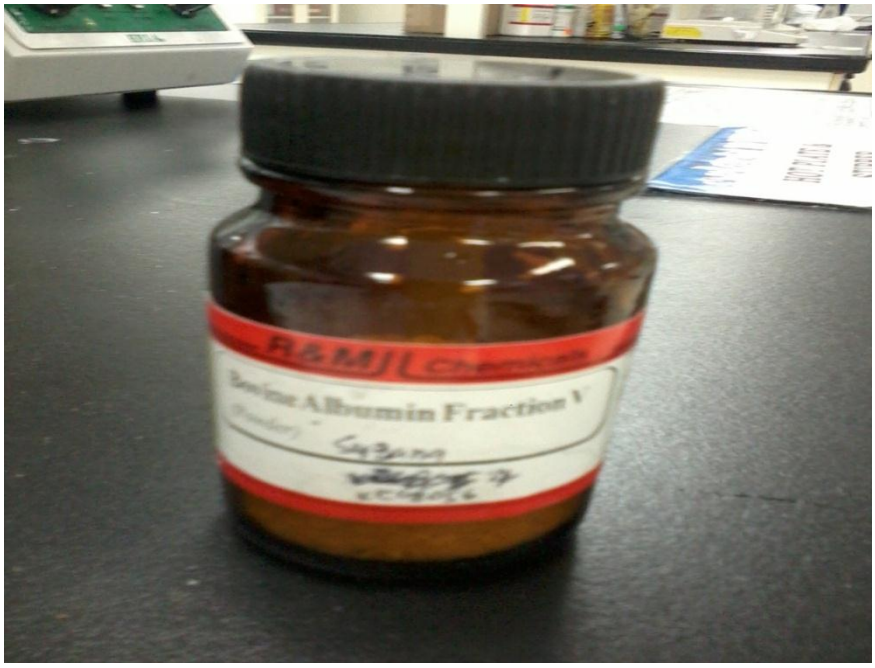


Figure 6.4: BSA sample

Figure 6.5: Preparation of standard curve

Figure 6.6: Folin & Ciocateu's Phenol Reagent

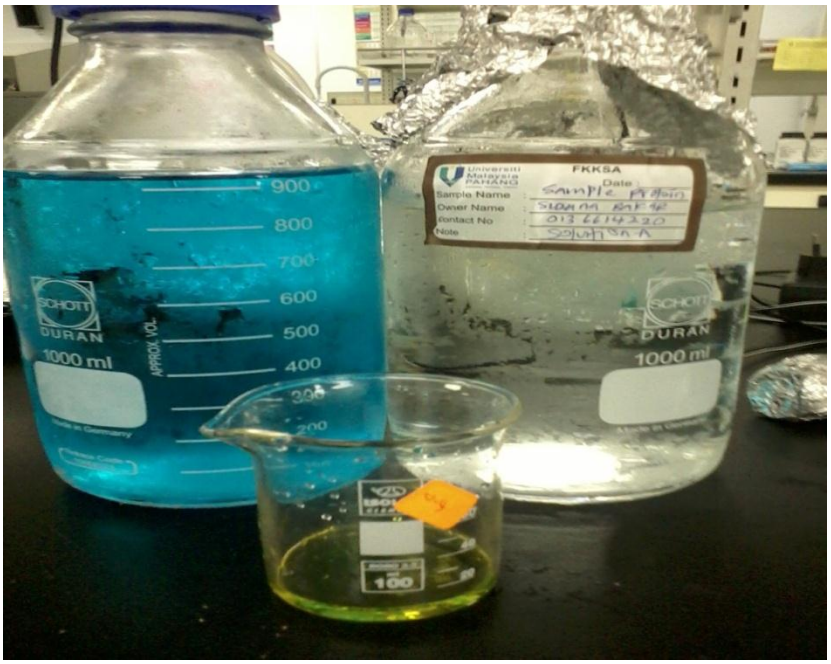


Figure 6.7: Lowry Solution

Figure 6.8: Chemical used

Figure 6.9: Sample being weighed

Figure 6.10: Sample inside Incubator Shaker

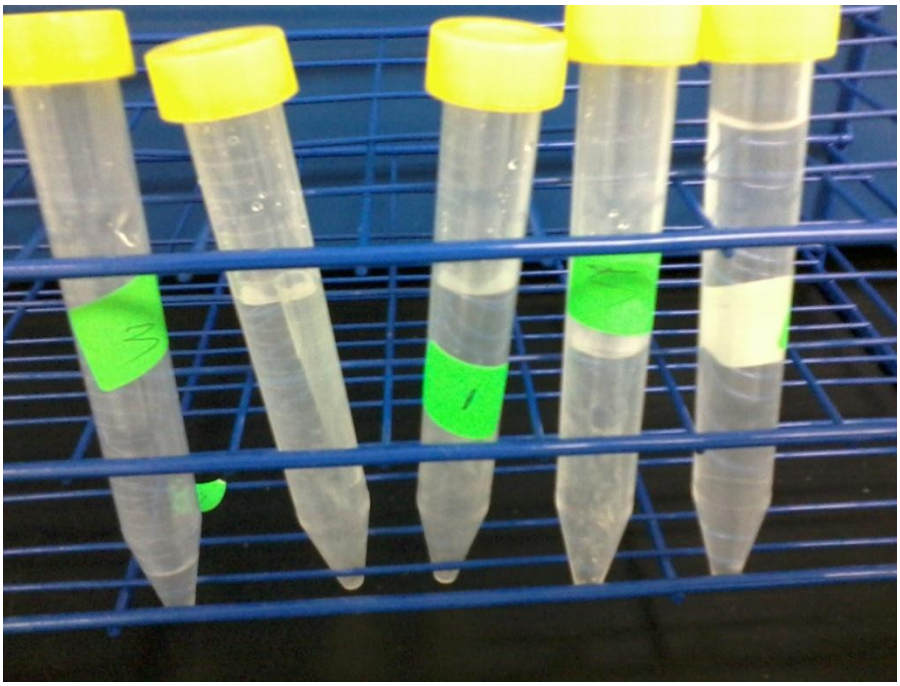


Figure 6.11: Sample Being Centrifuged

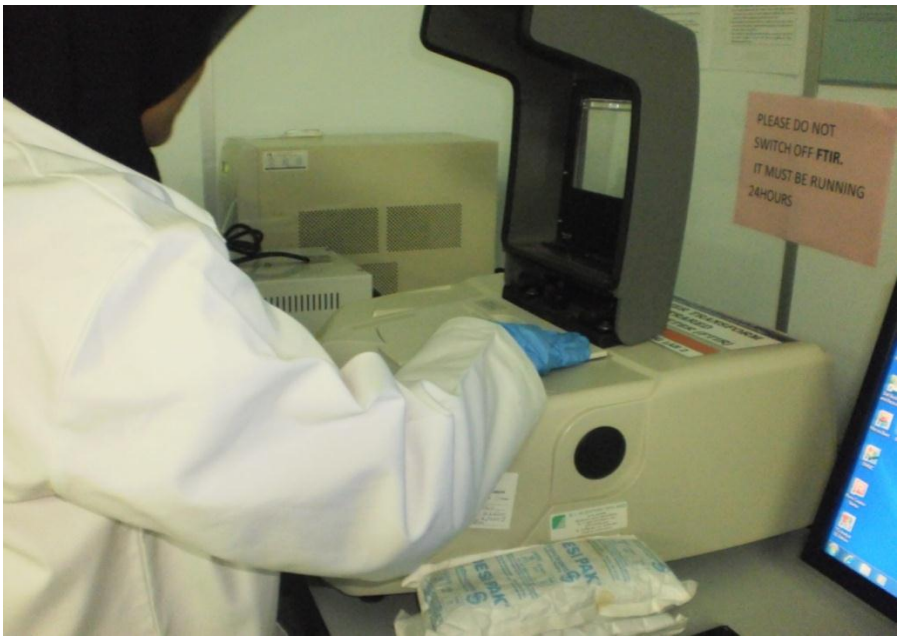


Figure 6.12: FTI-R

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JUDUL : CHARACTERIZATION AND PROCESS OPTIMIZATION OF *COLLOCALIA FUCIPHAGA* EXTRACT

SESI PENGAJIAN : 2011/2012

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Tarikh : **25 JANUARY 2012** Tarikh: **25 JANUARY 2012**

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