KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

COLLEGE OF SCIENCE

PROTEIN EXTRACTION FROM FERN AND ITS PHYSICOCHEMICAL PROPERTIES

A THESIS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF PHILOSOPHY IN FOOD SCIENCE AND TECHNOLOGY

BY

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CERTIFICATION PAGE

I hereby declare that this submission is my own work toward the MPhil and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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ABSTRACT

Fern is a seedless vascular plant that reproduces via spores and has various usefulness. This study was carried out to optimize conditions for protein extraction from two defatted fern types namely *Nephrolepis biserrata* and *Arthropteris orientalis*. The extraction of defatted fern protein was conducted using alkaline, alcohol and saline treatments method under various conditions such as variation of treatments concentration and time of agitation. Central composite design of response surface methodology was used for identification of the best condition and extraction yield optimization. Result showed that alkaline treatment of 0.1 M NaOH and agitation time of 30 min produced the highest extraction yield with maximum protein recovery of 5.11 mg/mL and 2.03 mg/mL for *Nephrolepis biserrata* and *Arthropteris orientalis* respectively. *Nephrolepis biserrata* fern type gave relatively higher maximum protein yield, giving about more than twice the values obtained for the *Arthropteris orientalis. Nephrolepis biserrata* showed the highest foaming capacity (65%) and solubility (55.9%). For each isolate, water and oil holding capacity were not significantly different ($p>0.05$) with *Nephrolepis biserrata* recording 2.13 mL/g and 2.73 mL/g and *Arthropteris orientalis* recording 2.39 mL/g and 2.93 mL/g respectively. Protein types such as β-galactosidase, phosphorylase and lysozyme were found to be present in the fern protein isolates in terms of their molecular weight.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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DEDICATION

To my family Mr. Douglas Essuman, Madam Clara Tsiquaye, Richard Crispin Essuman and Augustina Arita Essuman.

CHAPTER ONE

1.0 Introduction

Ferns have lived with human beings for a long time and are one of a group of about 12,000 species of plants (Chapman, 2009). Unlike mosses, they have xylem and phloem (making them vascular plants). They have stems, leaves and roots like other vascular plants but do not have either seeds or flowers; they reproduce via spores (Smith *et al*., 2006). They influence millions of human lives as traditional medicinal cures or treatments for cold, diarrhea, burn, trauma bleeding, as food additives to improve its quality and more in many countries (Benjamin and Manickam, 2007).

Some ferns used as food by some tropical people include the fiddleheads of bracken, *Pteridium aquilinum*, ostrich fern, cinnamon fern and *Diplazium esculentum*. Licorice fern rhizomes were chewed by the natives of the Pacific Northwest for their flavor. The mosquito fern is used as a biological fertilizer in the rice paddies of Southeast Asia, taking advantage of their ability to fix nitrogen from the air into the soil as compounds that can then be used by other plants (Allen, 1999).

Many modern pharmaceuticals are derived from chemicals produced by plants, there is little scientific evidence that ferns are useful as treatments for these or other ailments although herbalists have advocated some fern species for treatment of ulcers, rheumatism, intestinal infections and various other ailments (Jones, O., 1987).

In Ghana, fern together with other leafy plants are used in making palm-nut soup and given to lactating mothers. The protein in the fern is believed to enrich the blood of lactating mothers. According to some perspectives, (Shin, 2010; Wei, 2010; Thakur *et al*., 1998; Copeland, 1942) leaves of fern may play a significant role as a

potential source of food protein. The interest in this crop is developing due to its several advantageous properties.

Plant proteins play significant roles in human nutrition, particularly in developing countries where average protein intake is less than required (Khalid *et al*., 2003). Insufficient supply of food protein has led to a constant search for unconventional legumes as new protein sources for use as both functional food ingredients and nutritional supplements (Onweluzo *et a*l., 1994). In recent years, there has been serious worry over lack of adequate protein to meet the nutritional requirements of a large segment of world's population. This accounts in part for protein deficiency which prevails among the general population as recognized by Food and Agricultural Organization (Akubugwo *et al*., 2007). Many of the local vegetable materials are under exploited because of inadequate scientific knowledge of their nutritional potentials. Though several works reporting compositional evaluation and functional properties of various types of edible wild plants in use in the developing countries abound in literature, much is still need to be done. Many workers according to Ekop (2007) have reported the compositional evaluation and functional properties of various types of edible wild plants in use in the developing countries.

Functional properties of food protein are important in food processing and food product formulation. Protein isolates are intended to be used as additives in food products for improving functional properties (Yada, 2004). Some of these properties are solubility, water holding capacity, oil binding, emulsification, foaming properties, bulk density and viscosity (Jung *et al*., 2005). The importance of these properties varies with the type of food products in which the isolated protein is to be used (Yada, 2004). Protein isolates with high oil and water binding capacities are

desirable for use in meat, sausages and bread, while proteins with high emulsifying and foaming properties are good for salad dressing, confectionaries, frozen foods and soups (Ahmedna *et al*., 1999). Protein isolates obtained from corn flour (Siddiq *et al*., 2009), sesame seed (Lopez *et al*., 2003) as well as wheat and soybean proteins (Ma *et al*., 2007; Karki *et al*., 2009), have been added to a variety of products, usually as replacements for egg albumin due to their high foaming properties.

Protein isolation is a very important step when incorporating proteins from oil-producing plants into food products. Raw materials with high level of oil need to be defatted prior to protein isolation in order to prevent emulsion formation during protein extraction and produce oil free protein materials. To obtain samples that are free from fat, raw materials are first defatted with solvents such as hexane (Kumagai *et al*., 2002) and petroleum ether (Sathe *et al*., 2002). Various extraction conditions such as pH, solvent types (NaOH, NaCl or Ethanol), solvent concentration, extraction time, solvent/flour ratio, may affect the final properties of the extracted protein from fern leaves.

When many factors and interactions affect desired responses in a certain process designs, Response Surface Methodology (RSM) becomes an effective tool for optimizing the process. It is a statistical technique used to design experiments that yield the relevant information in the shortest time with the least cost and also obtain rapid and efficient development of new products and processes (Pericin *et al*., 2008). In addition, to analyze the effects of the independent variables, the experimental methodology also generates a mathematical model that describes the overall process (Batista, 1999). Response surface experiments aim to recognize the response that can be thought of as a surface over the explanatory variables experimental space,

usually uses an experimental design such as Central Composite Rotatable Design (CCRD) to fit an empirical, full second order polynomial model. RSM has successfully been applied for the optimization of the different extraction conditions (Tiezheng *et al*., 2010; Yi *et al*., 2011; Wani *et al*., 2006).

1.1 Statement of the Problem

Attention on plant protein isolates has been focused mainly on cotton seed, peanut, rapeseed, soya protein and sunflower seed and in some areas commercial preparations are available (Schenz and Morr, 1996). This has led to the neglect of other equally important edible leafy vegetable like fern. Meanwhile, it is used in Ghana in combination with other leaves to improve the quality and quantity of breast milk in lactating mothers. Although, physicochemical and functional properties of isolated rhizome starch from fern (*Pteridium aquilium*) has been studied (Zhang *et al*., 2011), that on the fern fronds have not yet received any investigation. Moreover, very little is known in literature about the characterization of protein types present in the fern fronds. This makes it difficult to use it as a substitute or supplements for other leafy vegetables or crops.

1.2 Justification of Work

Proteins are very important in foods, both nutritionally and as functional ingredients. They play an important role in determining the texture of foods. By expanding leafy vegetable production through the extensive conservation and use of deserted and underutilized fern plants, the study has the capacity to add significantly to reducing the current protein deficiencies that limit the growth and performance of many people in West Africa, especially women, children and the poor.

Protein with a vegetable origin is an alternative to animal protein for food applications due to the widespread variety of sources, such as legumes, oilseeds, cereals, and leafy vegetables. Animal diseases, such as mad cow disease, global shortage of animal protein, strong demand for healthy (cholesterol free and low in saturated fat) food and for economic reasons, there is a pressure for the direct consumption of vegetable proteins in food products. High meat prices have prompted the food industry to produce non meat proteins. Vegetable proteins have a lower price than muscle proteins and consequently, can reduce the cost of the meat product. An important reason for the increased acceptance of vegetable proteins, such as Texturized Soy Protein (TSP), is their low cost (Singh *et al*., 2008).

There is a growing trend toward widespread use of protein ingredients in food formulation and fabrication. An understanding of the relationships between the physical properties of proteins, their behaviour in food systems and utilization of protein isolates in various foods is based on their highly desirable food functional properties such as solubility, emulsification and foaming (Yada, 2004). Protein isolate from fern leave can therefore be used as cost effective ingredients applied to the manufacturing of various functional foods and healthy products for human consumption and also as an additive in fortifying livestock and fish feeds to enhance production especially in peasant communities (Oloyede *et al*., 2010). An additional reason for using fern proteins is because the ferns are common and measures can also be put in place to ensure the plants' availability.

Studies to characterize the interaction effects of pH, time and solvent types on the extraction of protein from fern leaves flour will provide useful information for food processors wishing to use it for specific food applications.

1.3 Goal

The goal of this study was to identify the optimum conditions suitable to maximally and efficiently isolate proteins from fern fronds for industrial usage.

1.3.1 Objective

The specific objective of this work was:

1. To investigate conditions necessary to optimize protein extraction from fern fronds.
2. To establish the utility of the fern protein isolate in the food industry by determining its physicochemical properties.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

Plants have been a source of medicine and a major resource for health care since ancient times, with some traditional herbal medicines having been in use for more than 2,000 years (Lee and Shin, 2011). Next to flowering plants, the dominant vegetation on the earth is formed by the non-flowering vascular plants called Pteridophytes. There are about 1200 species of Pteridophytes and like flowering plants some Pteridophytes are grown or gathered by other countries for food, as ornamental plant and medicine (Chapman, 2009).

Fern, like any of several non-flowering vascular plants, possesses true roots, stems, and complex leaves and reproduce by spores are known to belong to the family Davalliaceae. Davalliaceae is in the major group Pteridophytes in the order Polypodiales. It is sister to the largest family of ferns, Polypodiaceae, (Schuettpelz and Pryer, 2007) and shares some morphological characters with it (Kramer, 1990). Davalliaceae is native to tropical and subtropical regions of the Pacific, Australia, Asia, and Africa (Smith *et al.*, 2008). Fronds from Pteridophytes provide excellent ornamental qualities (Jones, O., 1987) and are known worldwide due to their ornamental value and several studies have been conducted for optimizing *in vitro* conditions such as sucrose and pH, not only to contribute for the propagation of ornamental species in commercial scale, but also to understand the species biology (Fernández *et al.*, 1999). Among the Pteridophytes species used as ornamental foliages in commercial and residential environments, we can find those of the genus *Nephrolepis* (Morton 1958; Zimmerman and Jones 1991). It is a highly distinctive

genus characterized by abundant stolons, disarticulate, inequilateral and usually entire pinnae, hydathodes in the upper surface of the pinnae and spores with a relative thin and tuberculate-rugose perispore (Tyron and Tyron, 1982). The number of fern species is about 9,000 but estimates have ranged to as high as 15,000, the number varying because certain groups are as yet poorly studied and because new species are still being found in unexplored tropical areas.

2.2 Evolution and Classification of Fern

Ferns first appear in the fossil record in the early carboniferous period. One problem with fern classification is the problem of cryptic species. A cryptic species is a species that is morphologically similar to another species, but differs genetically in ways that prevent fertile interbreeding. Ferns have traditionally been grouped in the class Filices, but modern classifications assign them their own phylum or division in the plant kingdom, called Pteridophyta, also known as Filicophyta. Traditionally, three discrete groups of plants have been considered ferns and two groups of eusporangiate ferns-families Ophioglossaceae (adders-tongues, moonworts, and grape-ferns) and Marattiaceae and the Leptosporangiate ferns. The Marattiaceae are a primitive group of tropical ferns with a large, fleshy rhizome, and are now thought to be a sibling taxon to the main group of ferns, the leptosporangiate ferns (Pryer *et al*., 2004).

2.3 Distribution and Abundance of Fern

Geographically, ferns are most abundant in the tropics (McHugh, 1992). Arctic and Antarctic regions possess few species. On the other hand, a small tropical country such as Costa Rica may have more than 900 species of ferns, about twice as many as are found in all of North America and north of Mexico. The finest display of fern

diversity is seen in the tropical rainforests, where in only a few hectares more than 100 species may be encountered, some of which may constitute a dominant element of the vegetation (Jones, 1987). Also, many of the species grow as epiphytes upon the trunks and branches of trees. A number of families are almost exclusively tropical (e.g., Marattiaceae, Gleicheniaceae, Schizaeaceae, Cyatheaceae, Blechnaceae and Davalliaceae). Most of the other families occur in both the tropics and the temperate zones. Only certain genera are primarily temperate and Arctic (e.g., *Athyrium*, *Cystopteris*, *Dryopteris*, and *Polystichum*), and even these tend to extend into the tropics, being found at high elevations on mountain ranges and volcanoes (Cobb, 1975).

Because of their ability to disperse by spores and their capacity to produce both sex organs on the same gametophyte and thus to self fertilize, it would seem logical to assume that ferns possess higher powers of long distance dispersal and establishment than do seed plants. Although genetic tests have shown that many, if not most, fern species tend to have an outcrossing breeding system, some other species are involved in the case of ferns with remote disjunctions separated growing regions **(**Schuettpelz, 2007). There are interisland and intercontinental disjunctions, east and west, as well as wide north-south disjunctions including species found in the Northern and Southern hemispheres that skip the tropics. Some disjunctions seem to follow the pattern of prevailing winds and the main centre of distribution of a species often may lead to downwind groups consisting of one or a few small populations sometimes hundreds or thousands of kilometers away (McHugh, 1992). Examples of species exhibiting west-to-east transcontinental disjunctions in North America are Wright's cliffbrake (*Pellaea wrightiana*), mountain holly fern (*Polystichum scopulinum*), and forked spleenwort (*Asplenium septentrionale*); all of

these ferns are well known in the western United States, and they exist as tiny populations in the mountains of the eastern states as well. Some species are disjunct between continents, such as between New Zealand and South America (*Blechnum penna-marina* and *Hypolepis rugosula*) or South Africa and Australia and New Zealand (*Todea barbara*). Some disjunct patterns, such as similar plants growing in Asia and in Eastern North America, are not the results of long-range dispersal but rather are the remnants of an ancient continuous flora, the intervening areas having been changed over time (Encyclopedia Britannica, 2011).

2.4 Uses of Indigenous Edible Wild Plant

In developing nations, numerous types of edible wild plants are exploited as sources of food hence provide an adequate level of nutrition to the inhabitants (Aberoumand and Deokule, 2010). Recent studies on agro pastoral societies in Africa indicate that these plant resources play a significant role in nutrition, food security and income generation (Abuye *et al*., 2003). Furthermore, Food and Agricultural Organization (FAO) report, at least one billion people are thought to use wild foods in their diet (Burlingame, 2000). In Ghana alone, the leaves of over 300 species of wild plants and fruits are consumed (Aberoumand and Deokule, 2010). In Swaziland, wild plants provide a greater share of the diet than domesticated cultivars. In India, Malaysia and Thailand, about 150 wild plants species have been identified as sources of emergency food (Faruq *et al*., 2000). Similarly, in South Africa about 1400 edible plant species are used. In Sahel region of Africa, over 200 wild foods were identified to be used by the rural communities (Aberoumand and Deokule, 2008). In most reports, it was emphasized that nutritionally, these unconventional plants foods could be comparable to or even sometimes superior to the introduced cultivars. It is, therefore worthwhile to note that the incorporation of edible wild and

semi cultivated plant resources could be beneficial to nutritionally marginal populations or to certain vulnerable groups within populations, especially in developing countries where poverty and climatic changes are causing havoc to the rural populace.

2.4.1 Effect of processing temperature on nutrients of indigenous leafy vegetable

Leafy vegetables are often cooked before consumption. They can also be dried in preservation by sun drying in direct sunshine or under shade which is the common practice used in most parts of Africa to preserve vegetables for dry season consumption (Lyimo *et al.*, 1991). However, ways of food preparation and preservation may affect significantly the concentration and availability of minerals, vitamins and other essential compounds in food. Some reports have documented the losses of nutrients from vegetables during drying (Yadav and Sehgal, 1997) and cooking (Kachik *et al.*, 1992; Kidmose *et al.*, 2005). According to Ray-Yu *et al.* (2006), mild-heat drying process (50 °C/ 16 hrs) maintained most nutrients and bioactive in moringa leaves and could be achieved by low-cost household preparation as a simple and effective way for continuous nutrients/bioactive supply.

2.5 Fern Consumption and Utilization

Ferns (Pteridophyta) are an important component of many ecosystems (Tyron, 1986) and potentially provide an abundant food resource for consumers. The selection of food resources by herbivorous animals is influenced by a variety of factors. Preference determines which available foods are consumed and is mainly influenced by food size and handling time (Diaz, 1994), nutritional value (Schaefer *et al.*, 2003) and secondary compounds such as phenolics. Invertebrates such as gastropods and insects consume ferns to some extend but very few vertebrate species are known to

consume ferns regularly. Some exceptions are found amongst birds such as the Takahe (*Notornis mantelli*) and the Kaka (*Nestor meridionalis*) in New Zealand (Mills *et al*., 1980; O´Donell and Dilks, 1994), the cantabrian capercaillie (*Tetrao urogallus cantabricus*) in Spain (Rodríguez and Obeso, 2000) and the Azores bullfinch or priolo (*Pyrrhula murina*) in the Azores, Portugal (Ramos, 1994). The reasons behind the ubiquitous low consumption of ferns by vertebrates are not clear but it has been suggested to be related with the high concentration of diverse biochemical defences on ferns (Seigler, 1991; Moran, 2004; Marrs and Watt, 2006) and lignin contents (Cornelissen *et al*., 2004).

According to Ramos (1995), consumption of fern by vertebrate, Azores bullfinch (*Pyrrhula murina*), is a seasonal pattern. Sporangia of three species (*Woodwardia radicans, Culcita macrocarpa* and *Osmunda regalis*) are mainly consumed due to their higher lipid content. Young leaves are mostly consumed especially from *Pteridium aquilinum* and *O. regalis* in early spring since they possess high amounts of proteins and reasonable high values of calories. In view of this evidence, the pattern of fern consumption may be influenced by seasonal variations in leaf developmental stage or nutritional composition of the ferns. There are four different leaf developing stages namely crozier, expanding leaf, recently expanded leaf and fertile leaf (Arosa *et al*., 2009).

Apart from the fern being consumed by vertebrates, it is also used as medicine and consumed by some native people as food source in times of food scarcity as they contain significant amounts of protein, fiber, vitamins, and minerals. The most popular consumed fern is the wood rootstock. There are many other reports of the rootstocks being cleaned, the leaf bases being removed, peeled, and eaten one at a

time (Turner *et al*., 1992). Similarly, many reports refer to the eating of the rootstocks with oil, grease, lard, and or salmon roe. Kari (1987) notes that some Tanaina people state that only the "stem" portion of the underground part is eaten, others that only the "leaf bases" are eaten, and still others that both parts of the rootstock are eaten.

Some ferns, especially bracken, are known to contain ptaquiloside which is very carcinogenic in mammals, especially ruminants. However, bracken consumption in humans does not lead to carcinogenesis because people eat bracken in small quantities than animals and also not repetitively. Nevertheless, according to studies conducted by Ham (2004) reported that when people eat 350 g fiddlehead of bracken every day, it can cause cancer. The presence of antithiamin substances in brackens fern such as astragalin, isoquercitrin, rutin, caffeic acid, tannic acid, etc., are known as useful natural substances for anticancer or antioxidant in this present time (Cai *et al*., 2004; Katsube *et al*., 2006). While toxicities caused by carcinogenesis and antithiamin activities of bracken fern have been highlighted, the pharmacological effects of the fiddleheads or whole plants of ferns and fern allies are underestimated (Lee and Shin, 2011). However, several healthy effects of ferns and fern allies are currently well known. For example, the glycoprotein isolated from bracken fiddlehead has immune function (Park *et al*., 1998), and the acidic polysaccharides isolated from the hot water extract of dried bracken fiddlehead have anti-complementary activity (Oh *et al*., 1994). Also, the fiddlehead of *Athyrium acutipinulum* has strong antioxidant effects (Lee *et al*., 2005).

Table 2.1: Edible ferns often used worldwide

Scientific name	English name	Countries	Edible parts
Athyrium acutipinnulum		Korea	Fiddlehead
A. brevifrons		Korea	Fiddlehead
A. distentifolium	Alpine Lady-Fern	Korea	Fiddlehead
A. esculentum	Vegetable fern, Pako fern	Asia, Oceania	Fiddlehead
Diplazium squamigerum		Japan	Fiddlehead
Matteuccia struthiopteris	Ostric fern	Canada, China, Europe, Malaysia, India, Japan, USA	Fiddlehead
Osmunda cinnamomea	Cinnamon fern	East Asia	Fiddlehead
O. japonica	Flowering fern	East Asia	Fiddlehead
O. regalis	Royal fern	Worldwide	Fiddlehead
Pteridium aquilinum var. *latiusculum*	Bracken	Worldwide	Fiddlehead
Stenochlaena palustris	High climbing fern	South Pacific, India.	Fiddlehead
Ceratopteris	Swamp fern, water lettuce, water sprite	Asia, Australia	Leaves
Cyathea	Tree fern	Oceania	Young leaves, terminal bud

Source: (Copeland, 1942; Thakur, *et al*., 1998; Shin, 2010; Wei, 2010)

Current research in pollution control has been directed toward removing nutrients (e.g. N and P) and minerals (e.g. Zn, Ni, Cu, etc.) discharged into streams from sewage treatment plants (Upadhyay *et al*. 2007). Various aquatic plants have been used to remove nutrients and minerals from contaminated freshwater and also waste water. The ability of *Azolla* plants, also a fern, to uptake minerals from waste water was determined by analysing waste water before and after cultivation of *Azolla* (Mishra *et al*. 2007). The Fe, Zn and P contents all decreased, whilst those of Cu and Mn was not changed. Similarly, Zahrann *et al*. (2007) investigated the potentials of *Azolla* plants in polluted water. By measuring the amount of pollutant remove taking into account the time, findings showed that the *Azolla* plants may be used to purify polluted water (fresh or waste) for recycling. This evidence support the view that cultivation of *Azolla* plants can be used as green manure in many countries of the world since it can absorb high amounts of these minerals.

Fern among other crops like sunflower has been used as phytoremediation to remove dangerous quantities of arsenic that has reacted with other organic and inorganic compounds from the ground. Exposure to arsenic through drinking contaminated water, digging, breathing or coming into any kind of contact with contaminated dust may cause skin, liver, bladder and kidneys cancer. According to Kokkinidou (2011), gene isolated from a specific fern type, *Pteris vittata* has the ability to increase the tolerance against arsenic by up to 100 to 1,000 times more than other plants. This specific gene is capable of encoding a protein that stores the toxic metal away from the cytoplasm so as not to hurt the plant. The presences of the protein therefore enable the fern to act as a hyperaccumulator in order to absorb larger than normal amounts of heavy metals in soils (Kokkinidou, 2011).

2.6 Preservation of Fern

As recounted by Peter Kalifomsky of the Outer Inlet Tanaina (Kari, 1987), the rootstocks of edible fern were formerly regarded as a good survival or starvation food. In some European countries, the rootstock of fern is generally consumed during times when other foods are scarce or as medicinal treatment. It is therefore imperative to safeguard fern plants especially those of nutritional and medicinal importance. Fern, particularly fern root stocks, are stored by covering them with leaves after drying then under shed or cabin. In Alaska, Tanaina people are said to preserve wood fern rootstocks by placing them in an underground cache or by storing them in oil or lard (Kari 1987). The Yakutat people also stored them in a pit cache, and in general stored them in a way similar to potatoes. They also keep well for several months in a refrigerator, according to Ray (1980).

2.7 Nutritional Components of Fern

Non-cultivated indigenous leafy vegetables (ILVs) are widely consumed in Ghana and other parts of sub-Saharan Africa and Europe, particularly during periods of food scarcity. However, much remains to be learned about their content of essential nutrients. Ghana is an agriculturally productive region of West Africa and it is therefore common for people in rural communities to consume ILVs as part of their diet, either as food or medicine. In Ghana, leaves from the plant are added to soups where the plant is a valued potherb and consumed mostly by mothers before and after childbirth to ensure good health and also boost breast milk production, thus reflecting the local population's perception that the plant has nutritional value.

Babayemi *et al.* (2006) pointed out that the dry matter and crude protein content is higher in the leaves of aquatic fern than any part of the plant. Crude protein (CP) of the aquatic fern decreases as the plant matured. Such lowered crude protein at advanced age had also been noticed in other aquatic plants (Bagnall *et al*., 1973). Thus, when using the plant as forage, it could be strategically cut every twelve weeks to optimize its nutrient value. The level of the CP in the fern, especially in leaves is high enough to meet the protein requirement of small ruminants. Oldham and Alderman (1980) reported that sometimes *ad libitum* intake by animals is increased by an increase in crude protein content of diets.

Older fern contains more crude fat (CF) than the young plant probably due to lignifications of cells as the plant matures (Babayemi *et al*., 2006). Stems of fern are known to contain more CF than the leaves and the leaves plus stem. The reason for this might be due to the fact that as leaves mature, they fall off and are replaced by younger and more photosynthetically active ones while the process of lignifications

proceeds in the stem, thereby increasing the percent of old stem in the entire biomass. Older aquatic fern contain more ash than the young fern due to the high mineral deposition in the old plant. The absence of seasonal variation in the ash content of the aquatic fern suggests a balance of mineral content throughout the year for the animals consuming it (Babayemi *et al*., 2006).

2.7.1 Nutritional analysis of fern

Turner *et al*. (1992) investigated traditional foods carried out under the Nuxalk Food and Nutrition Programme, approximately 30 wood fern rootstocks were dug and this was compared with reported values for the common potato, baked in the skin. There was a reasonably good agreement between the two foods for water, protein, ash, carbohydrates (computed by difference between total weight minus the sum of protein, fat, moisture, fiber, and ash) and approximate energy computations. In contrast, mineral values in fern "root" were much higher for calcium, magnesium, zinc, copper, and manganese, but higher in potato for sodium and iron. The differing values may have been in part due to contributions from the skin of the roots, since it is known that skins of root foods are rich in minerals more than the starchy flesh. These minerals can migrate into the flesh during cooking.

2.7.2 Nutritional determination of sporangia and leaf of fern

Fern sporangia are high in protein, phenolic and caloric content of consumed fern species. However, different species have different caloric content. *Pteridium incomplete* has higher caloric content as compared to *Pteridium aquilinum* (Arosa *et al*., 2009). Ramos (1994) also found non consumed spores of fern species (*Dryopteris sp., Blechnum spicant*) to have less lipid content than those of consumed species (*Woodwardia radicans, Culcita macrocarpa, Pteris incomplete*). Ramos

(1995) again noticed that no nutritional differences exist between consumed and non consumed species of fern leaves.

Table 2.2: Nutrients in cooked fern root (*Dryopteris expansa*) in comparison to cooked potato, per 100 g edible portion

Nutrient	Fern Root	Potato
Water, g	68.4	71.2
Protein, g	2.5	2.3
Fat, g	1.0	0.1
N.D. Fibre, g	3.7	n.a.
Ash, g	0.8	1.2
Carbohydrate, g	23.6	25.2
Calcium, mg	56.3	10.0
Phosphorus, mg	62.6	57.0
Sodium, mg	1.4	8.0
Magnesium, mg	44.4	27.0
Iron, mg	0.8	1.4
Zinc, mg	1.5	0.3
Copper, mg	1.5	0.3
Manganese, mg	3.2	0.2

Source: Turner *et al*. (1992)

The amount of lignin, an important component of the plant cell wall, determines to a great extent leaf digestibility (Cornelissen *et al*., 2004). In most cases, digestibility decreases as leaves age as a consequence of lignifications (Lowman and Box, 1983; Hill and Lucas, 1996), which may contribute to explain why old fern leaves were never consumed by the Azores bullfinch. Digestibility of fern leaves from several species consumed by the Roosevelt elk (*Cervus elaphus roosevelti*) ranged from 23 to 46 %, whereas that of grasses ranged from 55 to 76 % (Hutchins, 2006), meaning that fern leaves are likely to be less suitable for herbivores than grasses. *Polystichum*

speciosissimus, *P. hancockii*, *P. parvipinnulum*, *P. prionolepis* and *P. deltodon* are totally devoid of phloroglucinols (Widén *et al*., 2001). According to Widén *et al*. (1983), only *P. tsus-simense* and *P. rigens* have been found to contain some phenolics.

2.7.3 Total amino acid compositions of tropical ferns

Leaves of many plant species are consumed directly as vegetables or used as forages. In addition, they have been exploited for the production of leaf protein concentrates for human consumption or used as protein supplement in animal feed. Several studies have pointed out that leaves of tropical and subtropical plants may be good source of proteins. Amino acid data of these plants have also revealed good balance of nutritionally essential amino acids and chemical scores of greater than 75% are reported. There is evidence of some correlation between the amino acid data and taxonomy of plants. Such correlation is potentially useful in the search for plants rich in certain types of amino acids (Yeoh and Wee, 1998).

Among ferns, the Pteridaceae as a group partially overlap the Polypodiaceae and the Dryopteridaceae was separated from these two groups. In terms of differences in amino acid profiles, members of the Dryopteridaceae exhibited higher levels of glycine and tyrosine as compared to those of Pteridaceae and Polypodiaceae (Arosa *et al*., 2009).

Table 2.3: Amino acid composition of fern fronds

Species[1]	Amino acid composition (g % total amino acids)																		Total amino acids
	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val	Met	Lle	Leu	Tyr	Phe	His	Lys	Trp	Arg	
Ophioglossaceae																			
Ophioglossum pendulum	11.3	4.7	6.1	15.3	5.9	5.4	6.2	0.1	4.7	2.2	3.5	8.5	4.5	5.3	2.4	6.6	0.6	6.7	1.4
Ophioglossum reticulatum	10.8	4.6	6.6	19.2	5.7	5.4	6.5	0.1	4.2	1.5	3.3	8.0	4.7	5.1	2.2	5.6	0.3	6.2	3.2
Marattaceae																			
Angiopteris evecta	11.9	5.5	5.5	13.4	5.4	7.0	6.2	0.0	4.9	2.0	3.4	9.0	5.2	6.2	2.7	5.8	0.0	5.9	1.0
Schizaeaceae																			
Lygodium japonicum	10.2	5.2	5.3	16.0	6.4	5.7	7.1	0.1	4.9	2.1	3.2	9.3	4.8	6.2	2.2	5.4	0.2	6.1	6.9
Lygodium microphyllum	10.9	5.6	5.1	13.6	5.5	6.6	6.2	0.0	5.2	2.3	3.6	9.3	5.0	6.1	2.7	6.1	0.0	6.2	1.9
Gleicheniaceae																			
Dicranopteris linearis	11.4	5.7	4.9	13.5	5.2	6.0	6.2	0.0	5.8	2.3	3.9	9.7	4.7	6.3	2.7	5.8	0.0	5.7	2.5
Hymenophyllaceae																			
Trichomanes javanicum	11.6	5.0	5.6	12.8	6.3	7.1	6.5	0.0	4.6	2.0	2.9	9.1	5.5	6.2	2.9	6.3	0.0	5.7	2.0
Dicksoniaceae																			
Cibotium barometz	11.6	5.8	5.5	13.0	5.7	6.0	6.3	0.0	5.2	2.1	3.2	9.4	4.7	6.2	2.7	6.0	0.0	6.6	3.6

Cyatheaceae																		
Cyathea latebrosa	11.1	5.6	5.0	13.4	5.3	6.3	6.4	0.0	5.3	2.4	3.5	9.6	4.8	6.3	2.7	5.9	0.0	6.5	2.3
Pteridaceae																		
Acrostichum aureum	10.5	5.2	5.7	13.3	6.0	6.1	7.7	0.1	5.0	2.3	3.6	9.4	4.7	6.0	2.1	5.8	0.4	6.1	1.5
Adiantum latifolium	10.3	5.0	5.9	15.9	6.3	5.6	7.0	0.1	4.5	1.9	3.3	9.3	4.6	6.6	1.9	4.9	0.4	5.8	5.3
Adiantum trapeziforme	10.2	5.0	5.5	13.7	6.2	5.9	6.4	0.0	5.0	2.0	3.6	10.3	5.0	6.7	2.3	6.4	0.2	5.5	4.7
Ceratopteris thalictroides	10.9	4.8	5.5	13.9	5.8	5.6	7.8	0.0	4.9	2.3	3.6	9.9	4.3	5.5	2.0	6.2	0.2	6.8	3.6
Pityrogramma calomelanos	10.7	5.1	5.5	13.8	5.8	5.7	7.5	0.1	4.8	2.1	3.5	9.5	4.6	6.6	2.1	6.4	0.2	6.0	3.5
Pteris ensiformis	10.6	5.0	5.4	14.1	6.0	5.4	7.1	0.1	5.1	2.0	3.5	9.8	4.5	6.3	2.1	6.3	0.2	6.6	3.1
Pteris vittata	10.7	5.1	5.2	12.9	5.7	5.1	6.9	0.0	5.1	2.5	3.6	9.5	5.1	6.5	2.6	6.6	0.0	7.0	3.4
Vittariaceae																		
Vittaria ensiformis	10.1	5.0	7.4	12.7	5.9	7.5	7.3	0.1	4.7	1.8	3.4	8.8	4.4	5.5	2.8	6.7	0.3	5.5	1.6
Microlepia speluncae	10.2	4.9	5.5	15.6	6.4	5.7	6.9	0.1	4.5	2.2	3.4	9.4	4.8	6.4	2.2	6.0	0.2	5.7	1.1
Sphenomeris chusana	10.9	5.2	5.7	13.0	5.8	5.7	6.9	0.0	5.1	2.3	3.5	9.7	4.9	6.1	2.5	6.5	0.0	6.2	2.3
Thelypteridaceae																		
Christella subpubescens	11.3	5.2	6.2	12.7	6.4	5.4	6.9	0.0	4.9	2.2	3.4	9.0	5.1	6.5	2.5	6.1	0.0	6.2	3.1
Pronephrium repandum	10.1	4.7	5.7	18.9	8.4	6.0	6.1	0.1	4.3	1.8	3.3	8.3	4.3	5.6	1.8	5.0	0.2	5.1	4.9

Dryopteridaceae																			
Bolbitis heteroclita	11.4	4.8	6.2	12.1	6.2	8.4	6.6	0.0	4.5	2.0	3.2	8.6	5.6	6.1	2.8	5.8	0.0	5.7	2.9
Bolbitis x singaporensis	10.4	4.9	5.7	12.9	6.9	6.7	6.4	0.0	4.8	1.9	3.6	9.8	5.0	5.8	2.6	6.6	0.2	5.8	2.6
Tectaria griffithii	10.7	5.1	6.1	12.1	7.0	9.9	6.7	0.0	4.4	1.9	2.7	8.9	5.5	5.8	2.2	5.6	0.0	5.3	3.9
Tectaria singaporeana	11.5	5.7	6.6	13.3	7.2	6.6	6.5	0.1	4.6	1.8	3.2	8.8	5.3	7.0	2.1	4.4	0.2	5.0	3.3
Aspleniaceae																			
Asplenium longissimum	11.2	5.4	5.6	14.7	5.5	5.4	6.6	0.1	5.1	1.9	3.4	9.1	5.0	6.1	2.1	6.4	0.2	6.2	3.7
Asplenium nidus	10.4	5.1	6.0	14.9	5.3	5.8	7.1	0.1	5.0	2.2	3.4	9.3	4.9	6.0	2.4	5.3	0.4	6.3	1.2
Asplenium tenerum	10.5	5.4	6.7	14.6	6.1	5.9	6.5	0.1	4.7	1.9	3.2	8.5	4.9	6.0	2.1	6.9	0.3	5.8	3.6
Davelliaceae																			
Davallia denticulata	13.8	5.7	5.7	13.0	5.3	6.6	6.2	0.0	4.9	1.5	3.3	8.9	5.1	6.5	2.6	5.3	0.0	5.5	2.3
Davallia solida	10.1	5.0	5.6	14.1	6.7	5.7	7.1	0.0	4.9	2.1	3.6	9.6	4.5	5.7	2.3	6.2	0.2	6.7	2.8
Nephrolepis biserrata	11.6	5.7	5.4	14.0	5.2	6.1	6.4	0.0	5.0	2.3	3.5	9.0	5.1	6.8	2.7	6.0	0.0	5.4	1.3
Nephrolepis tuberosa	10.5	4.9	6.2	13.1	6.3	5.9	7.1	0.1	4.5	1.9	3.4	9.6	4.9	6.6	2.6	6.7	0.2	5.4	2.2
Blechnaceae																			
Blechnum orientale	11.2	5.3	5.6	12.9	6.1	5.4	7.0	0.0	5.1	2.3	3.3	9.3	5.0	6.4	2.5	6.2	0.0	6.5	3.2
Stenochlaena palustris	10.1	5.0	5.5	13.1	6.0	5.7	7.5	0.1	4.9	2.2	3.6	10.2	4.8	6.2	2.3	6.0	0.3	6.6	1.6

Polypodiaceae																			
Drynaria quercifolia	10.4	5.1	5.7	14.2	5.6	5.6	7.1	0.1	4.7	1.9	3.7	10.1	4.8	6.6	2.0	5.8	0.4	6.1	1.6
Goniophlebium verrucosum	11.4	5.1	6.1	13.6	5.3	5.4	6.5	0.1	4.8	2.0	3.5	9.1	4.8	6.1	2.4	6.7	0.3	7.0	3.1
Lecanopteris sinuosa	10.6	5.3	6.2	13.2	6.0	5.8	7.2	0.1	4.9	1.9	3.4	9.1	4.8	6.6	2.4	6.0	0.5	5.9	1.2
Microsorium punctatum	10.1	5.0	6.0	15.8	5.1	5.7	7.4	0.1	4.7	2.1	3.5	8.8	4.7	5.9	2.1	6.5	0.4	6.1	1.2
Platycerium bifurcatum	10.4	5.3	6.0	14.4	5.6	5.7	7.0	0.2	4.8	2.1	3.4	9.0	4.7	6.1	2.1	6.5	0.3	6.2	0.8
Platycerium coronarium	10.8	5.2	5.6	12.6	5.7	5.4	6.9	0.0	5.3	2.4	3.6	9.6	5.2	6.5	2.4	6.6	0.0	6.2	1.9
Platycerium ridleyi	11.0	5.0	5.8	15.5	5.3	5.8	7.3	0.1	4.6	1.9	3.2	8.9	4.3	6.1	2.3	6.4	0.5	5.9	1.4
Platycerium wandae	10.5	5.2	5.9	15.5	5.8	5.4	6.9	0.1	4.7	2.0	3.5	9.1	4.5	6.0	2.3	6.5	0.3	5.8	1.8
Pyrrosia lanceolata	10.5	4.8	7.4	15.0	6.7	6.1	6.1	0.6	5.5	1.8	2.2	8.6	4.1	6.1	2.3	8.2	1.2	3.0	0.4
Pyrrosia longifolia	8.9	4.7	7.2	13.5	4.6	5.2	5.9	0.2	4.5	1.9	3.6	9.2	5.2	7.2	2.8	7.5	1.5	6.3	0.4
Pyrrosia piloselloides	9.9	4.5	6.8	17.0	5.5	5.3	6.6	0.4	5.0	2.0	3.1	9.2	4.3	6.1	2.3	6.7	0.7	4.4	0.4
Marsileaceae																			
Marsilea crenulata	11.3	5.3	5.6	14.2	6.1	5.3	7.0	0.0	5.1	2.3	3.4	8.8	5.0	6.4	2.4	5.6	0.0	6.2	3.5
Salviniaceae																			
Salvinia molesta	11.3	5.4	5.0	16.0	4.8	6.5	6.2	0.0	4.9	2.1	3.6	9.0	5.1	6.2	2.6	4.9	0.0	6.3	0.8

[1]Classification according to Tyron and Tyron (1982). Source: Yeoh and Wee (1998)

2.7.4 Factors that inhibit nitrogen content in some fern species

Herz-Alla (1991) found that growth of *A. pinnata* was reduced after treatment with NaCl (0.10 %) and Na_2SO_4 (0.15 %). Similarly, Moore (1969) reported that *Azolla* plants died in rice fields when salt concentrations reached 0.15 - 0.19 % during the summer, and FAO (1978) suggest that the maximum salt level for growing *Azolla* is 0.10 %. Nitrogen fixation of the *Azolla anabaena* symbiosis is also clearly sensitive to salt stress, also reported (Herz-Alla, 1991) in *A. pinnata*. The N content of *A. filiculoides* was reduced when salt concentration was increased from 0.3 % to 0.9 % (Geshian *et al*., 1980). The sensitivity of growth and nitrogen fixation to salt stress may be attributed to Na^+ toxicity, because Rahoma (1985) found that the total N content of *Azolla* increased with increasing dilution of drainage water to reduce Na^+ concentration. Haller *et al*. (1974) found that *Azolla* plants died when cultured in soil containing about 1.3% salts (about 33 % of sea water). Other species of *Azolla* reduced water salt content by about 0.012-0.049 % and soil salt content by about 0.014-0.485 % (Lumpkin and Plucknett, 1980).

2.7.5 Physicochemical and functional properties of fern fronds and rhizome

Studies on chemical content of *Pteridium aquilinum* have demonstrated that they are abundant in protein, vitamin, and microelements necessary for human bodies like magnesium, zinc, germanium and so on (Zhang *et al*., 2008). In addition, fern rhizome starch has high value in food and medical applications. The vermicelli, cakes, and thick soup made from fern rhizome starch are ordinary processed foods in East Asian countries. From a pharmaceutical standpoint, fern rhizome starch has the functions of clearing fever and detoxification, and has special effects on the heatstroke prevention, epistaxis, toothache, and dysentery. Regular consumption of

fern rhizome starch can prevent cancer, jaundice hepatitis, tetanus, and other diseases (Chen, 1990).

Cao *et al*. (2007) reported that fern rhizome starch paste could be stretched and had a good toughness. The freeze thaw stability of fern rhizome starch was a little lower than that of potato starch and higher than that of maize starch. A study conducted by Zhang *et al*. (2011) reveals that viscosity measurement with Brabender viscograph, presented a higher peak viscosity for fern rhizome starch, which showed that it had more swelling power. Compared with corn and potato starches, fern rhizome starch had a lower transparency. Although fern rhizome starch has been used for a long time in food industry for the production of confectionary, bread, steaming bread, biscuits, and many other traditional foods, little information on the properties of this starch was reported.

2.8 Role of Protein

Proteins are nitrogen containing substances that are formed by amino acids. They serve as the major structural component of muscle and other tissues in the body. In addition, they are used to produce hormones, enzymes and haemoglobin. Proteins can also be used as energy; however, they are not the primary choice as an energy source. For proteins to be used by the body they need to be metabolized into their simplest form, amino acids. There have been 20 amino acids identified that are needed for human growth and metabolism. Twelve of these amino acids (eleven in children) are termed nonessential, meaning that they can be synthesized by our body and do not need to be consumed in the diet. The remaining amino acids cannot be synthesized in the body and are described as essential meaning that they need to be

consumed in our diets. The absence of any of these amino acids will compromise the ability of tissue to grow, be repaired or be maintained (Hoffman and Falvo, 2004).

2.9 Protein Assessment

The composition of various proteins may be so unique that their influence on physiological function in the human body could be quite different. The quality of a protein is vital when considering the nutritional benefits that it can provide. The quality of a protein is determined by assessing its essential amino acid composition, digestibility and bioavailability of amino acids (FAO/WHO, 1990).

2.10 Protein Sources

Protein is available in a variety of dietary sources. These include foods of animal and plant origins as well as the highly marketed sport supplement industry. Determining the effectiveness of a protein is accomplished by determining its quality and digestibility. Quality refers to the availability of amino acids that it supplies, and digestibility considers how the protein is best utilized. Typically, all dietary animal protein sources are considered to be complete proteins. That is, a protein that contains all of the essential amino acids. Proteins from vegetable sources are incomplete in that they are generally lacking one or two essential amino acids. Thus, someone who desires to get their protein from vegetable sources (i.e. vegetarian) will need to consume a variety of vegetables, fruits, grains, and legumes to ensure consumption of all essential amino acids. As such, individuals are able to achieve necessary protein requirements without consuming beef, poultry, or dairy (Hoffman and Falvo, 2004).

2.10.1 Animal source

Proteins from animal sources (i.e. eggs, milk, meat, fish and poultry) provide the highest quality rating of food sources. This is primarily due to the 'completeness' of proteins from these sources. Although protein from these sources are also associated with high intakes of saturated fats and cholesterol, there have been a number of studies that have demonstrated positive benefits of animal proteins in various population groups (Campbell *et al*., 1999; Pannemans *et al*., 1998). There have been a number of health concerns raised concerning the risks associated with protein emanating primarily from animal sources. Primarily, these health risks have focused on cardiovascular disease (due to the high saturated fat and cholesterol consumption), bone health (from bone resorption due to sulfur-containing amino acids associated with animal protein) and other physiological system disease (Hoffman and Falvo, 2004).

2.10.2 Plant source

Vegetable proteins, when combined to provide for all of the essential amino acids, provide an excellent source for protein considering that they will likely result in a reduction in the intake of saturated fat and cholesterol. Popular sources include legumes, nuts and soy. Aside from these products, vegetable protein can also be found in a fibrous form called Textured Vegetable Protein (TVP). TVP is produced from soy flour in which proteins are isolated. TVP is mainly a meat alternative and functions as a meat analog in vegetarian hot dogs, hamburgers, chicken patties, etc. It is also a low calorie and low fat source of vegetable protein. Vegetable sources of protein also provide numerous other nutrients such as phytochemicals and fiber that are also highly regarded in the diet (Hoffman and Falvo, 2004).

2.11 Effects of Animal and Vegetable Protein

The effects of various animals to vegetable protein ratio intake have been examined by Sellmeyer and colleagues (2001) in elderly women (> 65 y). They showed that the women consuming the highest animal to vegetable protein ratio had nearly a 4-fold greater risk of hip fractures compared with women consuming a lower animal to vegetable protein ratio. Amusingly, they did not account for any significant difference associated between the animal to vegetable protein ratio and bone mineral density. Feskanich *et al*. (1996), also showed similar results but in younger female population (age range = 35–59, mean 46). On the contrary, other studies examining older female populations have shown that elevated animal protein will increase bone mineral density, while increases in vegetable protein will have a lowering effect on bone mineral density (Munger *et al*., 1999; Promislow *et al*., 2002). Munger and colleagues (1999) also reported a 69% lower risk of hip fracture as animal protein intake increased in a large (32,000) postmenopausal population. Other large epidemiological studies have also confirmed elevated bone density following high protein diets in both elderly men and women (Dawson-Hughes *et al*., 2002; Hannan *et al*., 2000). Hannan and colleagues (2000) demonstrated that animal protein intake in an older population, several times greater than the RDA requirement, results in a bone density accruement and significant decrease in fracture risk.

High protein diets are associated with an increase in calcium excretion. This is apparently due to a consumption of animal protein, which is higher in sulfur based amino acids than vegetable proteins (Barzel and Massey, 1998). Sulfur based amino acids are thought to be the primary cause of calciuria (calcium loss). The mechanism behind this is likely related to the increase in acid secretion due to the elevated protein consumption. If the kidneys are unable to buffer the high endogenous acid

levels, other physiological systems will need to compensate, such as bone. Bone acts as a reservoir of alkali, and as a result calcium is liberated from bone to buffer high acidic levels and restore acid-base balance. The calcium released by bone is accomplished through osteoclast mediated bone resorption (Arnett and Spowage, 1996). Bone resorption (loss or removal of bone) will cause a decline in bone mineral content and bone mass (Barzel, 1976), increasing the risk for bone fracture and osteoporosis.

Dawson-Hughes *et al*. (2002), has also confirmed the effect of increased in animal protein intake leading to an increase urinary calcium excretion and lowering concentrations of the bone resorption. However, there is no evidence as to the amount of protein intake that will help alleviate this problem resulting in confusion regarding protein intake and bone. It is likely that other factors play an important role in further understanding the influence that dietary proteins have on bone loss or gain. For instance, the intake of calcium may have an essential function in maintaining bone. A higher calcium intake results in more absorbed calcium and may offset the losses induced by dietary protein and reduce the adverse effect of the endogenous acidosis on bone resorption (Dawson-Hughes, 2003).

2.12 Properties of Proteins in Food Systems

Through their provision of amino acids, proteins are essential to human growth, but they also have a range of structural and functional properties which have a profound impact on food quality. Proteins play a fundamental role not only in sustaining life, but also in foods derived from plants and animals. Foods vary in their protein content and even more so in the properties of those proteins. In addition to their contribution to the nutritional properties of foods through provision of amino acids

that are essential to human growth and maintenance, proteins impart the structural basis for various functional properties of foods (Yada, 2004).

2.12.1 Factors affecting properties of proteins in food systems

Intrinsic factors, extrinsic or environmental factors, and processing treatments or other intentional modifications can all contribute to influence the chemical and functional properties of proteins in food systems, as depicted in Table 2.3. Intrinsic factors include the basic chemical and physical properties of the amino acids comprising a particular protein. The distribution of nonpolar and polar or charged side chains on the surface protein plays a key role in the chemical properties and ultimately the functions of the proteins.

2.12.1.1 Protein interactions with other food constituents: Major food constituents such as water, other proteins, lipids and carbohydrates, as well as minor constituents such as salts, metal ions, acidulants, flavour components and phenolic compounds interact with proteins to shape chemical and functions of proteins (Yada, 2004).

Water molecules that are hydrogen bonded to protein molecules may be critically important for the structural stability of the protein (Damodaran, 1996). The balance between protein-water interactions versus protein-protein interactions is important in functional properties such as swelling, water binding capacity and solubility of protein ingredients, as well as their ability to form network structures such as gels or films including those surrounding foam bubbles.

Salts may promote either solubilization (salting-in) or precipitation (salting-out) of proteins, depending on the concentration and nature of the salt involved (Li-Chan, 1996). The increased β-sheet content that has been observed in the salt induced

aggregated state may be attributed to the relatively large surface area and opportunities for hydrogen bonding provided by the β-sheet structure (Przybycien and Bailey, 1991). Furthermore, the weaker strength of water hydration to β-sheet than to α-helix structures, due to different geometry of the water-carbonyl group interactions in these secondary structure conformations, may influence the changes in water-protein and protein-protein interactions that favour aggregate or network formation (Li-Chan and Qin, 1998).

Acidulants alter the net charge and isoelectric point of the protein molecule and may also affect the local distribution of positive or negative charges on the protein surface, again with possible alterations of the protein-solvent and protein-protein balance and associated properties. The specific binding of metal ions can also affect stability of proteins (Yada, 2004).

Carbohydrates, with their multiple hydroxyl groups, may contribute to the structural stability of proteins, either by exclusion of the carbohydrate from the protein surface resulting in preferential hydration of the protein, or by interaction of the carbohydrate with hydroxyl or ionic functional groups of the protein molecule (MacDonald *et al*., 2000). These carbohydrate-protein interactions affect stability of food proteins to processes such as thermal treatment, dehydration or frozen storage, and are the basis for addition of ingredients such as sucrose or sorbitol to stabilize fish muscle proteins during frozen storage or drying (MacDonald *et al*., 2000). Attractive (complexation) or repulsive (segregation) interactions may occur between proteins and anionic polysaccharides such as carrageenan or pectin, leading to precipitate or gel formation, depending on conditions such as biopolymer concentration, pH and ionic strength (Dickinson, 1998).

Nonpolar residues of proteins are primarily responsible for hydrophobic interactions with lipid molecules at oil water interfaces such as emulsions (Howell *et al*., 2001) although electrostatic, covalent, hydrogen and hydrophobic forces may all contribute to protein-lipid interactions (Alzagtat and Alli, 2002). Protein-lipid interactions may also be implicated in protein-protein interactions; for example, protein-lipid complexes in wheat gluten have been associated with the lipid-mediated aggregation of high and low molecular weight polypeptides in the gliadin fraction (Carcea and Schofield, 1996).

Table 2.4: Examples of structural or chemical properties and functional properties of food protein

Structural or chemical properties			Functional properties		
Intrinsic factors	Extrinsic factors	Processing	Surface or interface	Hydrodynamic	Bioactivity
amino acid composition 1^o, 2^o, 3^o, 4^o structures conjugates subunits	pH redox status temperature salt, other ions solvent other major or minor constituents	heating cooling, freezing drying concentrating storage shear force pressure chemical or enzymatic modification	solubility wettability dispersibility foaming fat binding flavour binding	viscosity thickening gelation coagulation film formation	enzyme hormone antimicrobial antihypertensive immunomodulatory antioxidant opioid

Source: Adapted from Damodaran (1996)

2.13 Chemical and Physical Properties of Food Proteins

Proteins play a fundamental role not only in sustaining life, but also in foods derived from plants and animals. Foods vary in their protein content and even more so in the properties of those proteins. In addition to their contribution to the nutritional properties of foods through provision of amino acids that are essential to human growth and maintenance, proteins impart the structural basis for various functional properties of foods (Yada, 2004).

2.13.1 Molecular forces affecting physicochemical and functional properties

The three-dimensional as well as quaternary structure of a protein in its native state is dependent not only on the covalent bonds that link the amino acid residues in sequence or that create disulfide bonds between cysteinyl residues, but also on the numerous non-covalent interactions that occur within the protein molecule, or between the protein molecule with solvent molecules (usually water in the case of food systems), or between proteins and other molecules in the food system. These molecular forces are instrumental in stabilization of the protein's folded structure in a specific native conformation. They influence molecular flexibility, size and shape of the protein molecule. Furthermore, these forces may be involved in intermolecular interactions including aggregation (Yada, 2004). Therefore molecular forces play an important role in determining the physicochemical and functional properties of the protein.

2.14 Response Surface Methodology

When many factors and interactions affect desired responses in a certain process designs, response surface methodology (RSM) becomes an effective tool for optimizing the process (Triveni *et al.*, 2001). RSM is a statistical design of experiments that involves a collection of mathematical and statistical techniques essential for modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response (Montgomery, 2005). The main idea of RSM is to use a sequence of designed experiments to obtain an optimal response. The most widespread applications of RSM are in the particular situations where several input variables potentially influence some performance measure or quality characteristic of the process. Thus

performance measure or quality characteristic is called the response. The input variables are sometimes called independent variables.

Independent variable and dependent variable are used to differentiate between two types of quantities being considered, assorting them into those available at the start of a process and those being created by it, where the latter (dependent variables) are dependent on the former (independent variables). The independent variable is typically the variable representing the value being manipulated or changed and the dependent variable is the observed result of the independent variable being manipulated. For example, the protein recovery from a plant is affected by certain factors including solvent types χ_1, solvent concentration χ_2, time χ_3 and pH χ_4. Therefore, solvent types, solvent concentration, time and pH can vary continuously. When treatments are from a continuous range of values, then a response surface methodology is useful for developing, improving and optimizing the response variable (Bradley, 2007). In this case, the protein recovery y is the response variable and it is a function of solvent type, solvent concentration, time and pH. This can be expressed as

$$y = f\,(x_1, x_2, x_3, x_4) + \varepsilon$$

The variables x_1, x_2, x_3 and x_4 are independent variables where the response y depends on them. The dependent variable y is a function of x_1, x_2, x_3, x_4, and represents the noise or error observed in the response y. The surface represented by f (x_1, x_2, x_3, *and* x_4) is called a response surface. The response can be represented graphically, either in the three-dimensional space or as contour plots that aid visualize the shape of the response surface. In addition to analyzing the effects of the independent variables, the experimental methodology also generates a mathematical model that describes the overall process (Batista, 1999).

The advantages of using RSM have been reported to include reduction in the number of experimental trials needed to evaluate multiple parameters and the ability of the statistical tool to identify interactions. Response surface methodology has been widely used for identification of the best condition and extraction yield optimization in preparing partially defatted peanut (Badwaik *et al*., 2012), protein from freeze dried fish waste (Nurdiyana *et al., 2008*), protein from palm kernel meal (Arifin *et al*., 2009) and protein from defatted corn (Jauricque *et al*., 2011).

2.15 Extraction and Isolation of Proteins

Extractable protein determines the amount of protein that can be made available from a particular source for food and non-food application. One of the preliminary factors that determine whether or not a protein source could be embraced for commercial using is the protein extraction efficiency of such protein (Liu, 1997). Protein extraction and isolation can be effective in reducing many of the antinutritional problems, while creating a protein rich commodity with marketable nutritional and functional properties. Protein isolation is a very important step when incorporating proteins from oil-producing plants into food products. Protein isolates are the most refined protein product containing the greatest concentration of protein, but unlike protein flour and concentrates, contain no dietary fiber. They are very digestible and easily introduced into foods such as sports drinks and health beverages as well as infant formulas (Hoffman and Falvo, 2004).

2.15.1 Factors that affect protein extraction

There have been different procedures in the extraction of proteins to produce a suitable mixture for use in food system. The first step in obtaining an enriched protein product is to extract the raw material with a suitable solvent which is usually

an aqueous solvent. Other factors apart from the source of raw material that intend to influence the extraction include the nature of the raw material and type of protein (Yada, 2004).

2.15.1.1 Source of raw material: Most raw materials contain oil which tends to influence the extraction process. Raw materials with high level of oil need to be defatted prior to protein isolation in order to prevent emulsion formation during protein extraction and produce oil free protein materials. To obtain samples that are free from fat, raw materials are first defatted with solvents such as hexane (Kumagai *et al*., 2002) and petroleum ether (Sathe *et al*., 2002). Those raw materials containing high levels of oil can be grind and mechanically press (Shrestha *et al*., 2002) before being defatted with hexane.

2.15.1.2 Nature of raw material: Some seed proteins contain high levels of phytate, phenolic compounds and other contaminants that may be toxic, interfere with protein isolation (reduction in yield), or contribute to discoloration, off-flavour, or reduced functionality of the isolated protein. Pawar and others (2001) emphasized the need for good isolation procedures that reduce antinutritional compounds such as phytates and polyphenols. The influence of phytates and polyphenols on the gelation of sunflower proteins was reported by Pawar and others (2001). There is not a unique methodology or protocol for extracting protein from oilseeds or flours leading to optimal products reviewed by Moure and others (2006). The presence of isoflavones in protein isolate products has been given considerably more attention than other compounds present in protein isolate. Isoflavones are thought to be beneficial for cardiovascular health, possibly by lowering low-density lipoprotein (LDL) concentrations (Crouse *et al*., 1999) increasing LDL oxidation (Tikkanen *et*

al., 1998) and improving vessel elasticity (Nestel *et al*., 1999). However, these studies have not met without conflicting results and further research is still warranted concerning the benefits of isoflavones.

The level of cell wall in some plant material influences protein extraction as seen in rice bran (Wang *et al*., 1999). This has call for the use of carbohydrases to break down the cell wall materials to facilitate preparation of protein isolates. To produce a rice bran protein isolate, the defatted rice bran is mixed with water, adjusted to pH 5.0 and treated with phytase and xylanase to degrade phytic acid and break down the cell walls, respectively (Wang *et al*., 1999).

2.15.1.3 Type of protein (solubility properties): Differential solubility of protein in different solutions depends on the pH and ionic strength of the solution. In order to enrich protein isolate, specific solution is normally design to extract sample leading to the removal of protein of interest while other proteins remain in solution or are left as residue. Preparative scale isolation of β-lactoglobulin from whey protein isolate (WPI) was achieved by taking advantage of the solubility of the protein at pH 2.0 in the presence of 7% (w/v) NaCl and at these conditions other whey proteins are insoluble (Mate and Krochta, 1994). The solubility properties of cottonseed protein isolate has been used to precipitate storage proteins and non-storage protein at pH 4.0 and pH 7.0 respectively (Uzzan, 1988). Both techniques make use of water to prevent proteolysis (Sathe and Salunkhe, 1981) and for the protein to precipitate by acidifying to adjust the pH.

2.16 Protein Solubilization

Protein extraction usually involves the use of acid, alkaline and saline solution (Eromosele *et al*., 2008). Different types of protein contained in raw materials would

favour certain treatment but the most common solvent seen in the literature is an aqueous alkaline solution and this has been used for Karkade (*Hibiscus sabdariffa*) (Abu-Tarboush, 1995), amaranth seeds (Ventureira *et al*., 2011), sesame seed (Bandyopadhyay and Ghosh, 2002). The use of alkali as a solvent for protein extraction is now well-liked due to its high degree of solubility that can be achieved. The fact that all these researchers made use of alkaline in their protein extraction, the pH of the solution is very important in order to obtain a higher protein yield. Higher pH values, especially pH 12 and above, (Deng *et al*., 1990) have been found to increase the formation of lysinoalanine. This has resulted in researchers preferring a lower pH alkaline solution to solubilize proteins.

Apart from NaOH, other solubilizing agents such as salt and enzymes are popular in solubilizing proteins. Salts including NaCl, phosphate salts, such as NaH_2PO_4 and sodium hexametaphosphate (SHMP), have also been used to enhance protein extraction (Bland and Lax, 2000). NaCl extraction has been used for a number of proteins including flax (Oomah *et al*., 1994), safflower (Ordorica-Falomir *et al*., 1990; Paredes-Loâpez, 1991), sunflower (Shamanthaka-Sastry and Narasinga Rao, 1990) and canola (Murray, 2001a; 2001b). Apart from NaCl being used as food ingredient, it avoids using pH extremes and the associated changes in protein structure.

Alternatively, enzymes including proteolytic enzymes, such as papain (Cai *et al*., 1996) and pectinase (Sen and Bhattacharyya, 2001) made protein soluble to be precipitated at pH 4.8-5.0. Protein fractions containing 78% protein and low levels of CGA and carbohydrates have been obtained with this approach (Parrado *et al*., 1991).

2.17 Protein Recovery

Researchers have used various procedures in precipitating proteins from solutions. Protein extracted using alkaline solution is mostly precipitated with pH adjustment. Maximum protein precipitation has been reported at pH 4.0 (Klockeman *et al*., 1997), with only about 53% of the protein precipitates (Chen and Rohani, 1992). For Karkade seed flour protein, a pH range of 3.0 to 5.0 was used (Abu-Tarboush, 1995). This finding agrees with data given by other investigators. Hang *et al*., (1970) reported that several bean proteins, namely mung bean, pea bean and red kidney bean have a common point of minimum precipitation at pH 4.0

The precipitate contains mostly the protein of interest and is collected by centrifugation (Kumagai *et al*., 2002). The protein isolate may be neutralized to pH 6.9-7.2 with dilute alkali (usually NaOH) to give a more soluble product called proteinate. The supernatant that remains after precipitated protein isolate has been removed contains soluble proteins that can be recovered by membrane processing. The supernatant is processed by ultrafiltration and diafiltration to remove low molecular weight substances, especially salts and peptides to produce a retentate, which is freeze dried as the soluble protein isolate (Xu *et al*., 2003).

2.18 Functional Properties of Proteins in Foods

Proteins have many useful functional properties in foods that enable it to perform a specific role or function in a food. For example, a protein with the ability to form a gel may be used in a food with the specific intention of forming a gel, as in use of gelatin to make jelly. Functional properties or roles of proteins in foods include solubility and nutritional value. They also may be used as thickening, binding, or gelling agents and as emulsifiers or foaming agents. The functional properties of a

specific protein depend on its amino acid composition and sequence since these determine the conformation and properties of the protein. Although no single protein exhibits all the functional properties, most proteins may perform several different functions in foods, depending on the processing conditions. Some proteins are well known for specific functional properties in foods (Vaclavik and Christian, 2008).

Protein isolation is only the first step towards incorporating proteins from oil producing plants into food products. To reap the benefits of these nutritious proteins, it is critical that their incorporation into food products be such that the food remains appealing to the consumer. Functional properties of proteins provide valuable information as to how effectively proteins will be able to do this. The functional properties that seem to receive the most attention when characterizing isolated proteins are solubility, water absorption capacity (water-holding capacity), fat-binding capacity, emulsification activity and stability, foaming capacity and stability and gelation. The tendency when evaluating functional properties of proteins from oil producing plants has been to compare them to soybean proteins or to evaluate changes in these properties as a function of conditions of preparation or handling. Comparison of functional properties of different oil producing proteins according to Arntfield (2004) is difficult because of the influence of the protein isolation technique and the variability in techniques used to evaluate functional properties.

2.18.1 Solubility and absorption capacity

Solubility is also affected by the type and concentration of salts in a food system. As salts increase in concentration proteins become more soluble. Solubility is often used as an indicator of other functional properties. However, solubility data for protein from oil producing plants is limited as solubility is often influenced by the

isolation method used. As many of these proteins are globulins (salt soluble), an evaluation of water solubility is not always beneficial. Nevertheless, in a study by Oomah and Mazza (1993), a low mucilage protein isolate from flax seed was shown to have better water-absorption properties than a soybean protein isolate. The pH used for protein precipitation affected the solubility of safflower protein with higher solubility when the protein was precipitated at pH 6 rather than pH 5 (Betschart *et al*., 1979). Testing protocol can also have an impact on solubility. Proteins in a concentrate prepared from sesame seeds, like most plant proteins, had reduced solubility around the isoelectric point, but if salt was included during the evaluation of solubility, protein solubility increased (Inyang and Iduh, 1996).

2.18.2 Fat-binding and emulsification properties

This pertains to the behaviour of the protein when incorporating proteins in mixed systems. Safflower proteins that had been neutralized to pH 7 following precipitation at pH 5 exhibited fat binding and emulsification properties that were equivalent to soybean (Betschart *et al*., 1979) while the fat absorption and emulsification properties of a low mucilage flax protein isolate were superior to those from a soybean protein isolate (Oomah and Mazza, 1993). Fat absorption and emulsification properties of canola protein were reported to be good, but were not compared to soybean (Thompson *et al*., 1982). Pawar and others (2001) demonstrated that oil absorption and emulsification properties can be influenced by the protein isolation technique, which can be improved when antinutritional factors, such as phytates and polyphenolic compounds, are reduced during isolate preparation. In a study by Inyang and Iduh (1996), emulsifying activity of a sesame protein concentrate increased as the pH or salt concentration during evaluation was increased and emulsion stability was also improved by including salt during testing.

2.18.3 Foaming properties

Foams are the result of the behaviour of proteins at air-water interfaces. Rapid diffusion of molecules to the interface followed by molecular rearrangement allows these films to entrap air. Foaming properties are influenced by the pH at which protein precipitate. Safflower protein precipitated at pH 6.0 exhibited good foaming properties than when precipitated at pH 5.0 (Betschart *et al*., 1979). Reduction of phytates and polyphenolic compounds during isolate preparation of sunflower protein had improved foaming capacity compared to isolates with higher levels of phytates and polyphenolics (Pawar *et al*., 2001).

2.18.4 Gelation

Among commercial proteins used in the food industry, gelatin has been regarded as both special and unique serving multiple functions with a wide range of applications in various industries (Karim and Bhat, 2008). Nonmeat proteins are often used as alternative gelling agents in comminuted meat products to enhance the yield and texture by improving water-binding properties (Pietrasik and others, 2007).

Isolated sesame globulins were shown to produce harder gels with less syneresis gels from soy protein (Yuno-Ohta *et al*., 1994), but gels from canola protein isolates were generally not as good as those from soybean (Owen *et al*., 1992). To produce gels from canola protein upon heating, at least 5.4% protein was required in the system to get any gel formation at pH 7 (Gill and Tung, 1976), and if the pH during testing was increased stronger gels were created (Leâger and Arntfield, 1993). The addition of phytates or phenolic compounds, however, resulted in weaker, less elastic gels even at pH 8.5 (Arntfield, 1996). Although the inclusion of salt was able to improve gel strength in the presence of phenolic compounds, the elasticity of the

network remained poor (Arntfield, 1996). A similar influence of the phytates and polyphenols on the gelation of sunflower proteins was reported by Pawar *et al.* (2001). These data emphasize the need for isolation procedures which reduce these compounds. Calcium content and hydrophobicity are predictors of gel strength (Kohnhorst and Mangino, 1985).

2.19 Effect of Drying Methods on the Functional Properties of Protein Isolate

Drying, the last step in the preparation of protein isolate could be carried out by various methods such as freeze drying, spray drying or vacuum drying. The method use in drying protein isolate after protein recovery could influence the functional properties. Cepeda *et al.* (1998) reported that the spray dried faba bean protein flour was better than a freeze dried product in terms of solubility. Similarly, Kalapathy *et al.* (1997) reported that spray dried SPIs treated with Na_2SO_3 had higher solubility, water-holding capacity and viscosity compared with freeze dried products. Their report concluded that freeze drying is usually used in laboratory experiments, while spray drying is utilized in industrial processes and vacuum drying is applied in both cases. Hence, the research results on functional properties of freeze dried protein isolate in laboratory experiments may not have practical applicability. Whilst these and other such studies report a positive correlation between spray drying and functional properties of protein isolate much has not been investigated on solar drying of protein isolate to see it effect on the functional properties. The direct evidence of the effects of various drying methods on the functional properties of protein isolate is based on experiments using the same drying method that is consistent with industrial practice. It is very important that the drying method must be taken into consideration in the research of the functional properties of proteins.

2.20 Protein Separation

To compare proteins from two different types of plants, scientists sometimes carry out an experiment to separate the proteins from each other, so they can be examined separately a process called electrophoresis. The most common type of electrophoresis performed with proteins is zonal electrophoresis in which proteins are separated from a complex mixture into bands by migration in aqueous buffers through a solid polymer matrix called a gel. Separation depends on the friction of the protein within the matrix and the charge of the protein molecule (Nielsen, 2010). The banding pattern that forms in an electrophoresis experiment is distinct for each set of proteins. Comparison of the same protein in different species can show different banding patterns in the different species. Researchers have used this sort of distinguishing information to identify to what plant species an individual plant belongs.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Source of raw materials

Two Ghanaian types of fern leaves samples were studied. They are *Nephrolepis biserrata* and *Arthropteris orientalis*. The *Nephrolepis biserrata* also known as aquatic fern was collected from a stream, along the Mango road, within the Kwame Nkrumah University of Science and Technology (KNUST) in one location. The *Arthropteris orientalis* was also harvested from palm trees along the Buroburo road of KNUST, Kumasi-Ghana.

3.1.2 Sample preparation

The fern leaves samples were sorted to remove unwanted materials, washed and solar-dried for two weeks. The dried fern leaves were milled into flour using MPE roller mills (Model GP-140 Grinder, Shanghai-China) at the Pharmacy Department, KNUST, Kumasi. The particle size was further reduced using blender and sieved to a particle size of 25 µm. The fern flour sample was stored in an airtight container under refrigeration at 4 ºC for further analysis.

3.1.2.1 Preparation of defatted fern flour (DFF): The flour was defatted using the cold extraction method by soaking the flour (tied in a cheese cloth) in hexane using a ratio of 1:10 w/v, with respect to flour/solvent. The set up was sealed and left for 48 h (2 days) at room temperature after which the defatted meal was solar dried to expel the residual solvent and stored in high density polyethylene bags under room temperature (25 ºC) until when needed.

***3.1.2.2 Preparation of sample for Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis* (SDS-PAGE):** About 0.5 g of protein isolate of each fern type was dissolved in 100 µL of 1x SDS sample buffer in a centrifuge tube. The centrifuge tube and its content was centrifuged (Model: Sorvall® RC 5C) at 5000 rpm for 10 min at 4 ^{O}C and heated for 3 min in dry bath (Fisher Isotemp® Dry Bath, model 154) at 95 ^{O}C. The tubes containing the samples were then stored in refrigerator for SDS-PAGE.

3.2 Methods

3.2.1 Proximate analyses

Analysis of fern leaves flour and its defatted fern flour was carried out according to AOAC (2005) as follows:

3.2.1.1 Moisture: About 2.0 g of flour was weighed and transferred into a previously weighed glass crucible and placed in a hot air oven to dry at 105 °C until a constant weight was obtained. Samples were cooled in a desiccator, weighed, and returned to the oven to dry to constant weight. Measurements were taken in triplicate. Loss in weight was calculated as percentage moisture (AOAC, 2005) as shown in Appendix 1.

3.2.1.2 Ash: About 2 g of sample in a pre-weighed porcelain crucible was combusted in a muffle furnace at 600 °C for 2 h. The crucibles containing ash were cooled and re-weighed. Loss in weight was calculated as percentage ash content (AOAC, 2005) as shown in Appendix 1.

3.2.1.3 Crude fat: The sample used for moisture determination was transferred into a paper thimble, labeled and put into a thimble holder for the crude fat

determination. About 200 mL of hexane was poured into a pre-weighed 500 mL round bottom flask and assembled on a semi-continuous soxhlet extractor and refluxed for 16 h. The hexane was recovered after removing the paper thimble from the thimble holder and the flask containing the fat was heated for 30 min in an oven at 103 ^{o}C to get rid of the residual hexane. The flask containing the fat was re-weighed after being cooled in a desiccator. The increase in weight was calculated as percentage crude fat (AOAC, 2005) as shown in Appendix 1.

3.2.1.4 Protein: The protein determination of fern flour was carried out by the Kjeldahl method in three steps: digestion, distillation and titration.

Digestion: About 2 g of fern flour sample was weighed into a digestion flask and mixed with 25 mL concentrated H_2SO_4, selenium catalyst and few anti-bumping agents. The flask and its content were digested by heating in a fume chamber till the colour of the solution turned clear.

Distillation: After the digestion has been completed the digestion flask was allowed to cool and the solution was transferred into a 100 mL volumetric flask and the volume made up to the 100 mL mark with distilled water. The distillation apparatus was flash out with water and 10 mL of digested sample was transferred into the distillation apparatus. The solution was neutralize with 18 mL NaOH and boiled under distillation water in a steam generator. Circulation was allowed for about 10 min. A conical flask was filled with 25 mL of 2 % boric acid and 3 drops of mixed indicator (methylene blue and methylene red) added. The conical flask and its content were placed under the condenser in a position where the tip of the condenser was completely immersed in solution for 10 min and end of condenser washed with distilled water.

Titration: The nitrogen content was then estimated by titrating the ammonium borate formed in the conical flask with 0.1 M HCl solution. Titre values of the replicate samples were recorded and percentage nitrogen calculated (AOAC, 2005) as shown in Appendix 1. A blank sample was run at the same time as the sample was being analyzed.

3.2.1.5 Crude fiber: A defatted sample of about 2.0 g was weighed and transferred into a flat bottom flask and 200 mL of 1.25 % H_2SO_4 was added. The flask was connected to condenser over a hot plate and refluxed for 30 min after the first drop of condensate. Flask was disconnected from condenser and filtered through a clean cheese cloth. The residue was then washed with lots of boiling water until filtrate is no longer acidic by checking with blue litmus paper. The residue was transferred quantitatively back into the flask and 200 mL of 1.25 % NaOH was added and refluxed for 30 min. The content was filtered through cheese cloth and washed with boiling water until filtrate is no longer basic by checking with red litmus paper. The residue was transferred into a dry porcelain crucible and dried in an air oven at 105 °C for 4 h. The dried sample plus the porcelain crucible after cooling in a desiccator was weighed and recorded. It was then placed in a muffle furnace preset at 600 ^{o}C and combusted for 2 h and re-weighed after cooling in a desiccator. The difference in weight was calculated as percentage crude fiber (AOAC, 2005) as showed in Appendix 1.

3.2.1.6 Available carbohydrate: The available carbohydrate content present was determined by subtracting all the other proximate determinations from 100 % (AOAC, 2005) as showed in Appendix 1.

All values were expressed as means of triplicates for all the analysis.

3.2.2 Extraction design of protein extract

3.2.2.1 Experimental design: Extraction of protein was conducted using the Design Expert software version 7.0 (2007). Response Surface Methodology (RSM) was used in this study to determine the optimum conditions for the extraction of protein from the defatted fern flour samples. For alkaline treatment, the effect of two independent variables i.e., NaOH concentration and time were investigated. The constraint of component proportion is shown in Table 3.1. For saline treatment, the effect of two independent variables i.e., NaCl concentration and time were investigated. The range of the variables is shown in Table 3.2. For alcohol treatment, the effect of two independent variables: ethanol concentration and time were investigated. The range of the variables is shown in Table 3.4.

Table 3.1: Constraint for alkaline treatment

Parameters	Lower limit	Upper limit
NaOH concentration (M)	0.01	0.1
Time (min.)	30	60

Table 3.2: Constraint for saline treatment

Parameters	Lower limit	Upper limit
NaCl concentration (M)	0.01	0.1
Time (min.)	30	60

Table 3.3: Actual level of independent variables for NaOH and NaCl

Runs	Independent variables	
	Solvent concentration (M)	Time (min.)
1	0.01	30
2	0.1	30
3	0.01	60
4	0.1	60
5	0.055	45
6	0.055	45
7	0.055	45
8	0.055	45
9	0.01	45
10	0.1	45
11	0.055	30
12	0.055	60
13	0.055	45
14	0.055	45
15	0.05	45
16	0.05	45

Table 3.4: Constraint for optimal extraction using NaOH

Parameters	Lower limit	Upper limit
NaOH concentration (M)	0.05	0.15
Time (min.)	10	60

Table 3.5: Actual level of independent variables for optimal extraction using NaOH

Runs	Independent variables	
	Solvent concentration (M)	Time (min.)
1	0.05	10
2	0.05	60
3	0.05	35
4	0.1	35
5	0.1	35
6	0.1	35
7	0.1	35
8	0.1	10
9	0.1	60
10	0.1	35
11	0.1	35
12	0.1	35
13	0.1	35
14	0.15	10
15	0.15	60
16	0.15	35

Table 3.6: Constraint for ethanol treatment

Parameters	Lower limit	Upper limit
Ethanol concentration (%)	50	70
Time (min.)	10	60

Table 3.7: Actual level of independent variables for ethanol

Runs	Independent variables	
	Solvent concentration (%)	Time (min)
1	50	10
2	50	60
3	50	35
4	60	35
5	60	35
6	60	35
7	60	35
8	60	10
9	60	60
10	60	35
11	60	35
12	60	60
13	60	35
14	70	10
15	70	60
16	70	35

3.2.3 Extraction of protein

Protein was extracted based on a method described by El-Tinay *et al*. (1988). The effect of time and solvent concentration on the extractability of protein from defatted fern flour was determined by dispersing 0.5 g of sample in 25 mL of the three solvents in conjunction with different levels of independent variables as shown in Tables 3.3, 3.5 and 3.7. Samples were agitated at room temperature for the specified extraction periods in an orbital shaker (Gallenkamp orbital shaker, London-UK) at 150 rpm. The solubilized liquor was separated from insoluble material by centrifugation at 2500 rpm for 15 min at room temperature (25 ^{o}C).

3.2.4 Protein determination

Aliquots were taken from the supernatant solutions (from the runs) for protein estimation (Table 3.3, 3.5 and 3.7) by means of standard curve according to the method of Bradford (1976) using the Coomassie protein assay reagent with egg albumin as the standard. The absorbance was measured at 595 nm using spectrophotometer (UV/VIS 1601, Shimadzu, Tokyo-Japan). The absorbance generated for the runs were converted to amount of protein extracted as shown in Table 4.2 – Table 4.6 using the equation generated from the standard curve (Appendix 2).

3.2.5 Protein isolation using optimized condition

Prior to maximum protein extraction, aliquots were taken from the supernatant of NaOH and ethanol extraction to determine the pH at which protein would precipitate for both NB and AO. The solvent that exhibited higher protein with its condition was used to extract more of the supernatant for protein isolate. The supernatant produced from the optimized condition after centrifugation was acidified using concentrated HCl to a pH range of 2.3 to 2.5 where protein precipitated after which it was centrifuged at 3000 rpm for 15 min. The precipitate was washed repeatedly with distilled water after which the precipitated proteins were freeze-dried (Labconco freeze system 12, Kansas-U.S.A.) and Kjeldahl analysis run (% N x 6.25) to obtain percentage protein content in isolate. The fern protein isolates were then refrigerated for further analysis.

3.2.6 Determination of functional properties

3.2.6.1 Protein solubility: The solubility of protein isolates as a function of pH was determined according to the method described by Dench *et al*. (1981) with slight

modification. One-hundred milligram samples of each freeze-dried protein isolate were suspended in 20 mL of distilled water and the pH of the suspensions was adjusted from 2.0-14.0 with an interval of 2 units using 0.1N HCl or NaOH solution. The suspensions were agitated with orbital shaker instead of magnetic stirrer (Dench *et al*., 1981) for 30 min at room temperature and then centrifuged at 4000 rpm for 30 min. The quantity of protein dissolved in supernatant was determined by Biuret method according to AOAC (1995) using egg albumin as the standard. Protein solubility was calculated as shown in Appendix 4. All samples were run in triplicates and means were reported.

3.2.6.2 Water holding capacity (WHC): To determine the water holding capacity of defatted fern flour, the method outlined by Diniz and Martin (1997) was followed. Triplicate samples (0.5 g) of each isolate were dissolved with 10 mL of distilled water in a graduated centrifuge tubes and vortex for 30 sec to disperse the proteins. After a holding period of 30 min at room temperature, dispersions were centrifuged at 3000 rpm for 25 min. The supernatant was filtered with cheese cloth and the volume of released fluid was accurately measured. The difference between initial volumes of distilled water added to the protein samples and the volume retrieved was calculated. The results were reported in triplicate as volume (mL) of water absorbed per gram of protein sample.

3.2.6.3 Oil holding capacity: Oil holding capacity of defatted fern protein was determined as the volume of edible oil held by 0.5 g of fern protein according to the procedure described by Shahidi *et al*. (1995). The protein sample in 10 mL of oil was shook using orbital shaker at 150 rpm and then centrifuged at 3000 rpm for 25

min. The volume of the supernatant was measured. The oil-holding capacity was expressed as volume (mL) of oil held by 0.5 g of protein sample.

3.2.6.4 Foaming capacity and stability: The foaming capacity was determined using the method outlined by Aruna and Parakash (1993) with minor modification. One hundred milliliters of deionised water (V_1) at different pH (2-14) were separately added to 1 g of defatted fern protein isolates. The suspension was blended for 3 min. using a high-speed blender instead of a shaker (Aruna and Parakash, 1993), poured into 250 mL graduated cylinder and the volumes of foam (Vt) were immediately recorded. FC was calculated as shown in appendix 4.

The foam stability was determined by measuring the decrease in volume of foam as a function of time at 10, 20, 30, 40, 50 and 60 min using the pH of the protein suspension that recorded the highest FC %.

3.2.7 SDS-PAGE of proteins

The molecular weights of the fern protein isolates were determined using polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulphate (SDS). It was performed on 12% polyacrylamide gels according to the procedure described by Laemmli (1970) in a Mini-Protean II dual slab cell (Bio-Rad Laboratories, Richmond, CA). The samples were loaded into the wells created in the electrophoresis apparatus set up. The first well was loaded with 20 µL pre-stained standard protein marker containing eight proteins ranging from lysozyme (18.5 kDa) to myosin (205 kDa) used for molecular weight determination. The subsequent wells were loaded separately with 20 µL of each prepared fern protein isolate sample. The remaining wells without samples were loaded with 1x SDS buffer to prevent spreading of samples into them. The whole setup (inner chamber) was placed in an

electrode tank (mini tank) and 10% of 10x electrode buffer (running buffer) was poured into the inner chamber to about half way between the two plates. Outside the inner chamber was filled with 200 mL of running buffer.

The whole setup was connected to an electrode and run for about 2.5 h at room temperature at 200 V. The gel with separated bands of proteins was stained with Coomassie brilliant blue R-250 in a plastic bowl and vortex for about 45 min on a shaker at 75 rpm. The stain solution was drained off and some amount of destain solution was poured onto the gel, vortex on a shaker for 4 h at 75 rpm. The gel was then put on a light box to read the bands. Molecular weights were determined by measuring the distances of migration in comparison with the standards.

3.2.7.1 Image analysis of electrophoregram: The electrophoregram from the SDS-PAGE was scanned using HP Deskjet F370 and analyzed using the ImageJ software according to the gel analyzer option as described by Ferreira and Rasband (2011).

3.2.8 Statistical analysis

The experimental results for chemical composition and functional properties were expressed as mean ± standard deviation (SD) of three replicates. Where applicable, the data were subjected to one way analysis of variance (ANOVA) and the difference between samples were determined by Tukey's test using Minitab 16 program. P-values < 0.05 were regarded as significant.

The response data obtained from the standard curve were loaded into the Design Expert (2007) statistical tool and run to generate regression parameters which were studied. The predicted values of protein yields were calculated using regression model and compared with experimental values. The most compatible estimation

model among the mean, linear, quadratic and cubic expressions of each response variable were identified based on all the statistical analysis which includes sequential model, sum of squares, lack of fit tests and the model summary statistics. When a model had been selected, an analysis of variance was calculated to assess how well the model represented the data.

Statistical significance of the terms in the regression model was examined by analysis of variance (ANOVA) for each response (Appendix 3). The p–values for the regression models as well as the interactions among the factors of extraction were tested against $p<0.05$.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Chemical Composition

The chemical composition of whole fern flour and defatted flour are shown in Table 4.1. There were general decreases in all the chemical composition after defatting for both *N. biserrata* and *A. orientalis* except for carbohydrate and protein content. The defatted flour of *A. orientalis* also showed a decrease in ash content.

The moisture content of a sample is known to reflect the amount of solid matter in the sample. Results show no significant differences in moisture content between whole flour of *N. biserrata* and *A. orientalis*. Moisture content of the flour decreased significantly ($p<0.05$) from 7.79 % to 4.22 % and 7.81 % to 3.92 % for *N. biserrata* and *A. orientalis* respectively after oil extraction. The trend in moisture content of the two fern types in this study was consistent with observation made by Tömösközi *et al.* (2008) who reported 13.36 % of moisture content of full fat flour of *Amaranthus hypocondriatus* but reduced to 12.00 % after defatting. Earlier report by Abu-Tarboush (1995) showed a different trend with increased in moisture content after defatting. According to Vaclavik and Christian (2008), controlling water level in foods is an important aspect of food quality as water content affects the shelf life of food.

There was a significant increase in protein and carbohydrate content after defatting by 2.14 % and 5.70 % for *N. biserrata* and 3.43 % and 7.53 % for *A. orientalis* respectively. This observation could be explained by the fact that when fat is removed from a food sample, the ingredients available to replace it are protein, carbohydrates, minerals or air (Vaclavik and Christian, 2008). These items increase

automatically and proportionally even if nothing new is added to the food sample (Glicksman, 1991). This phenomenon was consistent with observations Govardhan-Singh *et al*. (2011) and Abu-Tarboush (1995) made who also recorded increased in protein and carbohydrate contents as a result of defatting. The high protein content of the fern leaves suggest that it can supplement cereal and tuber flours which are not only deficient in amino acids but low in protein.

Table 4.1: Chemical composition of whole fern leaves flour and defatted flour obtained by the cold method of extraction

Composition	*Nephrolepis biserrata* (swamp)		*Arthropteris orientalis* (palm)	
(Mean %)	Whole flour	Defatted flour	Whole flour	Defatted flour
Moisture	7.79±0.18^{a}	4.22±0.21^{b}	7.81±0.02^{a}	3.92±0.64^{b}
Fat	3.03±0.58^{a}	0.27±0.25^{b}	3.78±0.33^{a}	0.53±0.05^{b}
Fiber	17.14±1.40^{a}	11.21±0.91$^{b\,c}$	12.21±1.30^{b}	9.00±0.59^{c}
Ash	5.59±0.48^{c}	6.09±0.52^{c}	12.72±0.67^{a}	10.39±0.27^{b}
Protein	23.42±1.62^{b}	25.57±2.62^{a}	19.28±1.49^{c}	22.71±0.43^{b}
Carbohydrate	43.01±1.53^{c}	48.71±1.18^{b}	44.21±0.65^{c}	51.74±0.92^{a}
*Protein isolate content	-	39.33±0.32^{a}	-	35.26±0.54^{b}

Values are means of triplicates ± standard deviation. The superscript showed that at $P < 0.05$, a significant difference exists. Means in row with different superscripts are significantly different ($P < 0.05$). *Percentage protein after isolation.

4.2 Alkaline Treatment

Relationship between time and concentration of extracted protein using alkaline treatment was established. From Table 4.2 the independent variables were analyzed to predict the maximum extracted proteins under the given range (Table 3.1).

Table 4.2: Central composite design for NaOH, variables and responses

Runs	Independent variables		Dependent variables	
	A: Solvent concentration (M)	B: Time (min.)	NB proteins (mg/mL)	AO proteins* (mg/mL)
1	0.01	30	2.023	0.420
2	0.1	30	6.182	2.159
3	0.01	60	3.489	1.307
4	0.1	60	3.068	1.943
5	0.055	45	2.682	1.227
6	0.055	45	3.114	1.886
7	0.055	45	3.261	1.148
8	0.055	45	3.170	1.432
9	0.01	45	1.989	0.761
10	0.1	45	3.841	2.239
11	0.055	30	3.352	1.318
12	0.055	60	3.511	1.398
13	0.055	45	3.284	1.114
14	0.055	45	3.852	1.466
15	0.05	45	3.750	1.295
16	0.05	45	4.966	1.125

* NB = *Nephrolepis biserrata*; AO = *Arthropteris orientalis*

The response surface graph for extracted protein from defatted *N. biserrata* as a function of time and NaOH concentration is shown in Figure 4.1.

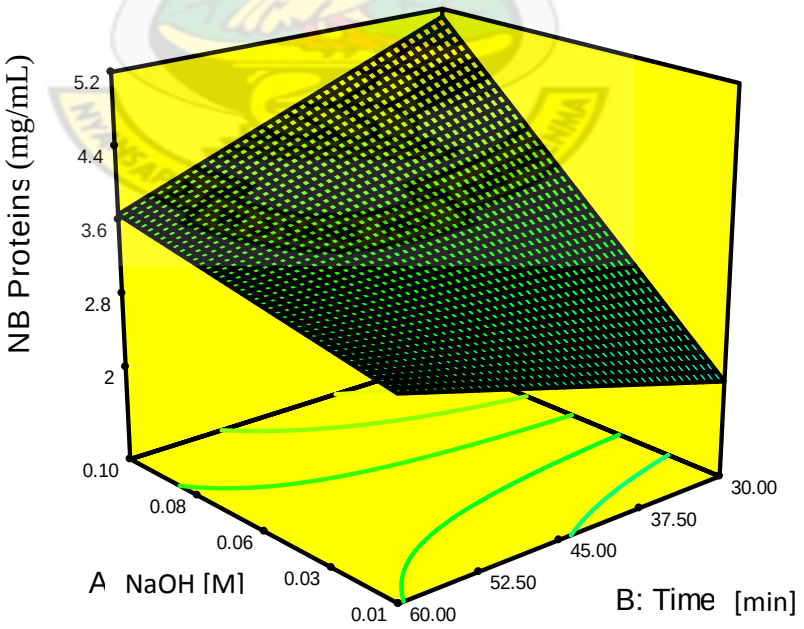


Figure 4.1: Response surface graph for amount of extracted protein as a function of time (min.) and concentration of NaOH solution (M) during protein extraction from defatted *Nephrolepis biserrata*

The graph indicates that the amount of extracted protein increased with decreasing time of agitation. The extracted protein was higher at the high alkaline concentration of 0.1 M and this was consistent with an observation by Batista (1999) when extracting proteins from hake and monk fish using NaOH. Again when NaOH concentration increased, there was a corresponding increase in extracted protein. However, increasing the agitation time showed a negative linear effect on extracted protein amount indicating that most of the proteins dissolve in the solution during the first 30 min of agitation, but the protein extraction was low after 45 min. This means that prolonged time of agitation causes protein denaturation which reduces the amount of protein in solution (Eke and Akobundu, 1993).

Since increasing NaOH concentration and decreasing time of agitation resulted in a high protein extraction, the concentration range of NaOH was further increased with decreasing time of agitation as shown in Table 3.4 and 4.3.

Table 4.3: Central composite design for optimal extraction using NaOH

Runs	Independent variables		Dependent variables	
	Solvent concentration (M)	Time (min.)	NB proteins (mg/mL)	AO proteins (mg/mL)
1	0.05	10	4.151	0.993
2	0.05	60	5.664	1.023
3	0.05	35	2.168	1.373
4	0.1	35	3.347	1.114
5	0.1	35	3.533	1.338
6	0.1	35	2.620	1.322
7	0.1	35	2.539	1.417
8	0.1	10	1.938	1.097
9	0.1	60	4.168	0.781
10	0.1	35	1.859	1.364
11	0.1	35	3.899	0.925
12	0.1	35	1.684	0.522
13	0.1	35	3.739	1.124
14	0.15	10	1.810	0.182
15	0.15	60	2.036	1.342
16	0.15	35	1.061	0.406

From Figure 4.2, increasing the concentration range of NaOH from between 0.01 M - 0.1 M to a concentration range from between 0.05 M - 0.15 M and decreasing agitation time range from between 30 min - 60 min to a range from between 10 min - 60 min did not follow the trend as predicted initially.

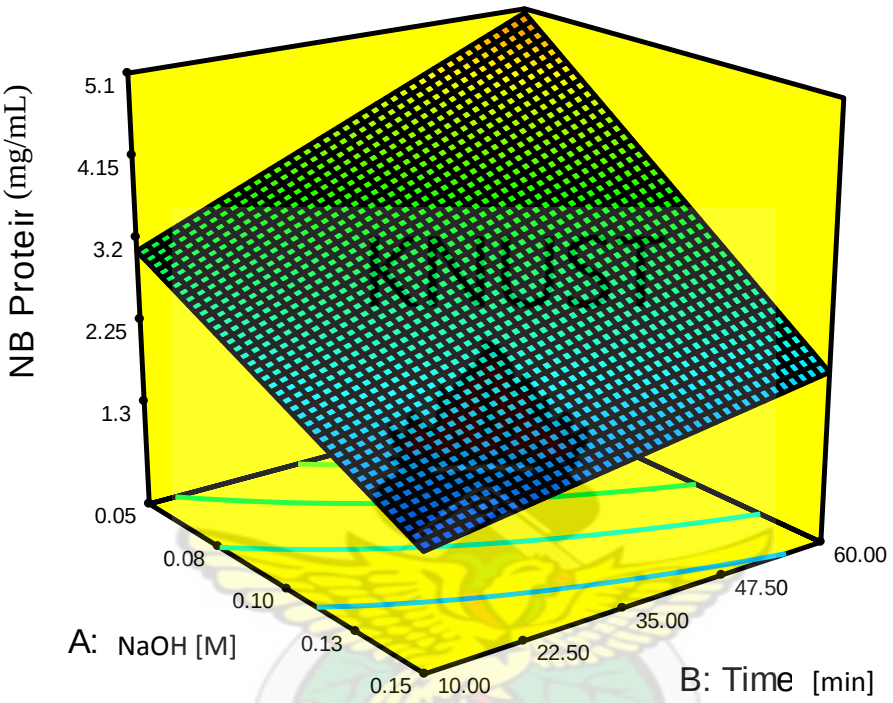


Figure 4.2: Response surface graph for amount of extracted protein as a function of reduced time (min.) and increased in concentration of NaOH solution (M) during protein extraction from defatted *Nephrolepis biserrata*

For NaOH concentration, *N. biserrata* protein extraction showed a decrease in the amounts extracted whilst increasing the concentration. This was also noticed in a study by Wani *et al.* (2006) where she observed a decrease in protein extracted from palm kernel meal as NaOH increased from 0.3 % to 1.5 %. This observation can be explained as decreasing time of agitation also affected protein extraction negatively since more of the proteins did not get solubilized into solution.

A similar trend was observed for proteins extracted from *A. orientalis* using NaOH as solvent concentration as compared to *N. biserrata.* However, there was no effect

of time on the amount of protein extracted and concentration of NaOH as shown by the response surface plot from the experimental design in Figure 4.3. This shows that time of agitation is not significant when extracting proteins from *A. orientalis.*

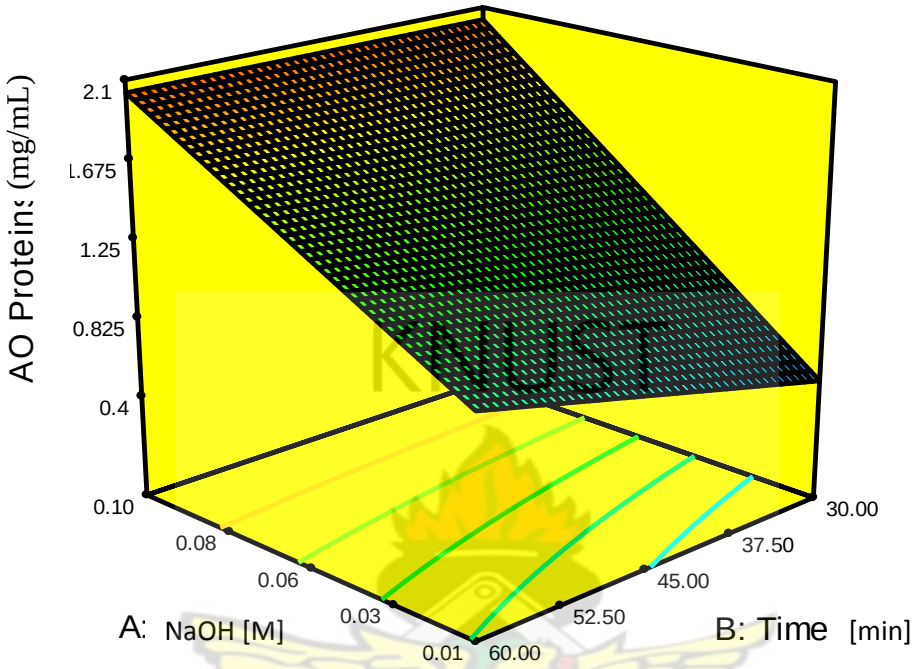


Figure 4.3: Response surface graph for amount of extracted protein as a function of time (min.) and concentration of NaOH solution (M) during protein extraction from defatted *Arthropteris orientalis*

Therefore since a high protein yield was required and high NaOH concentration resulted in an increased in protein extraction, a further study on the effect of NaOH concentration on *A. orientalis* with the response surface analysis was done to determine the optimum condition for extracting maximum proteins. The independent variables and their levels are shown in Table 3.4, 3.5 and 4.3.

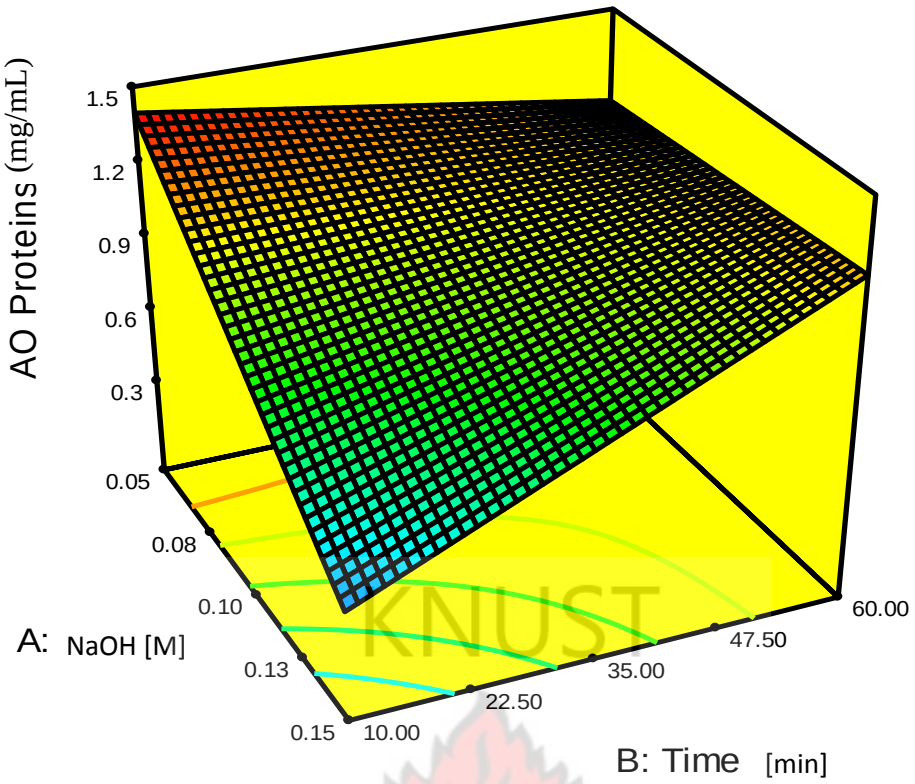


Figure 4.4: Response surface graph for amount of extracted protein as a function of reduced time (min.) and increased in concentration of NaOH solution (M) during protein extraction from defatted *Arthropteris orientalis*

From Figure 4.4, increased in protein extraction was observed as NaOH concentration falls below 0.08 M with agitation time of less than 45 min. Comparing this observation with the former (Figure 4.3), amount of protein extracted was observed to decreased from 2.24 mg/mL to 1.42 mg/mL when NaOH concentration range was increased from between 0.01 M – 0.1 M (Table 3.1) to the range of between 0.05 M – 0.15 M (Table 3.4).

4.3 Alcohol Treatment

Relationship between time and concentration of extracted protein using alcohol treatment was established. Figure 4.5 shows values of protein extracted from *Nephrolepis biserrata* by varying time and percentage ethanol while letting the flour / solvent ratio remain constant at 0.5: 25 (w/v).

Table 4.4: Central composite design for ethanol, variables and responses

Runs	Independent variables		Dependent variables	
	A: Solvent concentration (%)	B: Time (min)	NB proteins (mg/mL)	AO proteins (mg/mL)
1	50	10	1.505	0.201
2	50	60	1.980	0.157
3	50	35	1.749	0.180
4	60	35	2.817	1.778
5	60	35	2.039	2.080
6	60	35	1.786	0.643
7	60	35	1.442	0.792
8	60	10	2.742	1.515
9	60	60	2.335	0.901
10	60	35	2.802	1.126
11	60	35	3.064	1.275
12	60	35	2.561	0.865
13	60	35	2.701	1.136
14	70	10	2.508	3.370
15	70	60	4.749	2.939
16	70	35	5.792	3.232

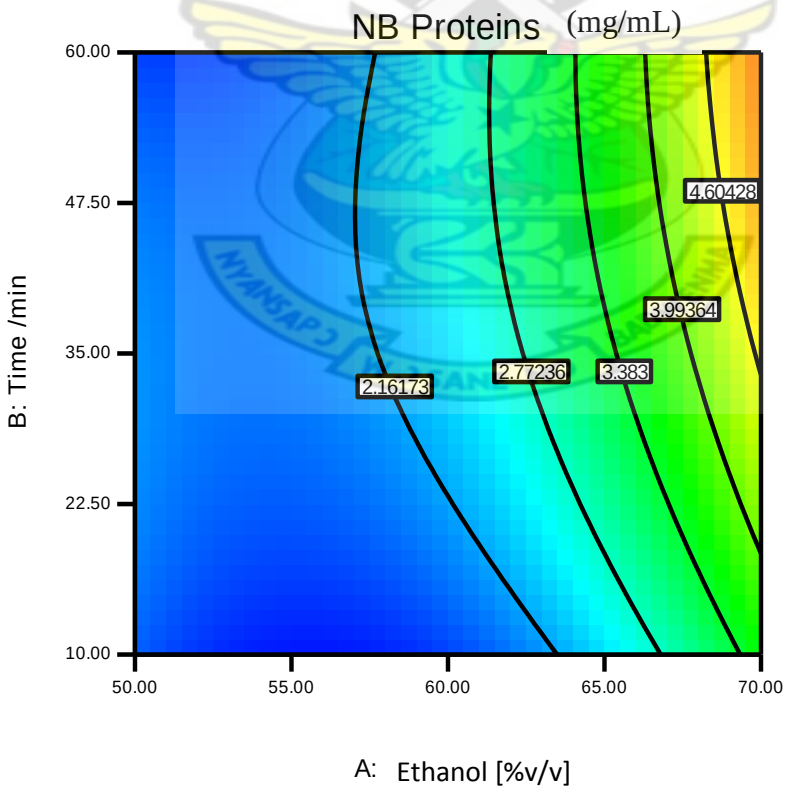


Figure 4.5: Effect of time and percentage ethanol on amount of protein recovery from *Nephrolepis biserrata*

Variation in time and percentage ethanol revealed that a maximum amount of extracted protein was obtained when time was more than 35 min and percentage ethanol was 70%. An increase in time of agitation with high concentration of ethanol increased the amount of protein extracted. Maximum protein observed was 5.12 mg/mL and the most favourable condition was 53 min of agitation and 70% concentration of ethanol.

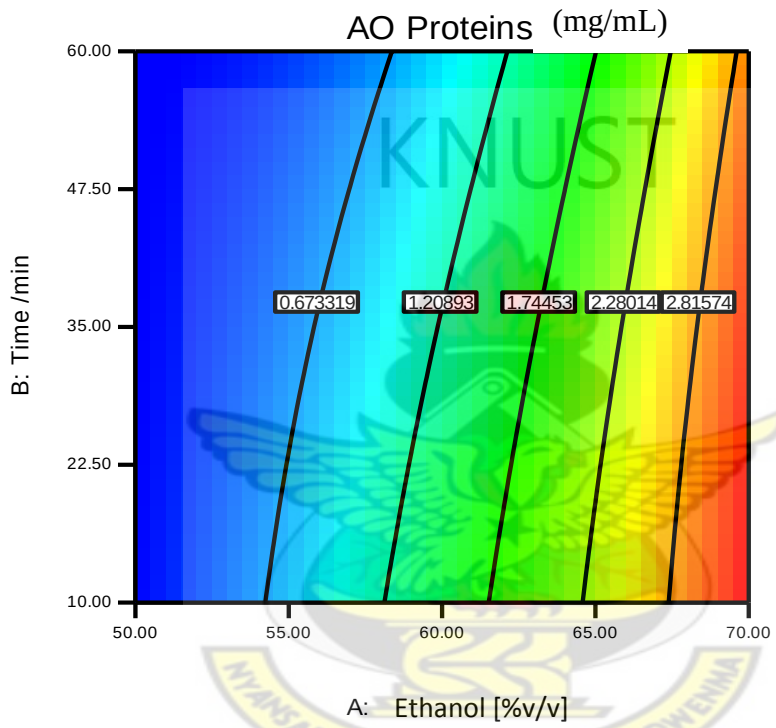


Figure 4.6: Effect of time and percentage ethanol on amount of protein recovery from *Arthropteris orientalis*

The optimum conditions for extracted protein recovery from *Arthropteris orientalis* were 70% ethanol and time of 10 min agitation. This yielded 3.32 mg/mL of protein extracted (Figure 4.6). This revealed that increase in percentage ethanol showed an increasing trend however, increasing time showed a negative linear effect on protein extraction.

4.4 Saline Treatment

The relationship between the variables and amount of protein extracted are as shown in Figures 4.7 and 4.8 for *N. biserrata* and *A. orientalis* respectively. Variation in time and salt concentration revealed maximum amount of extracted protein of 0.83 mg/mL at time of 53 min of agitation and NaCl concentration of 0.1 M (Figure 4.7). There was increased in protein extracted as NaCl concentration increase from 0.01 M to 0.1 M. This was significantly different from that observed in Figure 4.8 for *Arthropteris orientalis* but consistent with those reported by Govardhan-Singh *et al*. (2011).

Table 4.5: Central composite design for NaCl, variables and responses

Runs	Independent variables		Dependent variables	
	A: Solvent concentration (M)	B: Time (min.)	NB proteins (mg/mL)	AO proteins (mg/mL)
1	0.01	30	1.023	0.625
2	0.1	30	0.830	0.534
3	0.01	60	1.341	0.477
4	0.1	60	0.795	0.500
5	0.055	45	0.420	0.284
6	0.055	45	0.420	0.432
7	0.055	45	0.341	0.330
8	0.055	45	0.705	0.330
9	0.01	45	1.000	0.410
10	0.1	45	0.966	0.261
11	0.055	30	0.545	0.398
12	0.055	60	0.636	0.250
13	0.055	45	0.557	0.489
14	0.055	45	0.739	0.182
15	0.05	45	0.318	0.409
16	0.05	45	0.682	0.364

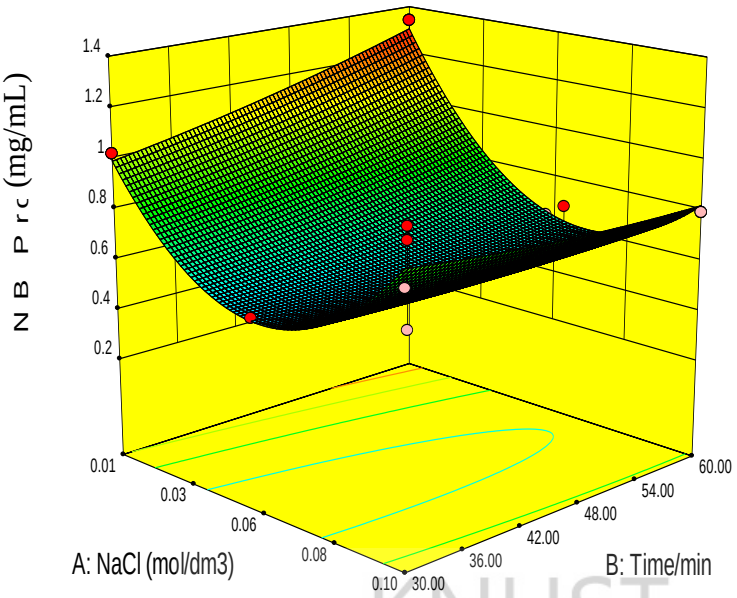


Figure 4.7: Response surface graph for amount of extracted protein as a function of time (min) and concentration of NaCl solution (M) during protein extraction from defatted *Nephrolepis biserrata.*

Though the experimental amount of extracted protein from *N. biserrata* ranges from 0.32 mg/mL to 1.34 mg/mL (Fig. 4.7), no significant effect was observed as time of agitation increased from 30 min to 60 min on the extracted protein but as NaCl concentration decrease, the amount of extracted protein also decreases.

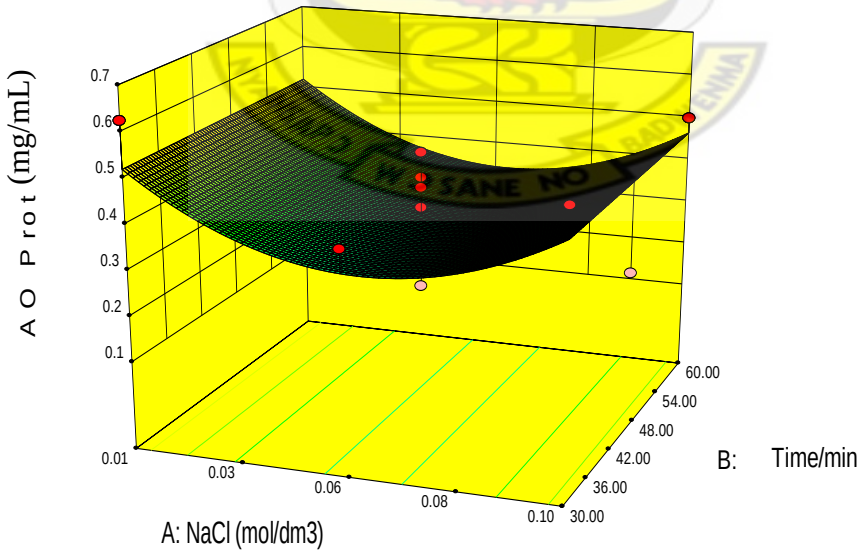


Figure 4.8: Response surface graph for amount of extracted protein as a function of time (min) and concentration of NaCl solution (M) during protein extraction from defatted *Arthropteris orientalis*

When the salt concentration was kept at 0.10 M (Figure 4.8), maximum amount of extracted protein of 0.47 mg/mL was observed at time of 45 min. The results also revealed that at 0.01 M concentration of NaCl, 0.52 mg/mL of protein was extracted and when the salt concentration was increased from 0.01 M to 0.08 M, the amount of protein extracted decreased to 0.35 mg/mL. But then there was a sharp rise in the amount of protein extracted from 0.35 mg/mL to 0.45 mg/mL peaking at 0.625 mg/mL. This reflection was contrary to the study conducted by Govardhan-Singh *et al*. (2011) who observed a consistent increase in protein extractability (from moringa seed flour) from 37.74 % to 84.72 % at 0.05 M to 0.75 M and a gradual decrease from 84.72 % to 71.21 % at 0.75 M to 2.0 M. This phenomenon could be explained by the fact that at low concentrations of salt, solubility of the protein usually increases slightly (salting in) (Arifin *et al*., 2009). But at high concentrations of salt, the solubility of the proteins drop sharply as a result of salting out and therefore precipitate since at high salt concentrations, the abundance of the salt ions decreases the solvating power of the salt ions thereby decreasing the solubility of the proteins and precipitation results (Arifin *et al*., 2009).

Time of agitation for *N. biserrata* and *A. orientalis* in salt solution had little or no effect on optimization result. This result agreed with the findings of Thompson (1977) for mung bean proteins, reporting that the time of extraction did not have much influence on nitrogen extractability. Jyothirmayi *et al.* (2006) had also reported that extraction of proteins increased till 35 min after which it remained constant.

4.5 Optimum Conditions for the Extraction of Defatted Fern Flour and Model Verification

By considering all the conditions in response to the amount of protein recovered from *N. biserrata* and *A. orientalis* (Table 4.6), it was evident that NaOH and Ethanol recorded the highest protein recovery for both fern types. *A. orientalis* recorded the lowest amount of protein for all the treatment with as low as 0.47 mg/mL for NaCl.

Table 4.6: Optimum protein recovery for both treatments

Treatment	Optimum conditions		Maximum protein recovery (mg/mL)	
	Concentration	Time (min.)	NB	AO
Alkaline (NaOH)	0.1 M	30	5.11	2.03
Alcohol (ethanol)	70 v/v	53	5.12	3.02
Saline (NaCl)	0.1 M	53	0.83	0.47

No protein precipitated for both NB and AO at extreme pH (pH of 1.8 and 13.8) for ethanol extraction but high protein precipitation was observed for NaOH extraction using supernatants of NB and AO at a pH range of 2.3 to 2.5. The pH of the supernatants for NaOH and ethanol prior to precipitation were 12.56 and 5.80 for NB and 12.60 and 5.70 for AO respectively (Table 4.7). The absence of protein precipitation upon the addition of concentrated or 0.5 M HCl to the ethanol extract might be due to little or no traces of prolamines in both supernatants of NB and AO. This is because in a less polar solvent such as ethanol, proteins are rarely noticeably soluble (e.g. prolamines) although prolamines are most soluble in alcohols.

Table 4.7: pH measurements of AO and NB protein extract

Solvent type	Supernatant	pH of supernatant	Precipitated pH
NaOH	AO extract	12.60	2.3 – 2.5
	NB extract	12.56	2.3 -2.5
Ethanol	AO extract	5.70	-
	NB extract	5.80	-

By comparing both treatments, it was noticed that the optimization condition of independent variables (0.1 M and 30 min of agitation) for alkaline treatment was most suitable for NB and AO protein extraction. Altering the range of independent variables for alkaline treatment (Table 3.4) yielded a protein recovery of 5.05 mg/mL and 1.13 mg/mL for NB and AO respectively at a time of 60 min and NaOH concentration of 0.05 M. This was quite lower than that shown in Table 4.6 with less time of agitation. Similar observation in a study by Beradi *et al*. (1967) and Martinez *et al*., (1967) showed that time above 30 min is not important to optimize protein extraction if the extracting solvent contains sufficient ions for maximum nitrogen solubility.

4.6 Model Fitting

The study utilized RSM to develop a prediction model for optimizing the extraction of protein from defatted fern flour (DFF). The independent and dependent values presented in Table 4.2 were analyzed to obtain a regression equation that could predict the responses within the given range. The regression equation for protein extraction is as follows:

NB protein extracted (mg/mL) = 3.43 + 0.93A – 0.25B -1.14 AB ….. (1)

AO protein extracted (mg/mL) = 1.44 + 0.64A + 0.13B – 0.28AB ….. (2)

Where, A is the coded value of variable NaOH concentration; B is the coded value of variable time.

The plot of experimental values of extracted protein (mg/mL) versus those calculated from Eq. 1 and Eq. 2 indicated a good fit, as presented in Appendix 5. Colour differences in the fit plotted indicated the level of extracted protein which represents red as the highest extracted protein while narrow down to blue colour was the lowest extracted protein.

The results of analysis of variance (ANOVA) gave a coefficient of determination (R^2) of 0.90 and 0.92; adjusted R^2 of 0.88 and 0.90 for NB and AO respectively. This implies that 90% and 92% of the variations could be explained by the fitted model, indicating the adequacy of the applied model which is a measure of degree of fit (Haber and Runyon, 1977). Joglekar and May (1987) suggested that, for a good fit model, R^2 should be at least 0.80. Therefore, the developed model could adequately represent the real relationship among the parameters chosen. The F-value for NB for NaOH, NaCl and ethanol were 43.33, 12.25 and 6.97 respectively, meaning the model suggested was significant at 95% probability level. AO recorded F-value of 49.44, 5.90 and 8.69 for NaOH, NaCl and ethanol respectively (Appendix 3).

4.7 Functional Properties and Characterization of Fern Leaves Protein

4.7.1 Water/oil holding capacity

Interactions of water and oil with proteins are very important in food systems because of their effects on the flavour and texture of foods (Kanu *et al.*, 2007). Water absorption is dependent on various parameters such as size, configuration, conformational characteristics, hydrophobic and hydrophilic balance of the protein

(Chavan *et al*., 2001). Water holding capacity is an important processing parameter and has implications for viscosity, bulking and consistency of products, as well as in baking applications. The ability of a protein matrix, such as protein particles, protein gels or muscle to imbibe and retain water against gravity is known as Water Holding Capacity (WHC). There was no significant difference between the fern species with respect to water holding capacity (Table 4.8) although *Arthropteris orientalis* protein isolate (2.39 mL/g protein) showed a better water holding capacity than *Nephrolepis biserrata* (2.13 mL/g protein). According to Kinsella (1979), an increase in the water holding capacity is due to the ability of a protein isolate to swell and unfold, exposing additional binding sites, whereas the carbohydrate and other components of the protein concentrate may impair it. This support the views of Bandyopadhyay and Ghosh (2002), who reported that protein concentrate exhibits poor water-binding capacity compared to that of the isolate.

Table 4.8: Water and oil holding capacity of defatted fern protein isolates

Protein isolates	Water holding capacity (mL/g protein)	Oil holding capacity (mL/g protein)
Nephrolepis biserrata	2.13±0.00[a]	2.73±0.23[b]
Arthropteris orientalis	2.39±0.23[a]	2.93±0.42[b]

Means within each column followed by the same letters are not significantly different ($p>0.05$).

Another important parameter that influences water holding capacity is pH. The pH of a system markedly influences its ability to bind water due to changes in the surface charges on a protein as the pH is altered. Increasing or decreasing the pH away from the isoelectric point will result in increased water holding capacity by creating a charge imbalance. Bora and Ribeiro (2004) reported best water absorption of 1.64 mL/g of defatted macadamia (*Macadamia integrofolia*) kernel flour protein isolate at a pH of 7.2, followed by 1.55 mL/g protein at a pH 12.0 and least of 1.02

mL/g protein at a pH of 2.0 as a result of conformational difference in protein structure caused by the pH of extraction medium. These values were less than that observed in this study at a precipitated pH of 2.3 to 2.5.

An important functionality that influences taste of the product that is required in various food industries is the ability of protein to absorb oil. The oil holding capacity of defatted fern protein was 2.73 mL/g and 2.93 mL/g for *Nephrolepis biserrata* and *Arthropteris orientalis* respectively. Though the *Arthropteris orientalis* protein isolate showed a higher oil holding capacity more than the *Nephrolepis biserrata* but were not significantly different ($p<0.05$). The oil holding capacity of the isolate followed the same trend as that of water holding capacity for both *Arthropteris orientalis* and *Nephrolepis biserrata* (Table 4.8). The oil holding capacity for *Nephrolepis biserrata* and *Arthropteris orientalis* was higher than that reported by Bora and Ribeiro (2004) but compared favourably with that reported by El-Adawy (2000) who recorded oil absorption values of 1.60 mL/g, 2.72 mL/g and 2.71 mL/g - 2.81 mL/g for fava bean, mustard seed and lupin seed protein isolates respectively.

4.7.2 Foaming capacity and stability

The capacity of proteins to form stable foams with gas by forming impervious protein films is an important property in the production of a variety of foods. Foam capacity is influence by the hydrophobic interactions of protein (Dia-Moukala and Zhang, 2011). Foam capacity of defatted fern protein as shown in Figure 4.9 was pH dependent and was found to be low at pH within the area of it isoelectric point (pH 2-8).

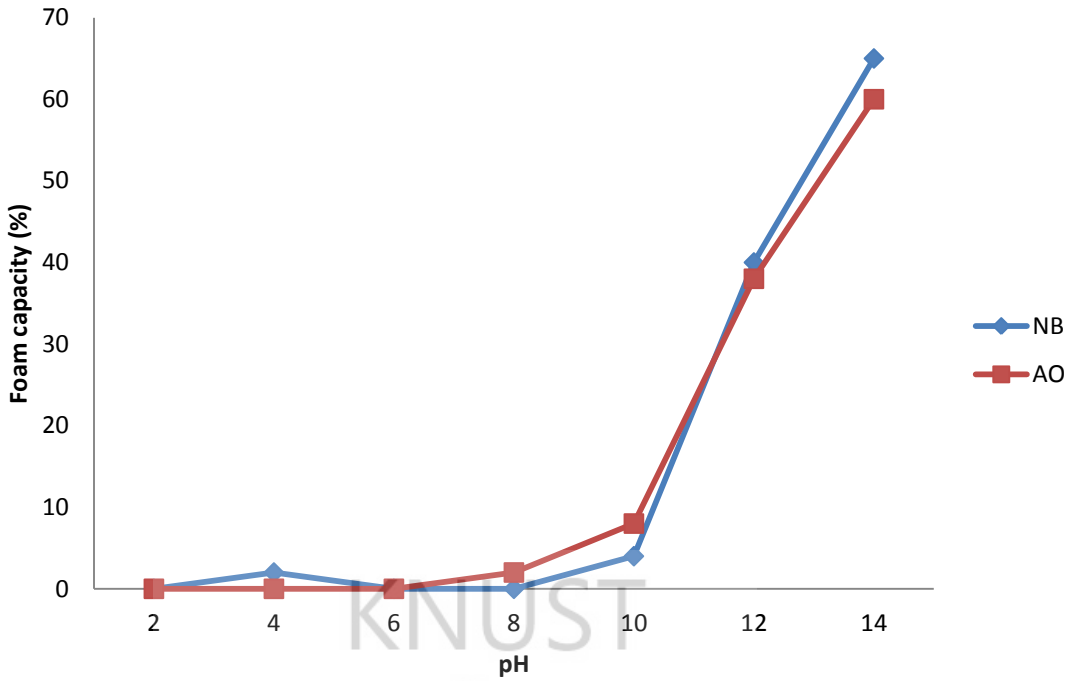


Figure 4.9: Foaming capacity of defatted fern protein isolate at different pH treatment

The lower foaming capacity could mainly be attributed to reduction in molecular flexibility as a result of high disulfide bonds (Kim and Kinsella, 1987). Away from pH 8, foaming capacity significantly increased but higher for *Nephrolepis biserrata* than *Arthropteris orientalis.* Foaming capacity of 65% and 60% was observed for *Nephrolepis biserrata* and *Arthropteris orientalis* respectively at pH 14. The higher foaming capacity at this pH was likely due to the increased net charges on the protein, which weakened the hydrophobic interactions but increased the flexibility of the protein allowing the protein to diffuse more rapidly to the air-water interface to encapsulate air particles (Wierenga and Gruppen, 2010; Aluko and Yada, 1995) and the more easily it is denatured there, the more it is able to foam (Belitz *et al*., 2009). Kim and Kinsella (1987) have also reported that the reduction of disulfide bonds would increase the molecular flexibility of protein and then result in the improvement of its foaming properties.

To have foam stability (Figure 4.10), protein molecules should form continuous intermolecular polymers enveloping the air bubbles, since intermolecular cohesiveness and elasticity are important to produce stable foams (Kamara *et al*., 2009). A sharp drop of foam stability was observed within 10 min for both *Nephrolepis biserrata* and *Arthropteris orientalis* but was more stable for *Arthropteris orientalis* within the time range of 20 min - 60 min.

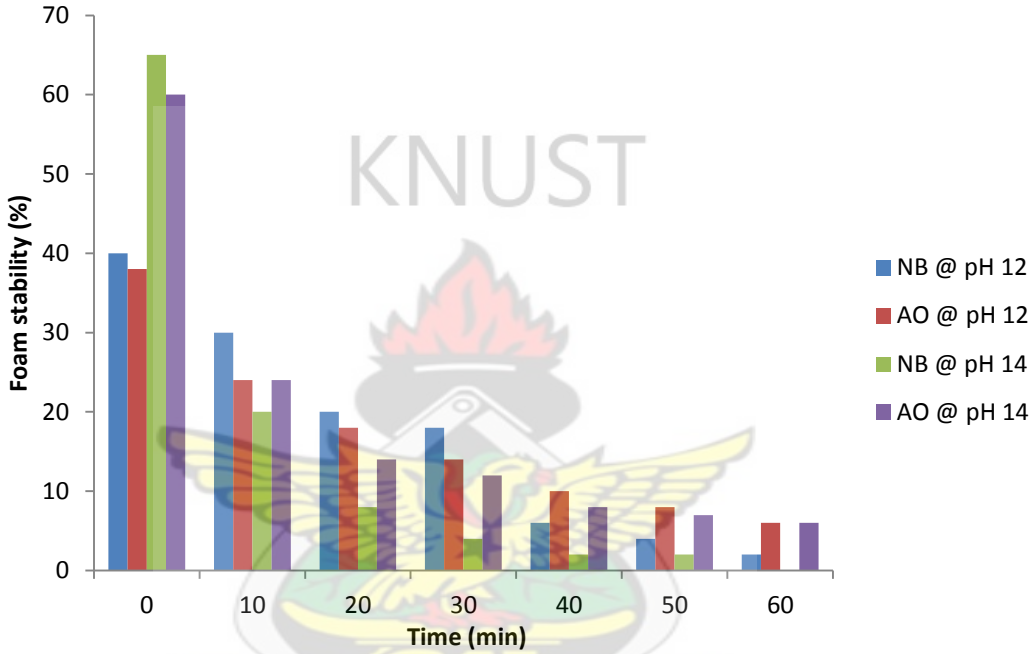


Figure 4.10: Foaming stability of defatted fern protein isolate at pH 12 and 14

4.7.3 Protein solubility

Solubility is considered the most important functional attribute for its contribution to other functional properties like gelling and emulsification (Sikorski, 1997). The general shape of solubility curves (U-shaped) when solubility is plotted against pH (Hu *et al*., 2010; Sikorski, 1997) disagree with the solubility curve observed in this study as depicted in Figure 4.11. Both *Nephrolepis biserrata* and *Arthropteris orientalis* isolates showed a minimum solubility of 0.5% and 2.2% respectively in the region of between pH 2-4. This is lower than the reported isoelectric points (pI)

of pH 4-5.5 of sesame protein isolate (Kanu *et al*., 2007) and the pH of minimum solubility of most vegetable protein isolates (Vani and Zayas, 1995). The low solubility at that pH is due to the isoelectric point (pI) of the fern protein isolates since at that pH there is no electrostatic repulsion between the molecules. Above the pI region which was observed to be above pH 4, the solubility for the *Arthropteris orientalis* protein isolate increased but < 45% between pH 4-10 and for *Nephrolepis biserrata* protein isolate within that region of pH it was observed showing a solubility profile of up to 55.9% at pH 8 and drop to 52.1% at pH 10. The observed increased in protein solubility is dependent on pH. At pH values above the pI of the protein isolates, the proteins carry a net charge (due to repelling of the positive or negative ions) and ionic hydration promote solubilization of protein.

Other research works have observed increases in solubility at pH values below or above the pI of a protein. This was contrary to the observation in this study where only increase in solubility was observed above the pI of the fern protein. Solubility was low at the extreme ends of the pH tested and the maximum solubility was shown at pH 8 and 10 for *Nephrolepis biserrata* and *Arthropteris orientalis* respectively. This outcome disagrees with observations made by Hu *et al*. (2010) and Tömösközi *et al*. (2008). The decreased in solubility above pH 8 and 10 might be due to denaturation of the proteins in the fern at that pH.

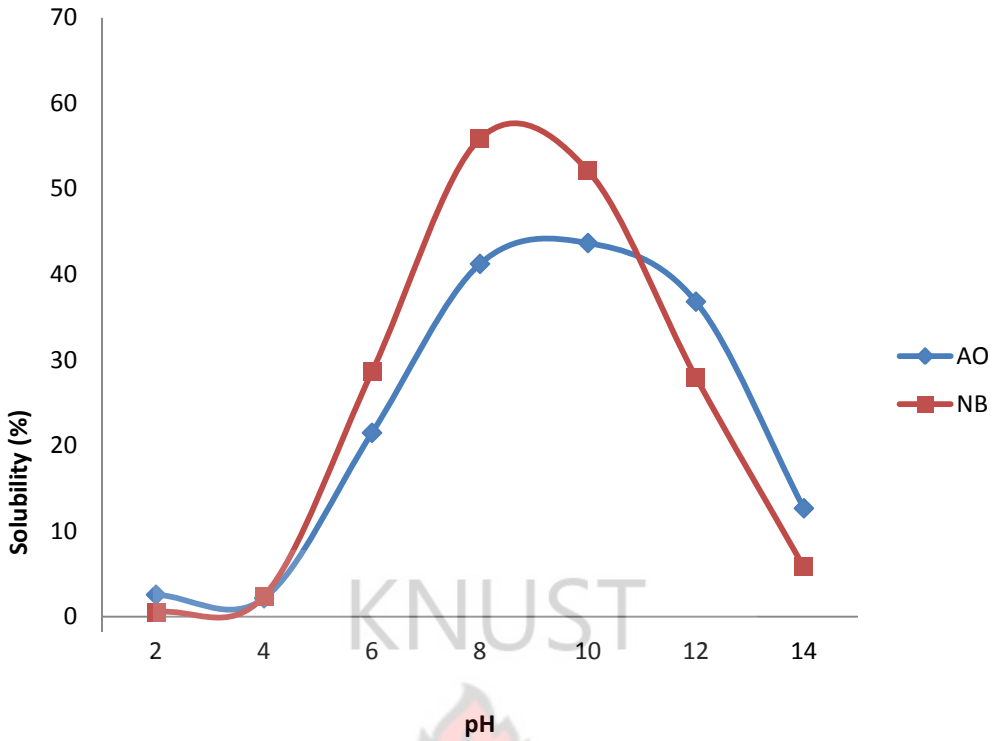


Figure 4.11: Solubility of fern protein isolate at various pH levels

4.8 SDS-PAG electrophoresis of proteins

SDS-PAGE pre-stained marker and two proteins extracts from fern leaves gave multiple bands separation as presented by the electrophoregram (Figure 4.12). The separation of proteins by electrophoresis is based on the fact that charged molecules will migrate through a gel matrix upon application of an electric field. The pre-stained protein marker gave eight bands from lysozyme with 18.5 kDa to myosin with 205 kDa. *Nephrolepis biserrata* showed eight bands with a molecular weight range from 23-205 kDa while *Arthropteris orientalis* showed eleven bands with molecular weight range from 18.5-111.5 kDa. *Nephrolepis biserrata* showed one prominent peak of about 97.66 % of protein extracted with a molecular weight of 23 kDa and the remaining bands constituting as low as 0.02 % (Appendix 4). A similar trend was also observed for *Arthropteris orientalis* with 97.19 % peak area and a molecular weight of 18.5 kDa.

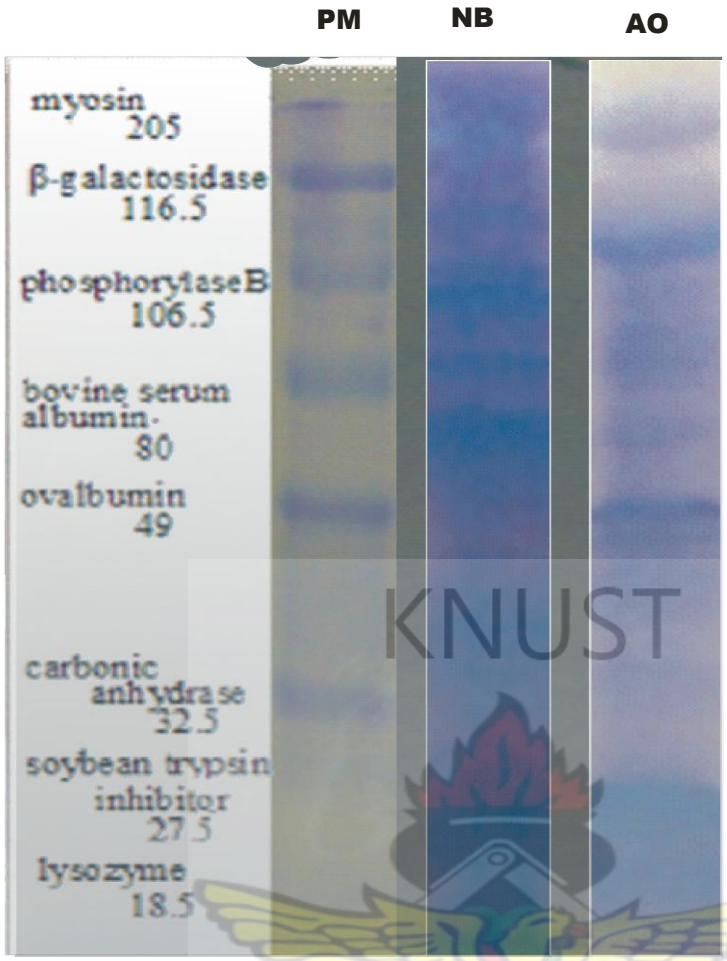


Figure 4.12: A 12 % SDS-polyacrylamide gel stained with Coomassie brilliant blue. Lane 1 (PM), marker protein. Lane 2 (NB), *Nephrolepis biserrata* protein extract. Lane 3 (AO), *Arthropteris orientalis* protein extract

The gel shows that the protein banding patterns of the two types of ferns are different enough from each other that perhaps they should be recognized as different species of fern. Banding patterns of the same protein (a protein with the same function) may be different in different species (Wiltfang *et al*., 1991). The reason being that over evolutionary time, the DNA of the two species accumulates differently due to mutation. The proteins in the two types of fern may therefore have slightly different sequences, which accounts for different banding patterns on the gel, but still retain the same or similar function.

The two fern types can therefore be characterized as having similar properties of protein types such as β-galactosidase (116.5 kDa), phosphorylase (106.5 kDa), bovine serum albumin (80 kDa) and lysozyme (18.5) in terms of their molecular weight. The presence of these types of proteins in the fern fronds could play a useful functional activity for human health. Lysozyme for example is an antimicrobial protein that plays an important role in pulmonary host defense (Nash *et al*., 2006).

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The output of proteins from defatted fern was optimized using Response Surface Methodology. Among the treatment methods used, defatted fern protein was effectively extracted by alkaline. The extraction of protein from both fern types (*Nephrolepis biserrata* and *Arthropteris orientalis*) were significantly influenced by variation in NaOH concentration and agitation time. The best condition to optimize protein extraction from *Nephrolepis biserrata* and *Arthropteris orientalis* was found to be 0.1 M NaOH and agitation time of 30 min. *Nephrolepis biserrata* fern type gave relatively higher maximum protein yield, giving about more than twice the values obtained for the *Arthropteris orientalis.*

Fern protein isolates showed good functional properties that confirmed that the procedure used in it production was good enough and it could be used in protein supplementation in various food systems particularly for developing countries where protein deficiencies remain a major health problem for children.

Bands observed for both *Arthropteris orientalis* and *Nephrolepis biserrata* after running electrophoresis showed their potentials of having similar protein types such as β-galactosidase, phosphorylase, bovine serum albumin and lysozyme in terms of their molecular weight.

5.2 Recommendation

The following are further recommended based on the findings of the study:

1. Further studies should be conducted to ascertain why using concentrated HCl rather than 0.5 M HCl brought about a good functional property but did not denature the proteins since this is known to affect solubility.

2. Functional properties of the fern protein isolate should be studied in real food systems to find the optimal utilization of fern or its components.

3. In order to determine the quality of the fern protein isolate, it is recommended that further studies should be conducted on the amino acid profile of the two fern type and also the protein digestibility analysis.

REFERENCES

Aberoumand, A. and Deokule, S. S. (2010). Composition analysis and evaluation of nutritional value of Polly dwarf, an emerging niche vegetable. *Electronic Journal of Environmental, Agricultural and Food Chemistry*, 9 (4): 790-795

Aberoumand, A. and Deokule, S. S. (2008). A Comparison of compounds of some edible plants of Iran and India, *Pakistan Journal of Nutrition.* 7 (4):582-585

Abu-Tarboush, H. M. (1995). Factors affecting protein extractability of defatted Karkade (*Hibiscus sabdariffa*) seed flour. *Journal of King Saud University*, vol. 7, Agricultural Science (2), pp. 179-186.

Abuye, C., Urga K., Knapp, H., Selmar D., Omwega, A., Imungi, J. and Winterhalter, P. (2003). A survey of wild, green, leafy vegetables and their potential in combating micronutrient deficiencies in rural populations, *East Africa Medcinal Journal*, 80: 247-252.

Ahmedna, M., Prinyawiwatkul, W. and Rao, R. M. (1999). Solubilized wheat protein isolate: Functional properties and potential food applications. *Journal of Agriculture. Food Chemistry*, 47:1340-1345.

Akubugwo, I. E., Obasi, N. A., Chinyere, G. C. and Ugbogu, A. E. (2007). Nutritional and chemical value of *Amaranthus hybridus* L. leaves from Nigeria, *Africa Journal of Biotechnology*. 6 (24), 2833-2839.

Allen, R. A. (1999). Ferns. Herbarium Intern.

Aluko, R. E. and Yada, R. Y. (1995). Structure-function relationships of cowpea (*Vigna unguiculata*) globulin isolate: Influence of pH and NaCl on physicochemical and functional properties. *Food Chemistry*. 53: 259-265

Alzagtat, A. A. and Alli, I. (2002). Protein-lipid interactions in food systems: a review. *International Journal of Food Sciences and Nutrition* 53 (3), 249-260.

AOAC (2005). Official Methods of Analysis of the Association of Official Analytical Chemist. 18th edn., Horwitz William Publication, Washington, DC., USA.

AOAC (1995). Official Method of Analysis. 16th edn., Association of Official Analytical Chemists, Washington, DC.

Arifin, B., Bono, A., Farm, Y. Y., Ling, A. L. L. and Fui, S.Y. (2009). Protein Extraction from Palm Kernel Meal. *Journal of Applied Sciences,* 9: 2996-3004.

Arnett, T. R. and Spowage, M. (1996). Modulation of the resorptive activity of rat osteoclasts by small changes in extracellular pH near the physiological range. Bone 18, 277-279.

Arntfield, S. D. (2004). Proteins from oil-producing plants. In: Yada, R. Y., Proteins in food processing. Boca Raton Boston. New York, Washington, DC. pp 146-175.

Arntfield, S. D. (1996). Effect of divalent cations, phytic acid, and phenolic compounds on the gelation of ovalbumin and canola protein. In: Macromolecular Interactions in Food Technology, Washington DC, America Chemistry Society, 82-92.

Arosa, M. L., Ramos, J. A., Valkenburg, T., Ceia, R., Laborda, H., Quintanilla, L. G. and Heleno, R. (2009). Fern feeding by the Azores bullfinch. Ardeola 56(1).

Aruna, V. and Parakash, V. (1993). Functional properties of the total proteins of sun flower (*Helianthus annuus* L.) seed. Effect of physical and chemical treatments. *Journal of Agriculturae, Food Chemistry*, 41: 18-23.

Babayemi, O. J., Bamidole, M. A and Omojola, A. B. (2006). Evaluation of the nutritive value and free choice intake of two aquatic weeds (*Neprolepis biserrata* and *Spirodela polyriza*) by West African Dwarf goats. Tropical and subtropical agroecosystems, año/vol. 6, número 001, Universidad Autónoma de Yucatán, Yucatán, México. Pp. 15-21

Badwaik, L. S., Prasad, K. and Deka, S. C. (2012). Optimization of extraction conditions by response surface methodology for preparing partially defatted peanut. *International Food Research Journal* 19(1): 341-346.

Bagnall, L. O., Shriley, R. I. and Hentages, J. F. (1973). Processing, chemical composition and nutritive value of aquatic weeds. Publication No 25. Water Resource centre. University of Florida, Gainsvills Florida.

Bandyopadhyay, K. and Ghosh, S. (2002). Preparation and characterization of papain-modified sesame (Sesamum indicum L.) protein isolates. *Journal of Agriculture, Food Chemistry*. 50: 6854-6857.

Barzel, U. S. and Massey, L. K. (1998). Excess dietary protein can adversely affect bone. *Journal of Nutrition* 128, 1051-1053.

Barzel, U. S. (1976). Acid-base balance in disorders of calcium metabolism. New York State, *Journal of Medicine* 76**,** 234-237.

Batista, I. (1999). Recovery of proteins from fish waste products by alkaline extraction. European Food Research and Technology, 210: 84-89.

Belitz, H. –D., Grosch, W. and Schieberle, P. (2009). Food Chemistry. 4th revised and extended edn. Heidelberg, Germany. Pp 62-63.

Benjamin, A. and Manickam, V. S. (2007). Medicinal Pteridophytes from the Western Ghats. *Indian Journal of Traditional Knowledge*. 6:611–618.

Beradi, L. C., Martinez, W. H., Fernandez, D. J. and Gazee, B. B. (1967). Extraction of nitrogenous constituents of cottonseed. In: Abstracts of papers. 154th meeting, Chicago: American Chemistry Society.

Betschart, A. A., Fong, R. Y. and Hanamoto, M. M. (1979). Safflower protein isolates: functional properties in simple systems and breads. *Journal of Food Science*. 44, 1021-1025.

Bland, J. M., and Lax, A. R. (2000). Isolation and characterization of a peanut maturity associated protein. *Journal of Agriculture, Food Chemistry*. 48, 3275-3279.

Bora, P. S. and Ribeiro, D. (2004). Influence of pH on the extraction yield and functional properties of Macadamia (*Macadamia integrofolia*) protein isolates. Food Science and Technology Int. 10: 263-267.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of Microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72:248-254

Bradley, N. (2007). The Response Surface Methodology. MSc. Thesis, Indiana University, South Bend

Burlingame, B. (2000). Comparison of total lipids, fatty acids, sugars and nonvolatile organic acids in nuts from *Castanea* species. *Journal of Food Comp. Anal*., 13: 99-100.

Cai, Y., Luo, Q., Sun, M. and Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Science, 74:2157–2184.

Cai, T., Chang, K. C. and Lunde, H. (1996). Physicochemical properties and yields of sunflower protein enzymatic hydrolysates as affected by enzyme and defatted sunflower meal. *Journal of Agriculture. Food Chemistry*. 44, 3500-3506.

Campbell, W. W., Barton Jr., M. L., Cyr-Campbell, D., Davey, S. L., Beard, J. L., Parise, G. and Evans, W. J. (1999). Effects of an omnivorous diet compared with a lactoovovegetarian diet on resistance-training-induced changes in body composition and skeletal muscle in older men. *American Journal of Clinical Nutrition* 70, 1032- 1039.

Cao, Q. M., Zhong, H. Y., Li, Z. H. and Sun, C. B. (2007). Research on *Pteridium aquilinum* starch paste properties. Food Science and Technology, 11, 36–39.

Carcea, M. and Schofield, J. D. (1996). Protein-lipid interactions in wheat gluten: reassessment of the occurrence of lipid-mediated aggregation of protein in the gliadin fraction. *Journal of Cereal Science* 24 (2), 101-113.

Cepeda, E., Villaran, M. C. and Aranguiz, N. (1998). Functional properties of faba bean protein flour dried by spray drying and freeze drying. *Journal of Food Engineering*. 36, 303–310.

Chapman, A. D. (2009). Numbers of Living Species in Australia and the World. Report for the Australian Biological Resources Study. Canberra, Australia. http://www.environment.gov.au/biodiversity/abrs/publications/other/species-numbers/index.html**.**

Chavan, U. D., McKenzie, D. B. and Shahidi, F. (2001). Protein classification of beach pea. In: Influence of pH on the extraction yield and functional properties of Macadamia (*Macadamia integrofolia*) protein isolates. *Food Science and Technology Int.* 10: 263-267.

Chen, M. and Rohani, S. (1992). Recovery of canola meal proteins by precipitation. Biotech. Bioeng. 40, 63-68.

Chen, X. Q. (1990). Chinese Plant Records, Science and Technology Press, Beijing, pp. 240-245.

Cobb, B. A. (1975). Field Guide to ferns and their related families: Northeastern and Central. North America. New York: Houghton Mifflin Company.

Copeland, E. B. (1942). Edible ferns. *American Fern Journal*. 32:121–126.

Cornelissen, J. H. C., Quested, H. M., Gwynnjones, D., Van Logtestijn, R. S. P., De Beus, M. A. H., Kondratchuk, A., Callaghan, T. V. and Aerts, R. (2004). Leaf digestibility and litter decomposability are related in a wide range of subarctic plant species and types. *Functional Ecology*, 18: 779-786.

Crouse, J. R., Morgan, T., Terry, J. G., Ellis, J., Vintolins, M. and Burke, G. L. (1999). A randomized trial comparing the effect of casein with that of soy protein containing varying amounts of isoflavones on plasma concentrations of lipids and lipoproteins. Archives of Internal Medicine 159, 2070-2076

Damodaran, S. (1996). Functional properties, in Nakai S and Modler H W, Food Proteins. Properties and Characterization, New York, VCH, 167-234.

Dawson-Hughes, B. (2003). Calcium and protein in bone health. Proclamations of the Nutrition Society 62: 505-509.

Dawson-Hughes, B. and Harris, S. S. (2002). Calcium intake influences the association of protein intake with rates of bone loss in elderly men and women. *American Journal of Clinical Nutrition* 75, 773-779.

Dench, J. E., Rivas, N. and Caygill, J. C. (1981). Selected functional properties of Sesame (*Sesamum indicum* L.). Flour and two protein isolates. *Journal of Food Science and Agriculture*. 32: 557-564.

Deng, Q. Y., Barefoot, R. R., Diosady, L. L. Rubin, L. J. and Tzeng, Y. M. (1990). Lysinoalanine concentrations in rapeseed protein meals and isolates. Can Institute of *Food Science and Technology Journal*. 23, 140-142.

Design Expert (2007). Stat Ease Inc., Hennepin Square, Suite 480, 2021E. Hennepin Ave., Minneapolis, MN55413-2726.

Dickinson, E. (1998). Stability and rheological implications of electrostatic milk protein-polysaccharide interactions. *Trends in Food Science and Technology* 9, 347-354.

Dia-Moukala, J. U. K. and Zhang, H. (2011). Defatted Corn Protein Extraction: Optimization by Response Surface Methodology and Functional Properties. *American Journal of Food Technology*, 6: 870-881.

Diaz, M. (1994). Variability in seed size by granivorous passerines: effects of bird size, bird size variability and ecological plasticity. Oecologia, 99: 1-6.

Diniz, F. M. and Martin, A. M. (1997). Effects of the extent of enzymatic hydrolysis on the functional properties of shark protein hydrolysate. Lebensmittel-Wissenschaft und-Technology, 30: 266-272.

Eke, O. S. and Akobundu, E. N. T. (1993). Functional properties of African yam bean (*Sphenostylis stenocarpa*) seed flour as affected by processing. *Food Chemistry*. 48: 337–340.

Ekop, A. S. (2007). Determination of chemical composition of *Gnetum africana* (AFANG) seeds. In: Comparison of the nutritive value, antioxidant and antibacterial activities of *Sonchus asper* and *Sonchus oleraceus*. Rec. Nat. Prod. 5: 1(2011) 29-42.

El-Adawy, T. A. (2000). Functional properties and nutritional quality of acetylated and succinylated mung bean protein isolate. *Food Chemistry* 70: 83–91.

El-Tinay, A., Nour, A. M., Abdel-Karim, S. H. and Mahgoub, S. O. (1988). Aqueous protein and gossypol extraction from glanded cottonseed flour: factors affecting protein extraction. *Food chemistry*, 29: 57-63.

Encyclopedia Britannica (2011). Encyclopedia Britannica, Online Academic Edition. http://www.britannica.com/EBchecked/topic/204819/fern. Accessed on 21st November, 2011.

Eromosele, C. O., Arogundade, L. A., Eromosele, I. C. and Ademuyiwa O. (2008). Extractability of African yam bean sphenostylis stenocarpa protein in acid salt and alkaline aqueous media. *Journal of Food Hydrocolloid*. 22: 622-628

FAO (1978). China *Azolla* propagation and small scale biogas technology. FAO Soils Bull. 41:120.

Faruq, U., Sani, A. and Hassan, L. (2000). Composition and distribution of deadly nightshade. *Nigerian Journal*. Basic Applied Science. 11: 157-164.

Fernández, H., Bertrand, A. M. and Sánchez-Tamés, R. (1999). Biological and nutritional aspects involved in fern multiplication. In: Interaction between sucrose and pH during in vitro culture of *Nephrolepis biserrata* (Sw.) Schott (Pteridophyta). Acta bot. bras. 18(4): 809-813.

Ferreira, T. A. and Rasband, W. (2011). The imageJ user guide (Ver) IJ 1.45, pp 129-137. http://rsbweb.nih.gov/ij/docs/guide. (Date accessed: October 09, 2012).

Feskanich, D., Willett, W. C., Stampfer, M. J. and Colditz, G. A. (1996). Protein consumption and bone fractures in women. *American Journal of Epidemiology* 143 472-479.

Food and Agriculture Organization/World Health Organization (1990). Protein quality evaluation; report of the joint FAO/WHO expert consultation. FAO Food and Nutrition Paper 52, Rome, Italy.

Geshian, S., Dai- X. and Zhi-Bao, S. (1980). Salt tolerance of *Azolla filiculoides* and its effects on the growth of paddy in Xinwei Haitu. Zhejiang Nongye Kexue 1: 17-24.

Gill, T. A. and Tung, M. A. (1976). Thermally induced gelation of the 12S rapeseed glycoprotein. *Journal of Food Science*, 43: 1481-1485.

Glicksman, M. (1991). Hydrocolloids and the search for the "Oily Grail." Food technology; 48(2): 142-152.

Govardhan-Singh, R. S., Ogunsina, B. S. and Radha, C. (2011). Protein extractability from defatted *moringa oleifera* Lam. seeds flour. *Ife Journal of Science* vol. 13 (1):121-127

Haber, A. and Runyon, R. (1977). General Statistics. 3rd Edn. Addison-Wesley, Boston.

Haller, W. T., Sutton, D. D. and Barlowe, W. C. (1974). Effects of salinity on the growth of several aquatic macrophytes. In: Propagation, taxonomy and ecophysiological characteristics of the Azolla-Anabaena symbiosis in freshwater habitats of Beni-Suef Governorate (Egypt). *Egyptian Journal of Biology*. Vol. 9: 1-12.

Ham, S. S. (2004). Wild vegetables: anticancer & healthy life. Human & Books, Seoul, p. 20.

Hang, Y. D., Steinkraus, K. H. and Hackler, B. R. (1970). Comparative studies on the nitrogen solubility of mung beans, pea beans and red kidney beans. *Journal of Food Science*, 35: 318-322

Hannan, M. T., Tucker, K. L., Dawson-Hughes, B., Cupples, L. A., Felson, D. T. and Kiel, D. P. (2000). Effect of dietary protein on bone loss in elderly men and women: The Framingham Osteoporosis Study. *Journal of Bone Mineral Research* 15: 2504-2512.

Herz-Alla, N. A. (1991). Some studies on *Azolla* propagation in Egypt. Ph.D. Thesis, Zagazig University (Benha Branch), Egypt.

Hill, P. A. and Lucas, P. W. (1996). Toughness and Fibre Content of Major Leaf Foods of Japanese Macaques *(Macaca fuscata yakui)* in Yakushima. *American Journal of Primatology*, 38: 221-231.

Hoffman, J. R. and Falvo, M. J. (2004). Protein-Which is best? *Journal of Sports Science and Medicine*. 3:118-130

Howell, N. K, Herman, H. and Li-Chan, E. C. Y. (2001). Elucidation of protein-lipid interactions in a lysozyme-corn oil system by Fourier transform Raman spectroscopy. *Journal of Agriculture*. *Food Chemistry*. 49 (3), 1529-1533.

Hu, X. –Z., Cheng, Y. –Q., Fan, J. –F., Lu, Z. –H., Yamaki, K. and Li, L.-T. (2010). Effects of drying method on physicochemical and functional properties of soy protein isolates. *Journal of Food Processing and Preservation* 34: 520-540.

Hutchins, N. R. (2006). Diet, nutrition and reproductive success of *Roosevelt elk* in managed forests of the Olympic peninsula, Washington. MSc. Thesis. Humboldt State University.

Inyang, U. E. and Iduh, A. O. (1996). Influence of pH and salt concentration on protein solubility, emulsifying and foaming properties of sesame protein concentrate. *Journal of American Oil Chemistry Society*, 73: 1663-1667.

Jauricque, U. K.-D.-M. and Hui, Z. (2011). Defatted Corn Protein Extraction: Optimization by Response Surface Methodology and Functional Properties. *American Journal of Food Technology*, 6: 870-881.

Joglekar, A. M. and May, A. T. (1987). Product excellence through design of experiments. Cereal Food World, 32: 857-868.

Jones, D. (1987). Encyclopedia of Ferns. Vol. 1. Portland, OR: Timber Press. <ahref="http://science.jrank.org/pages/2689/Ferns-Importance-humans.html">Ferns-Importance To Humans (accessed on 26th February, 2012)

Jones, O. L. (1987). Encyclopedia of ferns: An introduction to fern, their structure, biology, economic importance, cultivation and propagation. British Museum, 2: 12-22.

Jung, S., Murphy, P. A. and Johnson, L. A. (2005). Physicochemical and functional properties of soy protein substrates modified by low levels of protease hydrolysis. *Journal of Food Science*, 70: 180-187.

Jyothirmayi, T., Prabhakara Rao, P. G. and Walde, S. G. (2006). Nitrogen extractability and functional properties of defatted erythrina variegate flour. *Food chemistry. 96:242-247*

Kachik, F., Mudlagiri, B. G., Gary, R. B., Joanne, H., Lusby, W. R., Maria, D. T. and Barrera, M. R. (1992). Effects of food preparation on qualitative and quantitative distribution of major carotenoids constituents of tomatoes and several green vegetables. *Journal of Agriculture*. *Food Chemistry*. 40: 390-398.

Kalapathy, U., Hettiarachchy, N. S. and Rhee, K.C. (1997). Effect of drying methods on molecular properties and functionalities of disulfide bond-cleaved soy proteins. *Journal of American Oil Chemistry Society*. 74: 195–199.

Kamara, M. T., Zhu, K., Amadou, I., Tarawalie, F. and Zhou, H. (2009). Functionality, *in vitro* digestibility and physicochemical properties of two varieties of defatted foxtail millet protein concentrates. *International Journal of Molecular Science*, 10: 5224-5238

Kanu, P. J., Kerui, Z., Ming, Z. H., Haifeng, Q., Kanu, J. B. and Kexue, Z. (2007). Functional properties of Sesame (*Sesamum indicum* L.) protein isolate as influenced by pH, temperature, time and ratio of flour to water during its production. *Asian Journal of Biochemistry* 2 (5): 289-301.

Karki, B., Lamsal, B. P., Grewell, D., Pometto, A. L., Van Leeuwen, J. S., Khanal, K. and Jung, S. (2009). Functional properties of soy protein isolates produced from ultrasonicated defatted soy flakes. *Journal of American Oil Chemistry Society*. 86: 1021-1028.

Kari, P. R. (1987). Tanaína Plantlore, Denáina K′et′ una: An Ethnobotany of the Denáina Indians of Southcentral Alaska. National Parks Service, Alaska Region, Anchorage.

Karim, A. A. and Bhat, R. (2008). Gelatin alternatives for the food industry: recent developments, challenges and prospects. *Trends Food Science and Technology*. 19:644–56.

Katsube, T., Imawaka, N., Kawano, Y., Yamazaki, Y., Shiwaku, K. and Yamane, Y. (2006). Antioxidant flavonol glycosides in mulberry (*Morus alba* L.) leaves isolated based on LDL antioxidant activity. *Food Chemistry*. 97:25–31.

Khalid, E. K., Babiker, E. and El Tinay, A. H. (2003). Solubility and functional properties of sesame seed proteins as influenced by pH and/or salt concentration. *Food Chemistry.* 82: 361-366.

Kidmose, U., Yang, R. Y., Thilsted, S. H., Christensen, L. P. and Brandt, K. (2005). Content of carotenoids in commonly consumed Asian vegetables and stability and extractability during frying. *Journal of Food Composition and Analysis.*19: 562−571.

Kim, S. H. and Kinsella, J. E. (1987). Surface active properties of food proteins: Effects of reduction of disulfide bonds on film properties and foam stability of glycinin. *Journal of Food Science*. 52: 128–135.

Kinsella, J. E. (1979). Functional properties of soy proteins. *Journal of American Oil Chemistry Society*. 56: 242-246.

Klockeman, D. M., Toledo, R. and Sims, K. (1997). Isolation and characterization of defatted canola meal protein. *Journal of Agriculture, Food Chemistry*. 45: 3867-3870.

Kohnhorst, A. L. and Mangino, M. E. (1985). Prediction of strength of whey protein gels based on composition. *Journal of Food Science.* 50: 1403-1405.

Kokkinidou, A. (2011). Arsenic Exposure in Playgrounds? Plant Ferns! http://www.brighthub.com/engineering/civil/articles/117974.aspx (Accessed 5/12/2011)

Kramer, K. U. (1990). "Davalliaceae". In: The Families and Genera of Vascular Plants. Vol. 1. Springer-verlag: Berlin; Heidelberg, Germany.

Kumagai, H., Ishida, S., Koizumi, A., Sakurai, H. and H. Kumagai (2002). Preparation of phytate-removed deamidated soybean globulins by ion exchangers and characterization of their calcium-binding ability. *Journal of Agriculture, Food Chemistry* 50: 172-176.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Leâger, L. W. and Arntfield, S. D. (1993). Thermal gelation of 12S canola globuli. *Journal of American Oil Chemistry Society*. 70: 853 861.

Lee, C. H. and Shin, S. L. (2011). Functional activities of ferns for human health. *Korean J. Pant Res*. 24:347-359.

Lee, S. O., Lee, H. J., Yu, M. H., Im, H. G. and Lee, I. S. (2005). Total polyphenol contents and antioxidant activities of methanol extracts from vegetables produced in Ullung Island. *Korean Journal of Food Science and Technology*. 37:233–240

Li-Chan, E. C. Y. and Qin, L. (1998). The application of Raman spectroscopy to the structural analysis of food protein networks', in Sessa D J and Willett J L, Paradigm for Successful Utilization of Renewable Resources, Champaign, AOCS Press, 123-139.

Li-Chan, E. C. Y. (1996). Separation and purification. In Nakai, S. and Modler, H. W., Food proteins. Properties and characterization, New York, VCH, 429-503.

Liu, K. (1997). Certain functional properties of defaulted pumpkin seed flour. *Plant Foods Human Nutrition*. 42: 257-273.

Lopez, G., Flores, I., Galvez, A., Quirasco, M. and Farres, A. (2003). Development of a liquid nutritional supplement using a *Sesamum indicum* L. protein isolate. Lebensm.-Wiss.-Technology. 36: 67-74.

Lowman, M. D. and Box, J. D. (1983). Variation in leaf toughness and phenolic content among five species of Australian rain forest trees. *Australian Journal of Ecology*, 8: 17-25.

Lumpkin, T. A. and Plucknett, D. L. (1980). *Azolla*: botany, physiology, and use as a green manure. Econ. Bot. 34: 111-153.

Lyimo, M., Nyagwegwe, S. and Mukeni, E. (1991). Investigation of the traditional food processing, preservation, storage, and methods on vegetable nutrients; a case study of Tanzania, *Plant Food Human Nutrition*. 41:53-57

Ma, W., M. W. Sutherland, S. Kammholz, P. Banks, P. Brennan, W. Bovill and G. Daggard (2007). Wheat flour protein content and water absorption analysis in a doubled haploid population. *Journal of Cereal Science*. 45: 302-308.

MacDonald, G. A, Lanier, T. C. and Carvajal, P. A. (2000). Stabilization of proteins in Surimi: In Park, J. W., Surimi and Surimi Seafood, New York, Marcel Dekker, 91-125.

Marrs, R. H. and Watt, A. S. (2006). Biological Flora of the British Isles: *Pteridium aquilinum. Journal of Ecology*, 94: 1272-1321.

Martinez, W. H., Beradi, L. C., Gazee, B. B. and Fernandez, D. J. (1967). A comparative study on the preparation of cottonseed protein isolates. II. Characterization and color. In: Abstracts of Papers. 154th meeting, Chicago: American Chemistry Society.

Mate, J. I. and Krochta, J. M. (1994). β-Lactoglobulin separation from whey protein isolate on a large scale. *Journal of Food Science* 59: 1111-1114.

McHugh, A. (1992). The Cultivation of Ferns. North Pomfret, VT: Trafalgar Press.

Mills, J. A., Lee, W. G., Mark, A. F. and Lavers, R. B. (1980). Winter use by Takahe (*Notornis mantelli*) of the summer-green fern (*Hypolepis millefolium*) in relation to its annual cycle of carbohydrates and minerals. New Zealand *Journal of Ecology*, 3: 131-137.

Mishra, V. K, Upadhyay, A. R., Pandey, S. K. and Tripathi, B. D. (2007). Concentrations of heavy metals and aquatic macrophytes of Govind Ballabh Pant Sagar an anthropogenic lake affected by coal mining effluent. Environ Monitoring Assessment, in press.

Montgomery, D. C. (2005). Design and Analysis of Experiments: Response surface method and designs. New Jersey: John Wiley and Sons, Inc.

Moore, A. W. (1969). *Azolla*: biology and agronomic significance. Bot. Rev. 35: 17-35.

Moran, R. C. (2004). A natural history of ferns. *New Zealand Journal of Ecology*. Timber Press. Portland.

Morton, C. V. (1958). Observations on cultivated ferns. The species and forms of *Nephrolepis*. *American Fern Journal* 48: 18-27.

Moure, A., Sineiro, J., Domınguez, H. and Parajo, J. C. (2006). Functionality of oilseed protein products: a review. Food Res Int 39:945–63.

Munger, R. G., Cerhan, J. R. and Chiu, B. C. H. (1999). Prospective study of dietary protein intake and risk of hip fracture in postmenopausal women. *American Journal of Clinical Nutrition*. 69:147-52.

Murray, D. (2001a). Isolating protein from canola. Oils Fats Int, 17 (3), 19-21.

Murray, D., (2001b). Rapeseed: a potential global source of high quality plant protein. Asia Pacific Food Ind, April 2001, 30-34.

Nash, J. A., Ballard, T. N. S., Weaver, T. E. and Akinbi, H. T. (2006). The Peptidoglycan-Degrading property of lysozyme is not required for bactericidal activity In Vivo. *Journal of Immunology*. Vol. 177 (1), 519-526.

Nestel, P. J., Pomeroy, S., Kay, S., Komesaroff, P. Behrsing, J. Cameron, J. D. and West, L. (1999). Isoflavones from red clover improve systemic arterial compliance but not plasma lipids in menopausal women. *Journal of Clinical Endocrinology Metabolism*. 84: 895-898.

Nielsen, S. S. (2010). Food analysis: Protein separation and characterization. Food science text series. Fourth ed., pp 263-280

Nurdiyana, H., Siti-Mazlina, M. K. and Siti Nor Fadhilah, M. (2008). Optimization of protein extraction from freeze dried fish waste using response surface methodology (rsm). *International Journal of Engineering and Technology*, Vol. 5(1), pp. 48-56.

O'Donnel, C. and Dilks, P. (1994). Foods and foraging of forest birds in temperate rainforest, South Westland, New Zealand. 18: 87-107.

Oh, B. M., Kweon, M. H. and Ra, K. S. (1994). Isolation and characterization of acidic polysaccharides activation complement system from the hot water extracts of *Pteridium aquilinum* var. *latiusculum*. *Korean Journal of Food Nutrition*. 7:159–168.

Oldham, J. D. and Alderman, G. (1980). Recent advances in understanding protein-energy interrelationships in intermediary metabolism in ruminants. In: Protein and energy supply of high producing milk and meat. Pergamon Press. Oxford. pp 728.

Oloyede, F. A., Oloyede, F. M., Saliu, T. L. and Ogunwole, A. A. (2010). Chemical composition of a tropical fern *Ceratopteris cornuta* (Parkeriaceae, Pteridophyta) in Nigeria. Acta Botanica Hungarica 52(1-2), pp. 145-150.

Onweluzo, J. C., Obanu, Z. A. and Onuoha, K. C. (1994). Functional properties of some lesser known tropical legumes. *Journal of Food Science and Technology*. 31:302-306.

Oomah, B. D., Mazza, G. and Cui, W. (1994). Optimization of protein extraction from flaxseed meal. Food Res Int, 27, 355-361.

Oomah, B. D. and Mazza, G. (1993). Flax proteins. A review, *Food Chemistry*. 48: 109-114.

Ordorica-Falomir, C., Paredes-Loâ Pez, O. and Peza, R. J. (1990). Production of safflower protein isolates: Gel filtration and electrophoretic patterns. Lebensm Wissu Technol, 23, 122-125.

Owen, S. R., Tung, M. A. and Paulson, A. T. (1992). Thermorheological studies of food polymer dispersions. *Journal of Food Engineering* 16: 39-53.

Pannemans, D. L. E., Wagenmakers, A. J. M., Westerterp, K. R., Schaafsma, G. and Halliday, D. (1998). Effect of protein source and quantity on protein metabolism in elderly women. *American Journal of Clinical Nutrition*. 68: 1228-1235.

Parrado, J., Bautista, J. and Machado, A. (1991). Production of soluble enzymatic protein hydrolysate from industrially defatted nondehulled sunflower meal. *Journal of Agriculture, Food Chemistry*. 39: 447-450.

Paredes-Loâ Pez, O. (1991). Safflower proteins for food use, in Hudson B J F, Developments in Food Proteins. New York, Elsevier Applied Science, 1-33.

Park, H. A., Kweon, M. H., Han, H. M., Sung, H. C. and Yang, H. C. (1998). Effects of the glycoprotein isolated from *Pteridium aquilinum* on the immune function of Mice. *Korean Journal of Food Science and Technology*. 30:976–982.

Pawar, V. D., Patil, J. N., Sakhale, B. K. and Agarkar, B. S. (2001). Studies on selected functional properties of defatted sunflower meal and its high protein products. *Journal of Food Science and Technology*, India. 38: 47-51 (abstract only).

Pericin, D., Radulovic, L., Trivic, S. and Dimic, E. (2008). Evaluation of solubility of pumpkin seed globulins by response surface method. *Journal of Food Engineering*, 84: 591-594.

Pietrasik, Z., Jarmoluk, A. and Shand, P. J (. 2007). Effect of non-meat proteins on hydration and textural properties of pork meat gels enhanced with microbial transglautaminase. LWT-*Food Science and Technology*. 40: 915–20.

Promislow, J. H. E., Goodman-Gruen, D., Slymen, D. J. and Barrett-Connor, E. (2002). Protein consumption and bone mineral density in the elderly. *American Journal of Epidemiology* 155: 636-644.

Pryer, K. M., Schuettpelz, E., Wolf, P. G., Schneider, H., Smith, A. R. and Cranfill, R. (2004). Phylogeny and evolution of ferns (monilophytes) with a focus on the early leptosporangiate divergences. *American Journal of Botany* 91: 1582-1598.

Przybycien, T. M. and Bailey, J. E. (1991). Secondary structure perturbations in salt- induced protein precipitate, Biochim. Biophys. Acta 1076:103-111.

Rahoma, A. T. M. (1985). Relation of *Azolla* with some ecological factors. MSc.Thesis. Alexandria University, Egypt.

Ramos, J. A. (1995). The diet of the Azores Bullfinch *Pyrrhula murina* and floristic variation within its range. Biological Conservation. 71: 237-249.

Ramos, J. A. (1994). Fern frond feeding by the Azores Bullfinch. *Journal of Avian Biology*. 25: 344-347.

Ray, G. (1980). Root, Stem and Leaf: Wild Vegetables of Southeast Alaska. South East Regional Resource Center, Juneau.

Ray-Yu, Y., Lien-Chung, C., Jenn-Chung, H., Weng, B. B. C., Palada, M. C., Chadha, M. L. and Virginie, L. (2006). Nutritional and functional properties of moringa leaves-from Germplasm, to plant, to food, to health. American Chemical Society, Washington, D.C

Rodríguez, A. E. and Obeso, J. R. (2000). Diet of the Cantabrian Capercaillie: Geographic variation and energetic content. Ardeola, 47: 77-83.

Sathe, S. K., Hamaker, B. R., Sze-Tao, K. W. C. and Venkatachalam, M. (2002). Isolation, purification, and biochemical characterization of a novel water soluble protein from Inca peanut (*Plukenetia volubilis* L.). *Journal of Agriculture*, Food Chemistry. 50: 4096-4908.

Sathe, S. K. and Salunkhe, D. K. (1981). Solubilization and electrophoretic characterization of the great northern bean (*Phaseolus vulgaris* L.) proteins. *Journal of Food Science*. 46: 82-87.

Schaefer, H. M., Schmidt, B. and Bairlein, F. (2003). Discrimination abilities for nutrients: which difference matters for choosy birds and why? Animal Behaviour. 65: 531-541.

Schenz, T. W. and Morr, C. V. (1996). Viscosity in methods of testing protein functionality. Hall, G. M. (Ed), Blackie Academic and Professional, London, pp: 71-75

Schuettpelz, E. (2007). The evolution and diversification of epiphytic ferns. Duke University PhD thesis. http://dukespace.lib.duke.edu/dspace/bitstream/10161/181/1

Schuettpelz, E. and Pryer, K. M. (2007). "Fern phylogeny inferred from 400 Leptosporangiate species and 3 plastid genes". Taxon 56(4):1037-1050.

Sen, M. and Bhattacharyya, D. K. (2001). Hypolipidemic effect of enzymatically extracted sunflower seed protein fraction. *Journal of Food Science and Agriculture*. 81: 347-352.

Seigler, D. S. (1991). Cyanide and cyanogenic glycosides. In, G Rosenthal and B Berenbaum (Eds.): Herbivores: Their Interactions with Secondary Plant Metabolites, Vol 1, pp. 35-77. Academic Press. San Diego.

Sellmeyer, D. E., Stone, K. L., Sebastian, A. and Cummings, S. R. (2001). A high ratio of dietary animal to vegetable protein increases the rate of bone loss and risk of fracture in postmenopausal women. *American Journal of Clinical Nutrition* 73: 118-122.

Shahidi, F., Han, X. Q. and Synowiecki, J. (1995). Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). Food Chemistry. 53: 285-293.

Shamanthaka-Sastry, M. and Narasinga Rao, M. S. (1990). Binding of chrologenic acid by the isolate polyphenol-free 11S protein of sunflower (*Helianthus annuus*) seed. *Journal of Agriculture*, Food Chemistry. 38: 2103-2110.

Shin, S. L. (2010). Functional components and biological activities of Pteridophytes as healthy biomaterials. Ph.D. dissertation, Chungbuk National University, Cheongju, Korea.

Shrestha, M. K., Peri, I., Smirnoff, P., Birk, Y. and Golan-Goldhirsh, A. (2002). Jojoba seed meal proteins associated with proteolytic Y. and protease inhibitory activities. *Journal of Agriculture, Food Chemistry*. 50: 5670-5675.

Siddiq, M., Nasir, M., Ravi, R., Dolan, K. D. and Butt, M. S. (2009). Effect of defatted maize germ addition on the functional and textural properties of wheat flour. *International Journal of Food Propagation.* 12: 860-870

Sikorski, Z. E. (1997). Proteins, in Chemical and Functional Properties of Food Components. Second edition. Technomic Publishing Co. Inc., Lancaster, PA. pp. 142-144.

Singh, P., Kumar, R., Sabapathy, S. N. and Bawa, A. S. *(*2008). Functional and edible uses of soy protein products. CRFSFS 7:14–28.

Smith, A. R., Pryer, K. M., Schuettpelz, E., Korall, P., Schneider, H. and Wolf, P. G. (2008). "Davalliaceae". In: "Fern Classification". Biology and Evolution of Ferns and Lycophytes. Cambridge University Press. pp 417-467

Smith, A. R., Pryer, K. M., Schuettpelz, E., Korall, P., Schneider, H. and Wolf, P. G. (2006). A classification for extant ferns. Taxon 55 (3): 705–731.

Thakur, R. C., Hosoi, Y. and Ishii, K. (1998). Rapid in vitro propagation of *Matteuccia struthiopteris* (L.) Todaro – an edible fern. Plant Cell Rep. 18:203–208.

Thompson, L. U., Liu, R. F. K. and Jones, J. D. (1982). Functional properties and food applications of rapeseed protein concentrate. *Journal of Food Science*. 47: 1175-1180.

Thompson, L. U. (1977). Preparation and evaluation of mung bean protein isolates. *Journal of Food Science.* 42: 202-206.

Tiezheng, M., Qiang, W. and Haiwen, W. (2010). Optimization of extraction conditions for improving solubility of peanut protein concentrates by response surface methodology. LWT - Food Science and Technology 43: 1450-1455.

Tikkanen, M. J., Wahala, K., Ojala, S., Vihma, V. and Adlecrerutz, H. (1998). Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance. Proclamations of the National Academy of Science 95, P3106-P3110.

Tömösközi, S., Gyenge, L., Pelcéder, Á., Varga, J., Abonyi, T. and Lásztity, R. (2008). Functional properties of protein preparations from amaranth seeds in model system. *European Food Research and Technology*. 226:1343–134.

Triveni, R., Shamala, T. R. and Rastogi, N. K. (2001). Optimized production and utilization of exopolysaccharide from *Agrobacterium radiobacter*. Proc. Biochemistry, 36: 787-795.

Tyron, R. (1986). Biogeography of species, with special reference to ferns. Botanical Review, 52: 117- 156.

Tyron, R. M. and Tyron, A. F. (1982). Ferns and allied plants, with special reference to Tropical America. Springer-Verlag, New York. World Health Organisation (1985) *Energy and protein requirements*. World Health Organisation Technical Report Series 724, WHO Switzerland.

Turner, N. J., Gotiesfeld, L. M. J., Kuhnlein, H. V. and Ceska, A. (1992). Edible wood fern rootstocks of Western North America: Solving an ethnobotanical puzzle. *Journal of Ethnobiology*. Vol. 12 (1).

Turner, N. (1973). The ethnobotany of the Bella Coola Indians of British Columbia. Syesis 6:193-220.

Upadhyay, A. R., Mishra, V. K., Pandey, S. K. and Tripathi, B. D. (2007). Biofiltration of secondary treated municipal wastewater in a tropical city. Ecol. Engin. 30: 9–15.

Uzzan, A., (1988). Vegetable protein products from seeds: technology and uses in the food industry. In Hudson B. J. F., Developments in Food Proteins, New York, Elsevier Applied Science, 6, 73-118.

Vaclavik, V. A. and Christian, E. W. (2008). Essentials of food science. 3rd Edition, Springer science+Business media LLC. New York, USA.

Vani, B. and Zayas, J. F. (1995). Wheat germ protein flour solubility and water retention. *Journal Food Science* 60: 845–848.

Ventureira, J. L., Bolontrade, A. J., Speroni, F., Briand, E. D., Scilingo, A. A., Ropers, M. H., Boury, F., Añón, M. C. and Marc, A. (2011). Interfacial and emulsifying properties of amaranth (*Amaranthus hypochondriacus*) protein isolates under different conditions of pH. LWT - *Food Science and Technology* (45) 1-7.

Wang, M., Hettiarachchy, N. S., Qi, M., Burks, W. and Siebenmorgen, T. (1999). Preparation and functional properties of rice bran protein isolate. *Journal of Agriculture, Food Chemistry*. 47: 411-416.

Wani, A. A., Sogi, D. S., Grover, L. and Saxena, D. C. (2006). Effect of temperature, alkali concentration, mixing time and meal solvent ratio on the extraction of watermelon seed proteins-response surface approach. Biosystems Engineering 94(1): 67-73.

Wei, J. (2010). Edible ferns, nuts, and grasses. http://hubpages.com/hub/Edible-Ferns-Nuts and-Grasses. (Accessed on 27th February 2012)

Widén, C. J., Fraser-Jenkins, C. R., Reichstein, T. and Sarvela, J. (2001). A survey of phenolic compounds in *Dryopteris* and related fern genera. Part III. Phloroglucinol derivatives in subgenera *Erythrovaria* and *Nephrocystis* and related genera (Pteridophyta, Dryopteridaceae). Ann. Bot. Fennici 38: 99-138.

Widén, C. J., Sarvela, J. and Britton, D. M. (1983). On the location and distribution of phloroglucinols (filicin) in ferns. New results and review of literature. Ann. Bot. Fennici 20: 407-417.

Wierenga, P. A. and Gruppen, H. (2010). New views on foams from protein solutions. In: Defatted Corn Protein Extraction: Optimization by Response Surface Methodology and Functional Properties. *American Journal of Food Technology*, 6: 870-881.

Wiltfang, J., Arold, N. and Neuhoff, V. (1991). "A new multiphasic buffer system for sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins and peptides with molecular masses 100,000-1000, and their detection with picomolar sensitivity". Electrophoresis 12 (5): 352–366.

Xu, L, Lui, F., Luo, H. and Diosady, L. L. (2003). Production of protein isolates from yellow mustard meals by membrane processes. Food Res Int, 36, 849-856.

Yada, R. Y. (2004). Proteins in food processing. Boca Raton Boston. New York, Washington, DC.

Yadav, S. K and Sehgal, A. (1997). Effect of home processing on ascorbic acid and beta carotene content of bathua (*Chenopodium album*) and fenugreek (*Trigonella foenungraecum*) leaves. Plant Food Hum. Nutr. 50: 239-247.

Yeoh, H. H. and Wee, Y. C. (1998). Total amino acid compositions of tropical ferns and gymnosperms. *Asian Journal of Tropical Biology* 3(1), 13-18.

Yi, S., Bingjian, D., Ting, Z., Bing, H., Fei, Y., Rui, Y., Xiaosong, H., Yuanying, N. and Quanhong, L. (2011). Optimization of extraction process by response surface methodology and preliminary structural analysis of polysaccharides from defatted peanut (*Arachis hypogaea*) cakes. Carbohydrate Research 346: 305- 310

Yuno-Ohta, N., Maeda, H., Okada, M. and Ohta, H. (1994). Heat-induced gels of rice globulin: comparison of gel properties with soybean and sesame globulins. *Journal of Food Science*. 59: 366-370.

Zahrann, H. H., Abo-Ellil, A. H., Al-Sherif, E. A. (2007). Propagation, taxonomy and ecophysiological characteristics of *Azolla*-A*nabaena. Egyptian Journal of Biology*, Vol. 9, pp 1-12.

Zhang, S., Zhong, G., Liu, B. and Wang, B. (2011). Physicochemical and functional properties of fern rhizome (*Pteridium aquilinum*) starch. Starch Journal 63: 468-474.

Zhang, S. H., Qin, H. B., Hong, Y. (2008). Study on physical and chemical properties of fern root starch. Food Science. 29: 11–117 (In Chinese with English abstract).

Zimmerman, R. H. and Jones J. B. (1991). Commercial micropropagation in North America. In: P.C. Debergh & R.H. Zimmerman (eds.). Micropropagation: Technology and Application. Kluwer Acad.

APPENDICES

Appendix 1: Calculation of Proximate Analysis

Appendix 1a: Calculation of moisture content

$$\% \text{ Moisture} = \frac{(\text{weight of sample} - \text{weight of dry sample}) \times 100}{\text{weight of sample}}$$

Appendix 1b: Calculation of ash content

$$\% \text{ Ash} = \frac{(\text{weight after ashing} - \text{weight of empty crucible}) \times 100}{\text{weight of sample}}$$

Appendix 1c: Calculation of fat content

$$\% \text{ Fat} = \frac{(\text{weight of fat}) \times 100}{\text{weight of sample}}$$

Appendix 1d: Calculation of crude protein content

$$\% \text{ Nitrogen} = \frac{(S_t - S_b)\ 100 \times 0.1 \times 0.014 \times 100}{\text{Sample weight} \times 10}$$

S_t= Titre of sample
S_b= Titre of blank
Percentage nitrogen (%N) was converted to percent crude protein by multiplying by a factor of 6.25 (%Protein = % N x 6.25).

Appendix 1e: Calculation of crude fiber content

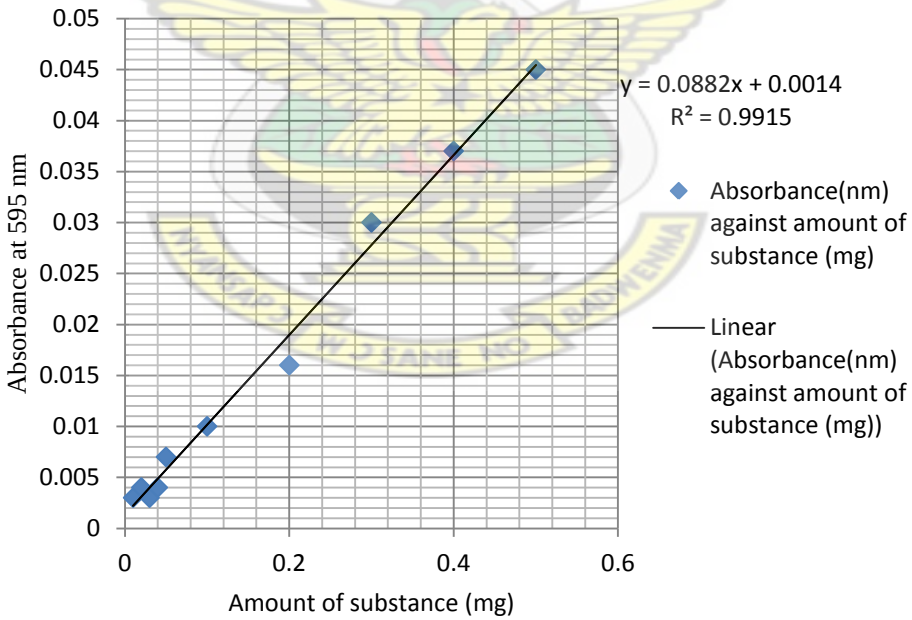
$$\%\text{Crude fiber} = \frac{(\text{weight of dry insoluble residue} - \text{weight of ash}) \times 100}{\text{weight of sample}}$$

Appendix 1e: Calculation of carbohydrate content

% Carbohydrate = 100 – (%moisture + %crude fat + %crude protein + %crude fiber + %ash)

Appendix 2: Standard Curve

Amount of Substance (mg)	Volume of stock solution (µL)	Volume of buffer (µL)	Mean absorbance(nm)
0.01	10	490	0.003
0.02	20	480	0.004
0.03	30	470	0.003
0.04	40	460	0.004
0.05	50	450	0.007
0.1	100	400	0.010
0.2	200	300	0.016
0.3	300	200	0.030
0.4	400	100	0.037
0.5	500	0	0.045



Appendix 3: ANOVA Tables

Appendix 3a: ANOVA for Response Surface Reduced 2FI Model for NB proteins using NaOH.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Block	0.039551	1	0.039551			
Model	69.54814	5	13.90963	43.3283	< 0.0001	significant
A-[NaOH concentration]	1.934573	1	1.934573	6.026168	0.0214	
B-Time	0.103349	1	0.103349	0.32193	0.5755	
C-NaOH	61.09521	1	61.09521	190.3107	< 0.0001	
AB	3.040354	1	3.040354	9.47066	0.0050	
AC	3.374656	1	3.374656	10.51201	0.0034	
Residual	8.025717	25	0.321029			
Lack of Fit	6.121695	13	0.4709	2.96782	0.0343	not significant
Pure Error	1.904022	12	0.158669			
Cor Total	77.61341	31				

Appendix 3b: ANOVA for Response Surface 2FI Model for AO proteins using NaOH.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob >F	
Block	0.07574	1	0.07574			
Model	10.68107	6	1.780179	48.80653	< 0.0001	significant
A-[NaOH concentration]	1.101928	1	1.101928	30.21118	< 0.0001	
B-Time	0.014732	1	0.014732	0.403897	0.5311	
C-NaOH	7.965945	1	7.965945	218.3995	< 0.0001	
AB	0.122175	1	0.122175	3.349635	0.0797	
AC	1.379175	1	1.379175	37.81236	< 0.0001	
BC	0.097118	1	0.097118	2.662655	0.1158	
Residual	0.875381	24	0.036474			
Lack of Fit	0.401175	12	0.033431	0.845993	0.6116	not significant
Pure Error	0.474206	12	0.039517			
Cor Total	11.6322	31				

Appendix 3c: ANOVA for Response Surface Quadratic Model for NB using ethanol

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Block	1.513879	1	1.513879			
Model	14.5242	5	2.90484	6.97058	0.0063	significant
A-[Etol]	10.18141	1	10.18141	24.43174	0.0008	
B-Time	0.88865	1	0.88865	2.132443	0.1782	
AB	0.779609	1	0.779609	1.870783	0.2046	
A^2	2.61168	1	2.61168	6.267101	0.0337	
B^2	0.217234	1	0.217234	0.521283	0.4886	
Residual	3.750558	9	0.416729			
Lack of Fit	2.591366	3	0.863789	4.47099	0.0566	not significant
Pure Error	1.159191	6	0.193199			
Cor Total	19.78864	15				

Appendix 3d: ANOVA for Response Surface Cubic Model (Aliased) for AO using ethanol.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Block	0.187204	1	0.187204			
Model	14.4777	7	2.068243	8.688748	0.0053	significant
A-[Etol]	4.658184	1	4.658184	19.56916	0.0031	
B-Time	0.188275	1	0.188275	0.790948	0.4033	
AB	0.037539	1	0.037539	0.157703	0.7031	
A^2	0.598018	1	0.598018	2.512289	0.1570	
B^2	0.004624	1	0.004624	0.019424	0.8931	
A^2B	0.047017	1	0.047017	0.19752	0.6701	
AB^2	0.001961	1	0.001961	0.008239	0.9302	
A^3	0	0				
B^3	0	0				
Residual	1.666259	7	0.238037			
Lack of Fit	0.054474	1	0.054474	0.202786	0.6683	not significant
Pure Error	1.611785	6	0.268631			
Cor Total	16.33117	15				

Appendix 3e: ANOVA for Response Surface Quadratic Model for NB using NaCl

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.982355	5	0.196471	11.96568	0.0009	significant
A-NaCl	0.176515	1	0.176515	10.75029	0.0095	
B-Time	0.029527	1	0.029527	1.79829	0.2128	
AB	0.06122	1	0.06122	3.728479	0.0855	
A^2	0.486734	1	0.486734	29.64357	0.0004	
B^2	0.001171	1	0.001171	0.071308	0.7955	
Residual	0.147776	9	0.01642			
Lack of Fit	0.043308	3	0.014436	0.829114	0.5244	not significant
Pure Error	0.104468	6	0.017411			
Cor Total	1.130131	14				

Appendix 3f: ANOVA for Response Surface Reduced Quadratic Model for AO using NaCl

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob>F	
Model	0.117188	2	0.058594	5.977334	0.0108	significant
A-NaCl	0.008071	1	0.008071	0.823324	0.3769	
A^2	0.109117	1	0.109117	11.13134	0.0039	
Residual	0.166645	17	0.009803			
Lack of Fit	0.103629	6	0.017271	3.014857	0.0539	not significant
Pure Error	0.063017	11	0.005729			
Cor Total	0.283833	19				

Appendix 4: Calculation of Functional Properties

Appedix 4a: % solubility

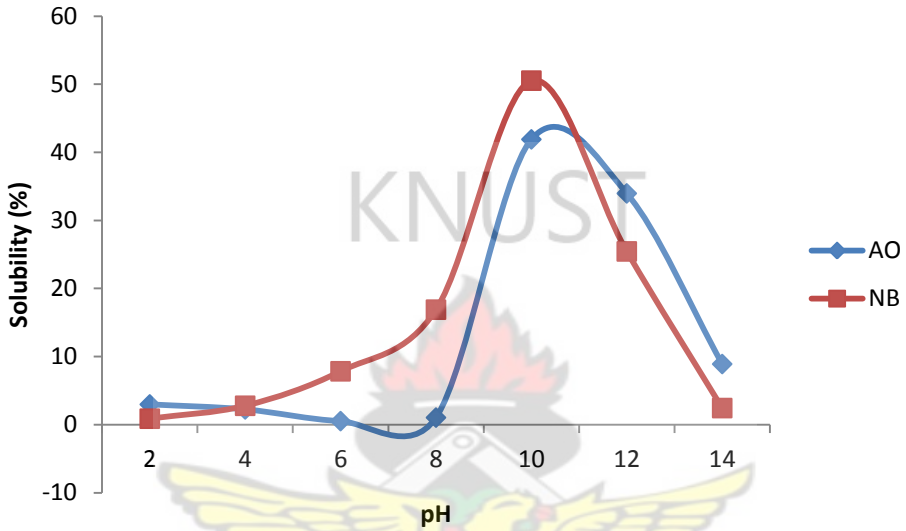
Solubility (%) = (protein content of supernatant /protein content of samples) x 100

Appendix 4b: % foam capacity

$$FC = \frac{Vt - V_1}{100} \times 100$$

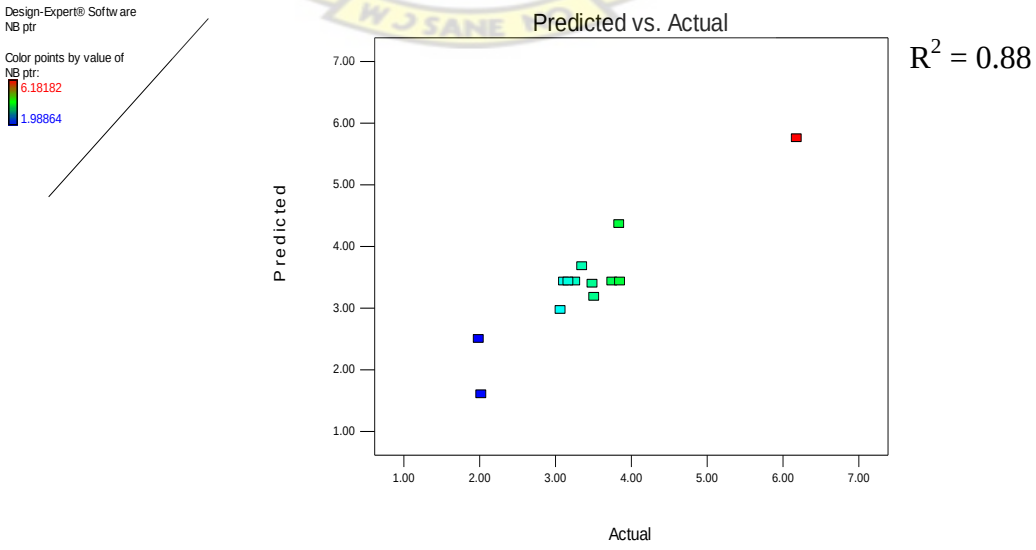
Where Vt = volume of foam, V_1 = volume of deionised water

Appendix 4c: % solubility precipitated at 0.5 M HCl

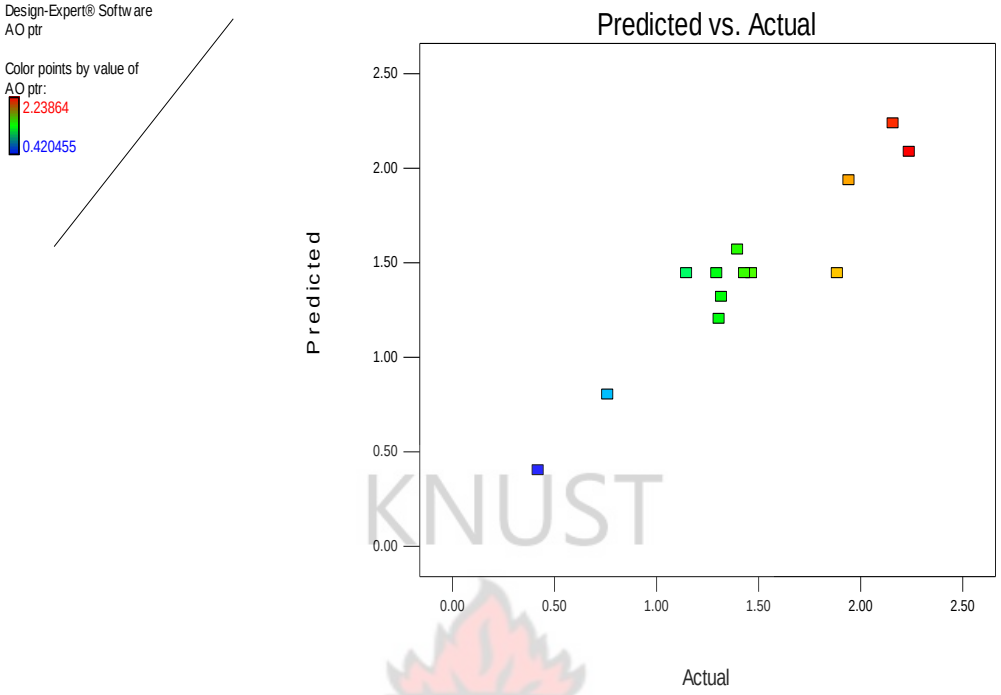


Appendix 5: Correlation between Predicted and Actual Response Value

Appendix 5a: Correlation between predicted and actual extracted protein (mg mL^{-1}) from NB



Appendix 5b: Correlation between calculated and experimentally extracted protein from AO.



$R^2 = 0.90$

Appendix 6: Percentage Band Area of Two Fern Types

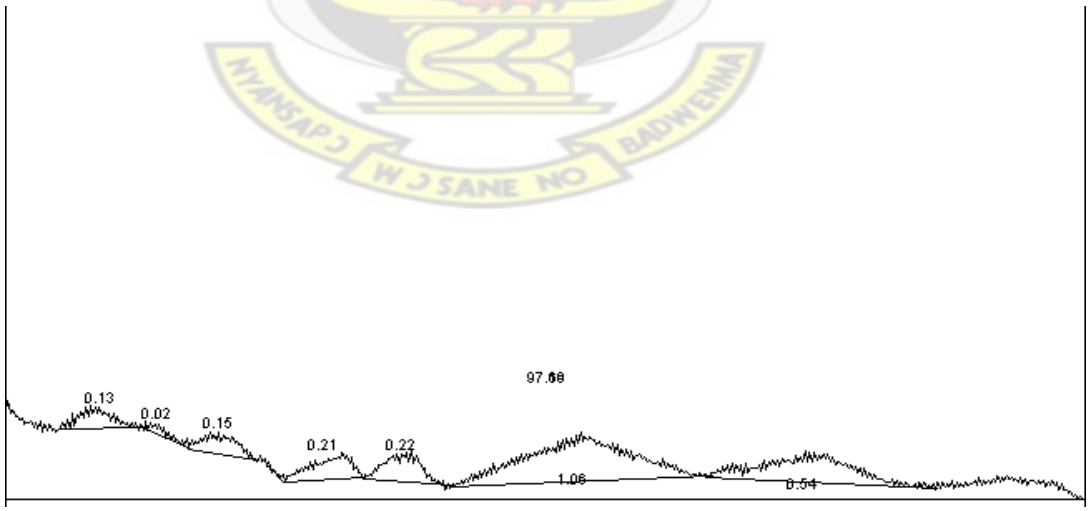
Appendix 6a : showing peak area of *Nephrolepis biserrata* and its percentage

Peak №	Area	Percent
1	194330.871	97.660
2	263.749	0.133
3	44.314	0.022
4	300.991	0.151
5	421.406	0.212
6	442.698	0.222
7	2111.116	1.061
8	1072.246	0.539

Appendix 6b: showing peak area of *Arthropteris orientalis* and its percentage

Peak №	Area	Percent
1	194323.063	97.190
2	1850.610	0.926
3	186.627	0.093
4	63.435	0.032
5	416.648	0.208
6	936.912	0.469
7	89.971	0.045
8	111.607	0.056
9	336.335	0.168
10	1503.711	0.752
11	123.263	0.062

Appendix 6c: Peaks showing percentage band area of the molecular weights of storage proteins as extracted from *Nephrolepis biserrata.*



Appendix 6d: Peaks showing percentage band area of the molecular weights of storage proteins as extracted from *Arthropteris orientalis.*