KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

COLLEGE OF SCIENCE

MODELING MICROWAVE PROTEIN-GLYCOCONJUGATES FORMULATIONS FROM PROTEIN-STARCH COMPOSITES

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

BY

MARTHA WUNNAM ALHASSAN (BSC. COMMUNITY NUTRITION)

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DECLARATION

I hereby declare that this submission is my own work toward the MPhil. Food Science and Technology 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.

MARTHA WUNNAM ALHASSAN …….…… ……..

(PG5016310) Signature Date

Certified by:

MR. ISAAC W. OFOSU …….…… ……..

(Supervisor) Signature Date

Certified by:

PROF. (MRS.) I. ODURO …….…… ……..

(Head of Department) Signature Date

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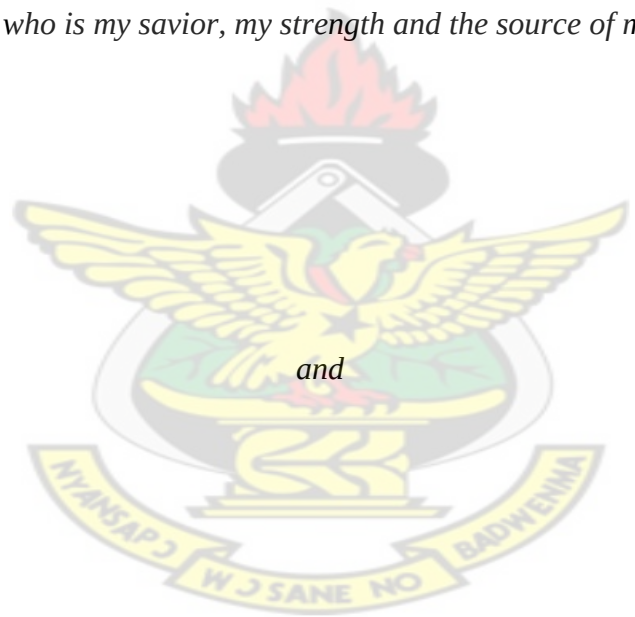
DEDICATION

I dedicate this thesis to

God, who is my savior, my strength and the source of my life



and

My parents: Mr. and Mrs. Nabia Alhassan, for their encouragement, and unflinching support.

ABSTRACT

Glycation of food proteins has the great potential of improving the functionality of food systems which can lead to increased utilization of food and ultimately contribute to the reduction of food insecurity. The aim of this project was to produce a glycated protein as food ingredient to effect food functional properties of food model systems. The glycation of two types of composites; Bambara protein-rice starch and Bambara protein-cassava starch was achieved by microwave heat treatment at varying composite ratios and microwave times. Conditions for glycation were optimized using the mixture composite design of response surface methodology. Composites of Bambara protein-cassava starch gave higher glycation values as compared to composites of Bambara protein-rice starch. The maximum glycation for Bambara protein-cassava starch composite was 51.9 (µg/10mg) whiles that for Bambara protein-rice starch composite was 10 (µg/10mg). The optimum conditions were found to be 0.7 g protein, 0.3 g cassava starch and 2.0 min microwave time for Bambara protein-cassava starch composites and 0.6 g protein, 0.4 g rice starch and 6.0 min microwave time for Bambara protein-rice starch composites. It was generally observed that for both types of composites, glycation generally increased with increasing protein content whiles a decrease in both protein content and microwave time led to a reduction in glycated protein content. Bambara proteins can be used in food industries especially in the formation of glycated food proteins to improve the functionalities of locally manufactured food systems with unique food functionalities.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

The glycation of food through Maillard reaction is an ubiquitous reaction which involves the condensation of a reducing sugar with amino groups of protein. Maillard reaction was first discovered by Louis-Camille Maillard (Maillard, 1912) and the first reaction scheme was discovered by Hodge (1953). Maillard reaction can be divided into three stages: early, advanced, and final stages. The early stage involves the condensation of a reducing sugar and a free amino group to form a Schiff base which undergoes an Amadori rearrangement to form the Amadori rearrangement product. The advanced Maillard reaction consists of dehydration and fission of the Amadori product into colourless reductones as well as furfurals (Hodge, 1953), which condense with themselves or with aldehydes formed by Strecker degradation of amino acids and form brown pigments (Morales and Van Boekel, 1997). The final stage is characterized by the formation of unsaturated, brown nitrogenous polymers and copolymers and nitrogen-free polymers (Ames, 1992).

The extent of glycation is affected by factors such as protein content, carbohydrate content, reaction temperature, time, and pH. It was observed by Olivier *et al.* (2006) that the sugar reactivity towards caseinate was faster with glucose, followed by fructose and lactose. This is because at an early stage of the Maillard reaction, terminal Ɛ-amino groups of peptides and Ɛ - NH_2 groups of lysine react with the carbonyl group of reducing sugars present in the reaction medium.

Maillard reaction is influenced by several factors whose regulation can control the glycation of food proteins. The control of Maillard reaction is very crucial because it is responsible for undesirable attributes that must be avoided and is also responsible for the desirable attributes that must be promoted. Silvan *et al*. (2011) were able to control the formation of early Maillard reaction products by the use of ferulic acid leading to the preparation of glycoproteins containing low amounts of advanced glycation end products with the potential to be used as functional food ingredients. Early Maillard reaction products and melanoidins are currently gaining a lot of attention due to their reported health-promoting properties and their potential use as functional food ingredients (Martin *et al*., 2009). On the other hand, studies have shown that a diet rich in advanced glycation end products induces inflammatory mediators and decrease insulin sensitivity, in both animals and humans (Lin *et al*., 2002).

The functional properties of food proteins can be greatly improved by glycation through Maillard reaction. Liu *et al*., (2012)a revealed that the emulsifying properties of the grafts of ovalbumin–dextran were much better than those of commercial emulsifiers. Recent studies have shown that functional properties of β-lactoglobulin, such as thermal stability and foaming capacity, are improved after modification by the Maillard reaction, depending on the sugar used during modification (Chevalier *et al* ., 2001).

The Maillard reaction, produced from an amino acid–sugar model system, has been known to be associated with the formation of compounds with pronounced antioxidant activity (Yoshimura *et al*., 1997). A study was carried out by Sun *et al*. (2006) revealed that α-lactalbumin glycated with a rare sugar (D- allose) possessed stronger antioxidant properties.

The physicochemical properties and structure of glycoconjugates are also greatly affected by glycation. Corzo *et al*. (2010) discovered that glycated sodium caseinate (with galactose/lactose/dextran) exhibited the increased viscosity with incubation time and the largest increase in apparent viscosity was observed. Glycation also results in modification of secondary structure of food protein and ultimately brings about the improved functional properties of food protein. The tertiary and quaternary structure of food protein also changes after glycation, resulting in the alteration of functional properties. This has received a lot of attention, but to what extent the tertiary/quaternary structure changes is unclear (Liu *et al*., 2012)b.

1.2 Problem Statement and Justification

Even though there has been an increase in production of agricultural foods, Africa's food insecurity and nutrition situation is growing worse (Frimpong, 2013). One of the reasons is the underutilization of most staples with Bambara groundnut as a typical example. Poor storage facilities coupled with insufficient processing of raw materials leads to most food stuffs running out during the dry season. This situation if not addressed appropriately could lead to an increase in nutritional deficiencies in the country in addition to a huge loss of income to farmers as well as the nation.

There is also a growing demand by consumers for foods with improved functionality such improved foaming in ice cream and improved emulsification in sausage, among others. Food scientists have therefore been searching for new and innovative methods of improving the functional properties of food systems. Furthermore, consumers are now becoming more

conscious of their health and are therefore demanding for processed foods with little or no chemicals or artificial additives.

Glycation endows food proteins with improved functional properties, such as solubility, water retention capacity, gelling capacity, and emulsifying properties. It occurs under mild and safe conditions and requires no extraneous chemicals (Liu *et al*., 2012). This makes glycation a good candidate to improve the functionality of food systems thereby increasing the utilization of food and reducing food insecurity and its associated effects. Since it does not involve the use of chemicals, glycation would also be readily acceptable to health conscious consumers.

1.3 Goal and objective

The goal was to contribute to finding a safer and simple technology that could convert most vegetable proteins into glycated proteins for commercial or industrial purposes. The objective for this project was to evaluate the glycation potential of sample starches using a microwave oven.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Protein Glycation

Various researchers have worked with major modifications of food such as chemical, physical or enzymatic treatments that can be used to improve the functional properties of food proteins. One of such modification is glycation of proteins through Maillard reaction. Maillard reaction, carried out under dry state and well controlled conditions (temperature, relative humidity and time), is known to be an adequate method for improving functionality of proteins without introducing important structural changes (Morgan *et al*., 1997).

Many studies have been carried out to detect and monitor protein glycation. The most successful method for the detection of non-enzymic protein glycation was developed by Resmini *et al.* (1990), who used ion-pair chromatography to detect furosine, resulting from the acid hydrolysis of Maillard reaction products. Pallini *et al*. (2001) also detected protein glycation using enzyme-linked immune sorbent assay methods. This was supported by Matsuda *et al*. (1992) who proposed the identification of protein glycation using immunological method. Lactose and poly-L-lysine were used for the reaction. The extent of the Maillard reaction was directly assessed as well as the ability of the Maillard reaction to raise the antibody capable of recognizing the lactulosyl-lysine. Lactulosyl-lysine is a stable intermediate produced at the early stage of the Maillard reaction between lactose and the Ɛ-NH_2 group of lysine. It was hypothesized that rabbit antibodies raised against lactosylated polymer would recognize the lactulosyl-lysine epitopes of the heated food model systems. Guan *et al*. (2006) also explored the use of microwave heating in protein graft reactions. The study was carried out to assess the reaction rate of graft reaction

between soy protein isolate and several sugars using microwave heating and was aimed at improving the reaction rate of the graft by the use of microwave heating.

The control of the glycation reaction is very important if the desired changes in functional properties are to be achieved. Silvan *et al*. (2011) assessed the feasibility of using controlled glycation conditions to obtain proteins containing early Maillard reaction products and minimal levels of advanced glycation end products. The possibility of using these products as ingredients in certain food systems was also explored. The effect of ferulic acid on the formation of early Maillard reaction products, fluorescent and non-fluorescent advanced glycation end products and melanoidins, were assessed using model glycation systems comprising of bovine serum albumin or soy glycinin and fructose. The extent of the Maillard reaction was estimated by the analysis of free amino groups, the incorporation of sugar into the protein backbone as well as the formation of $N^{\varepsilon-}$ (carboxymethyl) lysine, fluorescent advanced glycation end products and melanoidins.

Many studies have been documented on factors that affect the extent of glycation.The extent of the reaction is commonly affected by factors, such as reactants, temperature, time, and pH. Bourais *et al*., (2006) studied the effect of storage and intensity of heat treatment on protein glycation by use of an enzymatic method based on spectrophotometric measurement. A commercial kit (Gly-Pro), usually used for clinical diagnosis relative to diabetes, was used to estimate the effect of heat treatment applied to food and also protein–sugar model systems. The storage effects on product quality were evaluated by studying the evaluation of glycated protein in food samples and protein–sugar models. The influence of sugar type on the extent of glycation in model Maillard reaction systems was also investigated by Laroque *et al*. (2008). In their study,

they compared five reducing sugars (ribose, xylose, arabinose, glucose and fructose) with respect to their relative reactivity in the Maillard reaction with a shrimp hydrolysate. For each system, the extent of the Maillard reaction was assessed for a 24 hour period by monitoring browning intensity, free amino group disappearance and sugar consumption. The sugar reactivity was assessed on the basis of parameters such as browning development, decrease in available amino groups and sugar consumption.

The effect of parameters such as storage and heat treatment also influences the extent of glycation as observed by Birlouez-Aragon *et al*. (2004) in a study on the influence of typical composition of infant formula on protein modification and protein damage. Nutritional damage in 41 samples of commercially available infant formulas from Germany, France and Spain was evaluated by the analyses of lysine blockage and of tryptophan degradation after enzymatic hydrolysis of the milk proteins. For the protein modification, lactulosyllysine which is a product of early glycation was also measured in order to detect protein modification.

Many studies have suggested that glycation has a positive influence on the functional properties of food proteins. Glycation endows food proteins with improved functional properties, such as solubility, water retention capacity, gelling capacity, and emulsifying properties, and it occurs under mild and safe conditions and requires no extraneous chemicals (Liu *et al*., 2012). The effects of glycosylation reaction on the functional properties of peanut protein isolate-dextran conjugates were investigated in a research by Liu *et al*. (2012). A dry-heated Maillard reaction between peanut protein isolate and dextran under controlled temperature and relative humidity conditions was carried out. The effects of glycosylation reaction on functional properties of

conjugates were determined after a dry Maillard reaction between the peanut protein isolate and dextran. Some characteristics of the dextran-modified peanut protein isolate were also identified. Medrano *et al*. (2009) also found that glycation influenced the foaming properties of β-lactoglobulin. β-lactoglobulin was glycated with glucose and lactose and the influence of different conditions of reaction on the structural changes of the molecules and its foaming properties were investigated. It was revealed that the modification of β–lactoglobulin with glucose or lactose in the model systems employed resulted in products with improved foaming properties as compared to systems prepared without carbohydrates. Lactose led to the lowest degree of modification without significant differences by reaction time and by protein:sugar molar ratio. However, in the case of glucose, the degree of glycation increases with reaction time and molar ratio. The addition of lactose residues to β–lactoglobulin led to improved formation of foams as compared the addition of glucose residues to β–lactoglobulin. However, the modification of β–lactoglobulin with glucose resulted in foams that were more resistant to drainage than the non-glycated protein but less stable than lactose-modified proteins. This is supported by works of Medrano *et al*. (2012) in which a positive influence on the behaviour of β-lactoglobulin glycated with glucose and lactose at the oil water interface was found. B-lactoglobulin glycated with lactose emulsions showed greater stability to creaming than those prepared with β-lactoglobulin glycated with glucose. Flocculation and coalescence were however not influenced by the glycation.

The Maillard reaction, produced from an amino acid–sugar model system, has been known to be associated with the formation of compounds with pronounced antioxidant activity (Yoshimura *et al*., 1997) and affected by pH and temperature used (Mastrocola and Munari, 2000). This was

also observed by Lertittikul *et al*. (2007) in a study on the effect of pH on the characteristics and oxidative activities of Maillard reaction products from porcine plasma protein-glucose model system. Sun *et al*. (2006) also studied the extent of the formation of antioxidant activity in α-lactalbumin glycated with a rare aldohexose compared to two reference sugars (Fructose and Glucose). They went further to identify properties of the products that may possess some health benefits to humans. The results showed that the conjugation rate of α-lactalbumin modified with the rare aldohexose were faster than that of those modified with flucose and gructose, respectively. The glycated α-lactalbumin with the rare aldohexose also possessed stronger antioxidant properties than those modified with glucose and fructose. Hence, rare aldohexose was the best antioxidant of the three Maillard reaction products.

2.2 Protein–Carbohydrate Cross-Links in Food

The glycation reaction through Maillard reaction as reported by many scientific studies is influenced by many factors which explain the complexity of the reaction. Parameters of the Maillard reaction include: concentration and proportion of the reactants (amino and carbonyl compounds), water activity, heating time and temperature, pH, buffer type and concentration, presence of oxygen, or metal ions (Ames, 1992). In order to be able to control the reaction process, there is a need to regulate all of these factors. The type of heat used has also been found to have an influence on the reaction. In most cases, the conjugation of protein to the polysaccharide has been carried out by dry-heating techniques (Mu *et al*., 2011). However, it generally takes a considerably long time such as 10 h or even several weeks for the reaction to complete. It is for this reason that Guan *et al*. (2006) carried out protein graft reaction using microwave heating to find out if it could be an alternative to the dry heating. This was done by

performing graft reaction using both dry heating and microwave heating after which the two methods were compared. It was revealed from the study that compared to the classical heating, the microwave heating speeded up the graft reactions of soy protein isolate with sugars. However, microwave heating resulted in a reduction in lysine and arginine contents.

A study on the effect of the type of sugar involved in glycation on the extent of reaction was also conducted by Laroque *et al*. (2008) to determine whether or not the type of sugar was an important parameter in the glycation reaction and to determine the types of sugar which would be most suitable to for use in industries for the reaction. Five reducing sugars (ribose, xylose, arabinose, glucose and fructose) were compared with respect to their relative reactivity in the Maillard reaction with a shrimp hydrolysate. Results from the study indicated the prevailing propensity of pentoses over hexoses to react in the Maillard reaction. Results from both traditional kinetic parameters such as browning intensity and the disappearance of both reactants as well as chromatographic data highlighted the following order of reactivity: fructose <glucose < arabinose < xylose < ribose.

Since structural changes, such as those involved in denaturation processes, may influence the reactivity of proteins, it is important to know how different conditions may affect the susceptibility of different proteins to the Maillard reaction. Recent studies have shown that functional properties of β-lactoglobulin, such as thermal stability, emulsifying and foaming capacity, are improved after modification by the Maillard reaction, depending on the sugar used during the modification (Moreno *et al*., 2002). They have been conjugated with mono- and

disaccharides to improve its functional properties, such as solubility and emulsifying capacity (Chevalier *et al*., 2002).

An important area of concern to food scientist in the glycation reaction is the production of products of Maillard reaction. Early Maillard reaction products such as melanoidins are currently gaining a lot of attention due to their reported health-promoting properties and their potential use as functional food ingredients (Martin *et al*., 2009).

The formation of lactulosyl lysine as a result of glycation between lactose and amino groups of proteins has been found to cause loss of nutritional value due to blockage of the essential amino acid lysine. Further degradation can lead to the production of advanced glycation end products which has been implicated in the induction of inflammatory mediators (Vlassara *et al*., 2002). It was for these reasons that Birlouez-Aragon *et al*. (2004) investigated the role of composition and processing of infant formula in influencing protein modifications and protein damage.

Compounds that inhibit the formation of advanced glycation end products are therefore sought after in the quest to mitigate the deleterious effect of some of these Maillard reaction products. Very important in this quest will be components with no harmful properties that also have the ability to selectively act on certain reaction pathways leading to inhibition of undesirable substances while maintaining the formation of desirable attributes, including colour and antioxidant capacity. Investigations suggest that ferulic acid may be a good candidate to achieve this goal as it selectively inhibits the formation of advanced glycation end products and thus controls the formation of Maillard reaction products. Results derived from research conducted by

Silvan *et al.* (2011) confirmed the fact that ferulic acid can inhibit the formation of advanced glycation end- products which are harmful to humans by effectively reducing N^{ε}-(carboxymethyl) lysine and N^{ε}-(carboxyelthyl) lysine in model food systems (Srey *et al.*, 2010).

Oxidation reactions directly affect the food quality especially during storage. The prevention of lipid oxidation is therefore of great concern to the food industry. As an alternative to synthetic antioxidants, natural compounds with efficient antioxidative activity have been paid increasing attention (Lertittikul *et al*., 2007). Maillard reaction, produced from an amino acid–sugar model system, has been known to be associated with the formation of compounds with pronounced antioxidant activity (Yoshimura *et al*., 1997). Studies by Sun *et al*. (2006) have also revealed that Maillard reaction between α- lactalbumin and some rare and alimentary sugars can convey some antioxidative capacity on α-lactalbumin.

2.3 Analysis of Glycoproteins/Glycated Proteins

A commercial kit (Gly-Pro), usually used for clinical diagnosis of diabetes, was employed by Bourais *et al.* (2006) to estimate the effect of heat treatment applied to food and also protein–sugar model systems. The Gly-Pro kit employs two enzymatic reagents that react with glycated proteins resulting from the Maillard reaction; the hydrogen peroxide produced is then consumed in a colorimetric reaction. Food samples and model systems were heated and incubated at different temperatures and holding times. The enzymatic method was demonstrated to be useful for detecting early products of the Maillard reaction. However, the Gly-Pro kit seems to be specific for glucose binding proteins.

The effect of storage and intensity of heat treatment on protein glycation and on the Maillard reaction by use of an enzymatic method was monitored by Bourais *et al. (*2006), using spectro photometric measurement. In this study, a casein–glucose model system was selected to monitor the extent of casein glycation. The results obtained with the Gly-Pro assay, showed an increase in absorbance related to the color which was proportional to the amount of glycated proteins. The effect of the type of heating medium on the Maillard was also investigated by Guan *et al.* (2006). Microwave heating was compared with the classical heating in terms of the speed of protein-sugar graft reaction otherwise known as Maillard reaction in lactose, maltose dextran and soluble starch.

Another factor that plays an important role in food proteins glycation is water activity. Glycation is known to take place in both wet and dry conditions. However, studies have shown that the reaction proceeds at a faster rate under lower water activity conditions at optimum conditions ranging from 0.5 to 0.8 (Liu *et al*., 2012). The reaction is slower in aqueous solutions because of the lower concentration of the proteins and reducing carbohydrate. Reaction pH, amino: carbonyl ratio, origins of food proteins and properties of reducing carbohydrate also affect glycation, in terms of changes of functional properties, physicochemical properties and structure of glycol conjugates (Sanmartín *et al*., 2009).

Numerous studies have been conducted to confirm the glycation between food proteins and reducing carbohydrate using different methods. Glycation is also found to lead to an increase in molecular weight as compared to unglycated food protein Yajima *et al.* (2007). The weight of β-Lactoglobulin-xylobiose conjugate was increased by the Maillard reaction from 1 to 7 days.

Lactosylation of heated food model systems has also been monitored by Fogliano *et al*., (1997) using immunological approach. This was tested on bovine serum albumin-lactose and casein-lactose model systems.

2.4 Applications of Glycation of food Proteins

Food protein is an essential component of the diet of humans and animals as they are needed for survival. Their basic value in nutrition is to supply adequate amounts of needed amino acids (Friedman, 1996). Apart from their nutritional value, food proteins also provide unique functional properties, which affect their behavior in food systems during preparation, processing, storage, and consumption, and contribute to the quality and sensory attributes of food system. These functional properties of vital importance for proteins in food applications are solubility, swelling, water retention capacity, foaming properties, gelling capacity, emulsifying and fat binding properties (Zayas, 1997).

Both intrinsic factors such as molecular structure and composition and extrinsic factors such as temperature and environmental factors affect the functional properties of food proteins. When these factors are changed, food proteins functional properties are also altered especially during food processing. The functional properties of proteins are destroyed upon exposure to extrinsic factors. Different proteins have different conformational properties which are important for modification of functional properties of proteins. For example, the rigid structure of the proteins may suppress the formation of conjugates while the unfolding structure can accelerate their formation, because of the difference in the reactivity of the lysyl residues exposed outside between folded and unfolded proteins (Kato, 1996).

The functional properties of food proteins can be greatly improved by Maillard reaction through glycation. According to Chevalier *et al*. (2001), the Maillard reaction has been employed for improving food protein functional properties, such as solubility, heat stability and emulsifying properties. It is known that the Maillard reaction, carried out under dry state and well controlled conditions (temperature, relative humidity and time), is an adequate method for improving functionality of proteins without important structural changes (Morgan *et al*., 1997). This is supported by Junfeng *et al*. (2006) as it was suggested in their research that the Maillard reaction has also proved to be a reliable means for obtaining novel soy glycopeptides exhibiting enhanced gel forming, emulsifying and antioxidant properties. Recent studies have also shown that functional properties of β-lactoglobulin, such as thermal stability and emulsifying and foaming capacity, are improved after modification by the Maillard reaction, depending on the sugar used during modification (Moreno *et al*., 2002).

Many other studies have been carried out by various researchers on the effects of glycation reaction on functional properties of conjugates. Notably among them is that of Liu *et al*. (2012) on the Maillard reaction between peanut protein isolate and dextran and the effects of glycosylation reaction on functional properties of the conjugates. Recent studies have also shown that functional properties of β-lactoglobulin, such as thermal stability and emulsifying and foaming capacity, are improved after modification by the Maillard reaction, depending on the sugar used during modification (Chevalier *et al* ., 2001). Medrano *et al*. (2009) went further to evaluate the extent to which Maillard reaction with glucose and lactose as substrates can improve the foaming properties of β-lactoglobulin. The modification of β–lactoglobulin with glucose or

lactose in the model systems employed resulted in products with improved foaming properties as compared to systems prepared without carbohydrates.

Proteins, due to their amphiphilic nature, are surface active, and are commonly used as food emulsifiers. The stability of protein-stabilized emulsions critically depends on the ability of the protein to undergo interfacial denaturation and on its ability to form a viscoelastic film at the oil-water interface (Medrano *et al*., 2012). A research by Medrano *et al*. (2012) clearly indicates that glycated β-lactoglubulin samples formed more stable creaming emulsions than those formulated with proteins without glycation. Thus the glycation of β-lactoglobulin with both glucose and lactose causes an increase in the stability of oil water emulsions.

Other advantages of the Maillard reaction such as the formation of compounds with antioxidant activity and anti-bacterial properties have recently been discovered. It is also responsible for other properties such as improved anti- mutagenic and anti-carcinogenic properties. Some studies have also suggested that Maillard reaction products contain additional benefits such as increased immunity and decreased toxicity in some nitrosamines. They are also able to increase gut microbiota as it has been shown that some anaerobic bacteria, are able to use bread melanoidins as a source of carbon (Tuohy *et al.,* 2006). This is supported by a study carried out by Summa *et al*. (2008) which revealed that the Maillard reaction products in roasted cocoa beans were able to inhibit the growth of *Escherichia coli* and *Enterobactercloaceae*.

2.5 Sources of Glycated Proteins Materials

Various materials have been employed in our quest for the synthesis of glycated proteins. Obviously, these include starch and proteins. From literature these starches and proteins are sourced from various tubers, cereals, grains, legumes, etc.

2.5.1 Bambara groundnut

Inadequate supplies and high shortage of food protein in the world, especially in developing countries has necessitated the search for new sources to supplement or substitute the existing sources of protein (Ahmed *et al.,* 2010). Legumes are a group of foods that can be used as alternative sources of protein. They provide a relative cheaper source of protein as compared to that of animal proteins yet providing a comparable protein. Interest has shifted in recent years towards the utilization of legumes in the food industry to improve the functional properties of some widely consumed delicacies. One such legume is Bambara groundnut (*Vigna subterranean).*

Bambara groundnut is a legume that is in abundance in Ghana and most parts of Africa. It is known to be relatively resistant to drought and has the ability to produce a reasonable amount when grown on poor soils and under conditions where other legumes cannot thrive. The seeds contains 11.4 % protein, 53.1 % carbohydrate, 6.1 % fat, 6.1 % fibre, 4.4 % ash, 0.097 % calcium, 0.007 % iron, 1.2 % potassium and 0.003 % sodium (Amarteifio and Maholo, 1998) and chemical analyses also shows that it contains 32.72 % of total essential amino acids and 66.10 % of total non-essential amino acids (Amarteifio *et al.,* 2006). It also contains a fair

amount of iron (7.6 mg/100 g) which could be useful in areas where iron deficiency is a problem (De Kock, 2004).

In past years, many researchers have studied the effect of heat treatment under different conditions such as temperature, time, protein concentration, pH and ionic strength on functional properties such as solubility, water absorption, gelation, emulsification and foaming (Sorgentini *et al*., 1995). However, most of these investigations were conducted on soy protein isolates and little is heard of Bambara groundnut as regards this area of research.

2.5.2 Milk Proteins

Milk proteins have also been used in the glycation of foods. Both casein and whey proteins have been applied in this area of research.

There are two main types of proteins in milk, which can be separated based on their solubility at pH 4.6 at 20 ^{o} C. Under these conditions, some of the proteins precipitate; these are called caseins (Fox *et al.*, 2004). Caseins consist of four kinds of polypeptides: αs_1, αs_2, β, and κ caseins which represent 75, 22 and 3 percent of total casein, respectively. The other milk proteins are called whey proteins. This group includes β-lactoglobulin, α-lactalbumin, blood serum albumin, and immunoglobulins.

Maillard reaction affects the bioavailability, solubility, forming property, emulsifying property, and heating stability of milk proteins (Le *et al*., 2011). Since in Maillard reaction involving milk,

it is the lysine residue that reacts with lactose, it is no wonder that lysine loss by the Maillard reaction increases with severity of heat treatment.

Several modifications have been made involving milk proteins including glycation through Maillard reaction. This could be because of the the added benefit such as improved functionality and reduced cost that it brings. In a study by Mahran *et al*. (2011), it was discovered that the functional properties of buffalo milk casein can be positively modified through glysylation of food.

2.5.3 Starch

Starches including the root and tubers and some cereals are becoming increasingly popular as additions to dairy systems because of their relatively low cost, availability and other benefits. Even though starches have been studied extensively, little is heard of the interaction of milk protein and starch. In systems in which starches co-exist with milk proteins, knowledge on the synergistic interactions of the two components in food can be of great importance as it can give insights into the benefits that can be incorporated into food products as a result of this interaction.

Starchy foods represent the major source of carbohydrate in the human diet, estimated to comprise 80 % of the global average calorie intake (FAO, 1999). Starch contributes greatly to the textural properties of various foods and thus is applied in many industries as a thickener, colloidal, stabilizer, gelling agent, bulking agent, water retention agent and adhesive (Singh *et al.*, 2003). These properties play an important role in improving the quality of food.

The functionality of starches is controlled by its physicochemical properties. Some of which are; granular size, granule size distribution, amylose/amylopectin ratio, and mineral content. Starch swelling is a property of amylopectin, whereas, amylose has been known to restrict it (Park *et al*., 2007).

Yang *et al*. (1998) in their study, conjugated native potato starch, carboxymethyl potato starch, and corn starch phosphate monoester with lysine and poly(lysine) through the Maillard reaction, discovered that the conjugates gelatinized at higher temperatures and were difficult to retrograde than the two original starches. The digestibility of the conjugates on the other hand was reduced. The study also revealed that the modification was more drastic on amylose than amylopectin.

2.6 Treatment Methods

Temperature is one factor crucial for the glycation reaction to occur as the reaction requires high temperature. Both extrusion cooking, viscoanalysis of the Brabender viscoamylograph and microwave heating are methods by which food can be treated with temperature.

2.6.1 Extrusion

Extrusion cooking is a continuous process by which food biopolymers and ingredients are mixed, plasticized, cooked and formed by combination of moisture, temperature, pressure, and mechanical shear (Hauck and Huber, 1989). Extrusion is mostly applied in the production of solid products that have unique shapes that cannot be made easily using other processing methods. The cooking extruders have found many applications (Harper, 1981). These include breakfast cereals, snack foods, confectionery, texturized vegetable proteins and other products. During extrusion cooking the material receives a short input of mechanical and thermal energy

that is sufficient to bring about changes in structure and composition similar to those caused by conventional food processing operations, such as cooking, baking, roasting, and heat sterilization.

2.6.2 Brabender Viscoamylograph

Brabenderviscoamylograph is an instrument used for characterization of starch and starch containing products. The amylograph was originally used for the evaluation of rye flour and the control of amylase activity in malt supplemented wheat flour in the 1930's but it is now also applied in the analysis of the behavior of starch and starch containing foods under temperature.

The Brabender viscoamylograhs all operate under the same basic procedure in which starch suspension is heated at a rate of 1.5 ^{o}C/ min to 95 ^{o}C, held at 95 ^{o}C for a period ranging from 10 to 60 min then cooled at a rate of -1.5 ^{o}C / min to 50 ^{o}C and optionally held at 50 ^{o}C for and additional time (Deffenbaugh and Walker, 1989). The high temperature in the starch turns it into a plasticized mass. As the starch heats and cools, a graph of temperature against viscosity is plotted and the curves can be analyzed accordingly.

2.6.3 Microwave Heating

Microwave heating takes place due to the polarization effect of electromagnetic radiation at frequencies between 300 MHz and 300 GHz (Decareau, 1985). It has found its way into many applications in the food industry such as tempering of frozen foods, precooking of food for further processing and in the final drying of pasta products. In these applications, microwave heating has been found to be more efficient as compared to conventional methods in terms of reduction in process time and maintenance of the quality of food. However, it is less popular in

the food processing industry due to high cost of equipment and electricity. Yeo *et al*., (1991) made an observation that the short cooking temperatures in microwave do not usually promote Maillard reaction. Guan *et al*. (2006) also explored the use of microwave heating in protein graft reactions in a study to assess the reaction rate of graft reaction between soy protein isolate and several sugars using microwave heating. It was observed that the graft reaction using microwave heating was comparable to that of conventional heating methods.

2.7 Glycation of Food Proteins

2.7.1 Mechanism of Glycation

The glycation of food through Maillard reaction is a cascade of chemical reactions occurring during the processing and storage of foods containing reducing sugars and proteins. Maillard reaction was first discovered by Louis-Camille Maillard (Maillard, 1912). Hodge later brought out the first reaction scheme in 1953 which can be divided into three stages: early, advanced and final stages. In an early stage, a reducing sugar such as glucose condenses with a specific compound possessing a free amino group to form a Schiff base with the release of water. In most cases, it is the ε-amino group of the lysine residues in food proteins that are the primary source of reactive amino groups. Other groups such as the imidazole group of histidine, the indole group of tryptophan and the guanidino group of arginine residues also react but to a lesser extent (Ames, 1992). The Schiff's base then cyclizes to the corresponding N-substituted glycosylamine, which then undergoes an irreversible Amadori rearrangement to form the Amadori rearrangement product, 1-amino-1-deoxy-2-ketose (Ames, 1992).

The advanced Maillard reaction consists of dehydration and fission of the Amadori product into colourless reductones and furfurals (Hodge, 1953). These products then condense with themselves or with aldehydes formed by Strecker degradation of amino acids and form brown pigments (Morales and Van Boekel, 1997).

The final stage of the Maillard reaction is the stage in which most of the color is produced. This stage is characterized by the formation of unsaturated, brown nitrogenous polymers and copolymers (Ames, 1992). Several compounds result from Maillard reaction induced changes in the products. One of such compounds is the Amadori compound (N-substituted 1-amino-1-deoxy-2-ketose).

2.7.2 Factors that Influence the Extent of Glycation

The extent of glycation varies with different reaction conditions. It is commonly affected by several factors including reaction temperature, time, and pH. The type of sugar used in the glycation has also been found to influence the extent of the reaction.

The loss of available primary amino groups is an indicator used to compare the sugar reactivity in the Maillard reaction which directly affects the extent of glycation (Sun *et al.*, 2006). This is because, at an early stage of the Maillard reaction, terminal Ɛ-amino groups of peptides and Ɛ-NH_2 groups of lysine react with the carbonyl group of reducing sugars present in the reaction medium. This is supported by Laroque *et al*. (2008) in a study on the reactivity of sugars (ribose, xylose, arabinose, glucose and fructose) in Maillard reaction. The study revealed that the reactivity of sugars in the reaction was declining in the following order: ribose > xylose >

arabinose and glucose >fructose. It was also observed by Olivier *et al.,* (2006) that sugar reactivity towards caseinate was faster with glucose, followed by fructose and lactose.

The control of Maillard reaction is very crucial because is responsible for undesirable attributes that must be avoided as well as the desirable attributes that must be promoted. Maillard reaction is influenced by several factors whose regulation can control it. Parameters of the Maillard reaction include: nature, concentration and proportion of the reactants (amino and carbonyl compounds), water activity, heating time and temperature, pH, buffer type and concentration, presence of oxygen, light or metal ions (Ames, 1992).

2.7.3 Formation of Glycation End-products

Early Maillard reaction products and melanoidins are currently gaining a lot of attention due to their reported health-promoting properties and their potential use as functional food ingredients (Martin *et al*., 2009). Studies have shown that N^{ε}- carboxymethyl lysine levels are significantly higher in vegetarians whiles in an alternative nutrition group on the other hand, the fluorescent advance glycation end-products values are also significantly higher.

Silván *et al*. (2011) investigated how ferulic acid affects the formation of certain Maillard reaction products. Examples are early Maillard reaction products, fluorescent and non-fluorescent advanced glycation end products and melanoidins in model systems. Model glycation mixtures were prepared using bovine serum albumin and fructose in 0.2 % KOH in the presence or absence of ferulic acid. The extent of the Maillard reaction was estimated by the analysis of free amino groups, the incorporation of sugar into the protein backbone and the formation of N^{ε}-

carboxymethyl lysine, fluorescent advance glycation end products and melanoidins. The formation of melanoidins was estimated by measuring the absorbance at 420 nm of the isolated fractions containing glycated protein.

The presence of advanced glycation end products as contaminants in the preparation of novel glycated protein to be used as functional food ingredients would be undesirable. Their removal however, would require the employment of tedious and time consuming separation procedures (Silván *et al*., 2011). On the other hand, the use of components with the ability to selectively inhibit the formation of advanced glycation end products can favor the controlled formation of early Maillard reaction products. Lactulosyllysine has been found to be responsible for loss of nutritional value (Birlouez-Aragon *et al*., 2004). It is mostly formed when lactose reacts with amino groups of proteins. Thermal treatment can also result in further degradation of the primary reaction products to produce advanced glycation end-products. Oxidation reactions, which are catalyzed by iron, can also promote advanced glycation end products formation. Studies have also shown that a diet rich in advanced glycation end products induces inflammatory mediators and decrease insulin sensitivity, in both animals and humans (Lin *et al*., 2002).

2.7.4 Food Functionalities of Glycated Proteins

The diverse property of proteins is as a result of the contribution of both intrinsic and extrinsic properties. The intrinsic properties include molecular structure and composition of the proteins whiles the extrinsic properties are the external contributors such as temperature, pH, environmental conditions and chemicals. The functionality of food proteins are modified when these factors are changed. Glycation is an effective method for improving the functional

properties of food proteins and even endows them with novel functionality (Oliver *et al*., 2006). It is superior to other forms of food modification in the food industries because of the minimal use of chemicals in the reaction. Several reaction factors, such as temperature, time, pH, water activity, intrinsic properties of protein and sugar, and the amino group: reducing sugar ratio influence the yields and types of Maillard reaction products.

2.7.5 Antioxidant Effect of Glycation

Since the antioxidant activity of Maillard reaction products was first reported by Franzke and Iwainsky (1954), increasing interest has been directed towards the utilization of normal food constituents with antioxidant properties.

Synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene and tocopherol have been banned in many countries because some researchers have listed them as being potentially toxic. The use of non toxic and natural antioxidants is therefore in increasing demand in the food industry. Studies have shown that some of the products of glycation possess certain of anti-oxidative activity that is as good as conventional antioxidants.

The antioxidative properties of Maillard reaction products is as a result of mechanisms such as radical chain-breaking activity, metal-chelating ability, active oxygen species scavenging and hydroperoxide-destroying ability.

Maillard reaction products could exhibit antioxidant activities in both model lipid and food, inhibit the oxidative degradation of natural organic compounds and improve the oxidative

stability of food products (Mc Gookin and Augustin, 1991). According to Mastrocola and Munari (2000), the antioxidant activity of compounds resulting from Maillard reaction is affected by pH and temperature. The pH strongly influences the proportion of the amino acid in the unprotonated form which results in an increase in the initial condensation step of the Maillard reaction.

A study was also carried out by Sun *et al*. (2006) to determine the degree of α-lactalbumin conjugation with a rare sugar (D-allose) and two alimentary sugars (D-fructose, D-glucose) through Maillard reaction and the extent to which these reactions could convey antioxidant activity to α-lactalbumin. The extent of antioxidant activity was evaluated by using tetrazolium salt reducibility and radical cation -scavenging activity. The chemical characteristics of Maillard reaction products were considered important factors for evaluating antioxidant activity. Therefore, the formation of covalent conjugates and fluorescent substance, due to non-enzymatic glycation, were also characterized. The glycation was done by dissolving α-lactalbumin in carbonate buffer with the sugars at the ratio 1:13 of protein to sugar.

2.7.6 Structure and Physicochemical Properties of Glycated Proteins

The physicochemical properties and structure of glycoconjugates are also greatly affected by glycation. Electrospray interface and matrix-assisted laser desorption ionization mass spectrometry techniques were used by Oliver (2011) to understand the physicochemical and structural properties of glycoconjugates. During glycation, the surface hydrophobicity of food proteins changes, leading to increase or decrease of its value as observed by Tang *et al*. (2011). The study revealed that glycation led to increases in hydrophobicity.

After glycation, the food protein generally exhibits an increase in viscosity or viscoelastic property, and therefore improves the gelling capacity. Paraman *et al*. (2007) observed that the viscosity of rice endosperm protein was enhanced by glycation with glucose and xanthan gum. According to Baniel *et al*. (1992), the increase of surface hydrophilicity and partial unfolding of the quaternary structure of protein are responsible for the greater hydrodynamic volume and increased hydration properties.

Glycation also results in modification of secondary structure of food protein and ultimately brings about the improved functional properties of food protein. Casein-starch interactions is reported to affect the behavior of caseinate-potato system. Starch has been extensively studied for its use in the imitation of cheese. An increase or decrease in contents of secondary structure is subject to the molecular weight of sugar, glycation extent, and reaction time. The tertiary or quaternary structure of food protein also changes after glycation, resulting in the alteration of functional properties. This has received a lot of attention but the extent to which tertiary or quaternary structure changes is unclear (Liu *et al*., 2012).

2.8 Drawbacks of the Glycation Process

Despite the numerous benefits of glycation, it has been found from literature that if not well controlled, the reaction can also be undesirable. The negative aspects of the Maillard reaction may be considered from the nutritional, toxicological and physiological points of view.

When it comes to nutrition, Maillard reaction diminishes the bioavailability or destroys all of essential amino acids such as lysine, tryptophan and cysteine thus drastically reducing the quality

of proteins. In an assessment of available infant formulas for protein modification by Birlouez-Aragon *et al*. (2004), nutritional damage was evaluated. This was done by the analyses of lysine blockage and of tryptophan degradation after enzymatic hydrolysis of the milk proteins and the results was compared to similar processed milk products. It was found from the research that infant formula showed an increase in lysine loss by 6 folds as compared to similarly processed milk products.

The bioavailability of minerals can also be affected by Maillard reaction products. It was observed by Garcia *et al*. (2009) that there was a significant decrease in the availability of iron in diets rich in Maillard reaction products. Other studies have revealed that there is also a significant decrease in absorption of phosphorous and bioavailability of magnesium and calcium.

It is proposed that the accumulation of the advanced glycation end products and the activation of the receptor for advanced glycation end products in the retina could play a significant role in the initiation and progression of age-related macular degeneration and cataracts (Pawlak *et al.*, 2008). Mostafa *et al.* (2007) suggested that the level of advanced glycation end products in plasma proteins is increased in patients with diabetes. It has also been suggested by various researchers that advanced glycation end products are involved in neurodegenerative diseases, such as Alzheimer, arthritis, loss of bone mass and DNA modification (Kasimura *et al*., 1990). Tobacco smoke is also found to be a source of toxic reactive glycation products as studies have shown that reactive glycation products are present in aqueous extracts of tobacco and in tobacco smoke in a form that can rapidly react with proteins to form advanced glycation end products.

In the process of glycation, the amino groups of food protein are destroyed and covalent bonds are formed with carbonyl groups of reducing carbohydrate. Thus the primary structure of food protein changes during glycation and glycoconjugates are generated which eventually leads to improvement of functional properties. The modification of protein amino groups with carbonyl compounds is site-directed. Such a change in the primary structure of milk proteins has been widely investigated using several analyses such as electro spray interface and matrix-assisted laser desorption ionization mass spectrometry as reviewed by Oliver (2011).

2.9 Future Applications

The glycation reaction is one that has a promising future in the food industry as it is known to be an effective method for improving and modifying the functional properties of food proteins (Oliver *et al*., 2006) with minimal use of chemicals as compared to other forms of food protein modification. With glycation, functional properties such as solubility, swelling, water retention capacity, foaming properties, gelling capacity, emulsifying and fat binding properties are being modified (Zayas, 1997). Such favorable modification improves on the sensory quality of glycated food proteins.

Another advantage of glycation is the antioxidant activity as observed by Mastrocola and Munari (2000) which can help in the storage stability of such modified food proteins and can be used to replace some of the conventional artificial antioxidants. Other benefits of this process are anti-bacterial, anti-mutagenic and anti-carcinogenic properties.

Despite all these desirable attributes of glycation, it can also be quite undesirable in a number of ways that may make food scientist a bit skeptical about its application in the food industry.

Studies have shown that the reaction can drastically reduce the nutritional quality of food proteins through the reduction or loss of some essential amino acids. This is mainly due to the blockage of the essential amino acid lysine by the formation of lactulosyl lysine during the glycation process (Birlouez-Aragon *et al*., 2004). Some products of Maillard such as the advanced Maillard reaction are also able to cause DNA modification and interfere with the biological activity of tissues and serum proteins (Kasimura *et al*., 1990). Glycation can also cause structural changes to proteins and has also been implicated in many age related health complication including heart disease, vision impairment, Alzheimer's etc., while advanced Maillard reaction end-products are being implicated in the induction of inflammatory mediators in diabetic patients (Vlassara *et al*., 2002).

Even though Maillard reaction has been very beneficial in the food industry due to its numerous applications since its discovery, the formation mechanism of the reaction is very complex and is still unclear. The extent to which the tertiary or quaternary structure changes, is also not well understood (Liu *et al*., 2012). There is also very little literature on the bioactivity of dietary advanced glycation end-products.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Sources of Materials

Bambara groundnut (navred variety), cassava (Afisiafi variety) and rice (dadaba variety) were obtained from the Ayigya market. Defatting of the Bambara groundnut flour was done using hexane purchased from Ghana Nut Limited, *Techiman* in the Brong Ahafo Region of Ghana. A solid-liquid extractor (E1VS, France) was used to defat Bambara groundnut in a large scale. A spectrophotometer (UV/VIS 1601, Japan) was used for protein determination and analysis of glycation.

3.1.2 Sample Preparation

Bambara groundnut (navred) seeds were sorted to remove unwanted materials. They were then washed and solar-dried for three days in a solar dryer. The dried Bambara groundnut seeds were coarse milled into flour with a hammer mill (F8, England) to a diameter of 60 μm. The flour milled again using a Binatone blender (BLG 401, China) after which it was sieved to further reduce the size to 25 μm. Following that, it was stored in a refrigerator in an airtight plastic bag.

The pieces of cassava (Afisiafi) were washed and cut into smaller sizes. They were then dried for three days in a solar dryer and milled using a hammer mill (F8, England) to a diameter of 60 μm. The flour was further milled using a Binatone blender (BLG 401, China) and sieved to a

diameter of 25 μm. The dry cassava flour was then stored in an airtight plastic bag placed in a refrigerator until analysis was carried out.

The rice (dadaba) was also sorted and washed to remove impurities after which it was soaked for 24 h. It was then solar dried for two days and then milled to reduce the particle size to 60 μm using a hammer mill (F8, England). The flour was milled again with a Binatone blender (BLG 401, China), sieved to a diameter of 25 μm and stored in a refrigerator in an airtight plastic bag to await analysis.

3.1.2.1 Defatting of Bambara groundnut Flour

Dry Bambara groundnut flour was defatted using the solid-liquid extractor (E1VS, France) and hexane as the solvent. This was done at a meal to solvent ratio of 2:5 w/v for 3 h. The defatted Bambara groundnut flour was then air dried for 10 min to expel residual hexane after which it was stored in a refrigerator in an airtight plastic bag.

3.1.2.2 Extraction of Protein from Bambara groundnut.

Proteins were extracted from the dried defatted Bambara groundnut flour using a protocol described by Gomez-Brenes *et al*. (1983). The extraction was done using 0.01 M NaOH at a meal to solvent ratio of 1:10 w/v. The insoluble polysaccharides and residues were removed by centrifuging at 2500 rpm for 20 min. The supernatant produced after centrifugation was then acidified using 0.5 M HCl to a pH of 4 to allow the proteins to precipitate after which it was centrifuged for 15 min at 3000 rpm. The precipitate was washed repeatedly with distilled water,

freeze-dried (Labconco freeze system 12, U.S.A.) and stored in a refrigerator in an airtight container.

3.1.2.3 Determination of Protein Content of Bambara Protein Isolate

The protein content of the extracted Bambara protein was determined in order to verify the extraction process. This was done according to protocol described by Bradford (1976). A standard curve was generated prior to protein determination using bovine serum albumin as the standard. One milligram (1 mg) of the sample was then dissolved in 1 ml of 0.01 M NaOH after which 100 µl aliquot of the sample was measured into a beaker and 2.5 ml of Bradford reagent was added. The absorbance was read at 595 nm using a spectrometer and the protein concentration was calculated from the equation generated from standard curve.

3.1.2.4 Extraction of Starches from Cassava and Rice

Starches from both cassava and rice were extracted according to a protocol described by Baah *et al.* (2005). Twenty litres of water were each added to 5 kg of rice and cassava flours to dissolve the starch in both flours. The two solutions were then filtered separately using cheese cloth. Ten litres of water was gradually added to the residue of the two solutions to extract all starch from the solutions. The extracted starches were allowed to settle after which the supernatants were decanted. Following this, the sediment of starches were both dried in a solar dryer.

3.2 METHOD

3.2.1 Treatment Method

The Design Expert (2008) was used to determine the factors and the levels at which they were varied as shown in Table 3.1.

Table 3.1: Factors and levels of variation

Factors	Level of Variation
Bambara protein	0.6– 0.8 g
Starch	
• Cassava starch	0.2 – 0.4 g
• Rice starch	0.2 – 0.4 g
Microwave time	2.0 –6.0 min

3.2.1.1 Proximate Analysis

Bambara groundnut flour was analyzed for proximate composition according to AOAC (2005).

3.2.1.1.1 Moisture Content: An empty crucible was weighed and the measurement was recorded after which 2.0 g of Bambara groundnut flour was also weighed and transferred into the crucible. The sample was then dried in an oven at a temperature of 105 °C until a constant weight was obtained and then made to cool in a desiccator. The loss in weight was calculated as percentage moisture as shown in Appendix 1a.

3.2.1.1.2 Crude Fat: The crude fat content of Bambara groundnut flour was determined by the semi-continuous Soxhlet method (Soxhlet, 1879). The sample whose moisture content was previously taken was used for the fat analysis. A 500 ml round bottom flask was weighed and the measurement recorded. The sample was then transferred into a paper thimble and labeled appropriately. The labeled thimble was then placed into the thimble holder of the apparatus (B-811, Switzerland). Two hundred milliliters (200 ml) of hexane was poured into the round bottom flask and then reassembled on the Soxhlet extractor (B-811, Switzerland) after which it was refluxed for 16 h. After 16 h, the paper thimble was removed from the thimble holder and the round bottom flask was heated in an oven at 103 ^{o}C for 30 min. This was done in order expel residual hexane from the fat extracted. The round bottom flask was reweighed and the result recorded. The crude fat was calculated as the difference between the initial weight ant final weights of the round bottom flask expressed as percentage as shown in Appendix 1b.

3.2.1.1.3 Protein Content: The protein content of defatted Bambara groundnut flour was determined by Kjeldhal method (AOAC, 2005). A blank sample was taken through the procedure alongside the sample. Dry defatted Bambara groundnut was weighed into a digestion flask after which selenium catalyst and anti-bumping agents were also added. Twenty five milliliters (25 ml) of concentrated H_2SO_4 was added to the sample and digested in a fume chamber **(**BST-FH1200, India) until the solution was clear. The digested sample was allowed to cool in a desiccator after which it was transferred into a 100 ml volumetric flask and the volume made up to the mark with distilled water. An aliquot of 10 ml was transferred into the distillation apparatus and neutralized with 18 ml NaOH. After neutralization, the sample was boiled over

distillation water for about 10 min. A conical flask was then filled with 25 ml of 2 % boric acid with 2 drops of mixed indicator.

The total nitrogen content was then determined by titrating the content of the conical flask with 0.1 M HCl solution. The titre value of the titration was recorded and used to calculate the percentage nitrogen which was subsequently converted to protein content using the appropriate conversion factor as shown in Appendix 1c.

3.2.1.1.4 Ash: The total ash content of Bambara groundnut flour was determined by combusting 2.0 g of sample in a muffle furnace (FO410CR, Japan) for 2 h at 600 °C. The sample was cooled after which they were weighed again. The weight remaining was calculated as the percentage ash content as shown in Appendix 1d.

3.2.1.1.5 Crude Fiber: The defatted Bambara groundnut flour was used for crude fiber determination. This was determined by weighing 2.0 g of the sample into a flat bottom flask after which 200 ml of 1.25 % H_2SO_4 was added. The content of the flat bottomed flask was refluxed for 30 min whiles connected to a condenser over a hot plate (Model CB300, U.K). After refluxing, the content of the flask was filtered using a clean cheese cloth and the residue was washed off with boiling water from a water bath until the filtrate was no longer acidic. The acidity was verified by dipping a blue litmus paper into the filtrate. The residue was then transferred back into the flask and refluxed again for another 30 min in the presence of 200 ml of 1.25 % NaOH. The content of flask was again filtered using a cheese cloth after which it was washed with boiling water from a water bath until the filtrate was no longer basic. This was

verified by dipping a red litmus paper into the filtrate. The residue was dried in an air oven for 4 h at a temperature of 105 °C. The porcelain crucible containing the sample was weighed after cooling off in a desiccator. The sample was then combusted for 2 h at 600 ^{o}C in a muffle furnace (FO410CR, Japan) after which it was cooled again in a desiccator and reweighed. The crude fiber content was calculated as the loss in weight of the sample expressed as a percentage as shown in Appendix 1e.

3.2.1.1.6 Total Carbohydrate Content: The carbohydrate content of Bambara groundnut flour was determined by subtracting all the other proximate determinations from 100 % as all the other proximate contents were already determined.

3.2.1.2 Glycation of Protein-Starch Composites

Protein-Starch glycation reaction was done by microwave heating according to a protocol described by Guan *et al*. (2006) which was carried out in a microwave oven (CMX20, U.K) of 800 W power level. For each run, the protein and starch were separately weighed according to the ratios generated by the Design Expert (2008) as shown in Table 3.4 after which they were mixed together in a tightly capped centrifuge tube and agitated in a vortex mixer (SA7, U.K) for 2 min. The covers of the centrifuge tubes were opened and placed in a covered plastic container for 2 h alongside an opened beaker containing water. This was done to increase the moisture content of the samples. The samples were then heated by microwave irradiation at a temperature of 90 ^{o}C according to the time generated by the Design Expert (2008) for each run. The mixture was then cooled for 3 min in an ice bath to stop the reaction. Each experimental run of reaction mixtures was heat-treated in duplicates.

3.2.1.3 Determination of Soluble Proteins in Glycated Samples

Ten milligrams (10mg) of each sample was weighed into a centrifuge tube and 1 ml of Phosphate Buffered Saline (PBS) added to the sample. The solution was mixed by shaking the content of the centrifuge tube for 2 h in an orbital shaker (98001 Cat, USA) after which it was then centrifuged for 1 h at 2500 rpm. Hundred micro litres (100 µl) of the supernatant which contained the soluble proteins was taken for protein determination. The protein content was then determined for each run of sample according to the method of Bradford (1976) using bovine serum albumin as standard and Coomassie protein assay reagent. An aliquot of 100 µl of the supernatant for each run was measured into a test tube after which 2.5 ml of Bradford reagent was added and kept for 5 min for the reaction to come to completion. The absorbance of the each sample was measured at 595 nm using a spectrophotometer (UV/VIS 1601, Japan). An aliquot of 20 µl was injected into the spectrophotometer (UV/VIS 1601, Japan) and the absorbance was read in triplicates for each sample and the average taken. The absorbance was compared to that of the standard curve that was previously obtained. The protein content representing the total soluble glycated protein was then calculated from a standard curve generated using bovine serum albumin as the standard.

3.2.2 Statistical Method

Three factors were varied at different levels and randomized with the help of the Design Expert (2008). Two separate experimental designs were generated for each of the two different types of composites which were; Bambara protein-rice starch and Bambara protein-cassava starch composites. The Design Expert (2008) was used to generate 21 runs which were performed at different combinations for each experimental design. This was done in order to examine the combined effect of the mixture components (Bambara protein and cassava/ rice starch) and the

process factor (microwave time) on the response (glycated protein). The treatment conditions were applied on each sample after which glycation was measured. The response surface methodology was used predict best the model for the experiment as well as to optimize the conditions for glycation. Table 3.2 indicates the constraints for the glycation. The actual values for the factors in each run are shown in Table 3. 3.

Table 3.2: Model constraints for glycation of Bambara protein- (rice and cassava) composites

Parameter	Lower limit	Upper limit
Bambara protein	0.6	0.8
Rice/ cassava starch	0.2	0.4
Microwave time	2.0	6.0

Table 3.3: Factors and actual values of experimental runs for the glycation of Bambara protein – (rice and cassava) composites

Runs	Factors		
	A: Bambara protein /g	B: Rice starch /g	C: Microwave time /min
1	0.70	0.30	4.0
2	0.75	0.25	5.0
3	0.70	0.30	4.0
4	0.75	0.25	3.0
5	0.60	0.40	4.0
6	0.70	0.30	4.0
7	0.65	0.35	3.0
8	0.60	0.40	6.0
9	0.80	0.20	6.0
10	0.70	0.30	6.0
11	0.60	0.40	2.0
12	0.70	0.30	2.0
13	0.70	0.30	4.0
14	0.80	0.20	4.0
15	0.80	0.20	2.0
16	0.60	0.40	2.0
17	0.80	0.20	6.0
18	0.60	0.40	6.0
19	0.70	0.30	4.0
20	0.70	0.30	4.0
21	0.70	0.30	4.0

The Design Expert (2008) was used to optimize the glycation process using the constrained factors and responses. The variations between the factors and data obtained from the response were evaluated by determining the analysis of variance. The significance of the interaction among the factors and response were determined by the p-value and F-value. The R-squared was

used to determine the amount of variation among the factors and the response. It was also used to determine the fit of the model. The R^2 value is always between 0 and 1 and the closer the R^2 is to 1.0, the stronger the model and the better it predicts the response (Haaland, 1989).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSIONS

4.1 Proximate Composition of Bambara groundnut Flour

The proximate composition of the Bambara groundnut flour that was used for analysis is shown in Table 4.1.

Table 4.1: Proximate composition of Bambara groundnut flour

Composition	Whole flour (Mean %)
Moisture	10.01 ± 0.01
Fat	5.75 ± 0.03
Protein	16.44± 0.01
Carbohydrate	62.87± 0.03
Fibre	1.45 ± 0.05
Ash	3.10 ± 0.1

* Protein isolate was 86.90%

4.2 Data Analysis

The following regression equations were generated for the models of Bambara protein-rice starch and Bambara protein-cassava starch composites respectively.

Y= 8.35A + 6.67B - 9.04AB -0.61AC + 2.32BC -10.75ABC

Y=**7.62A + 16.92B + 76.68AB + 3.206AC + 3.30BC-18.04ABC**

Where: A=Bambara protein
B= Rice/ cassava starch
C= Microwave time

The results in Table 4.2.1 shows the actual values of the factors (Bambara protein, rice /cassava starch and microwave time) and the response (glycated protein) for the first combined composite design. It is also a summary of the experimental design used to the optimize conditions for glycation. The independent variables were analyzed and used to predict the maximum glycated proteins under the given range.

Table 4.2.2 shows the actual values of the factors (Bambara protein, cassava starch and microwave time) and the response (glycated protein) for the second combined composite design. It is also a summary of the experimental design used to the optimize conditions for glycation. The combination which gave the maximum glycated protein yield was predicted by analyzing the independent variables of the experimental design.

Table 4.2.1: Experimental design (actual values) and responses for glycation of Bambara protein-rice starch composite obtained by microwave heating

Runs	Independent variable			Dependent variable
	A: Bambara protein /g	B: Rice starch /g	C: Microwave time /min	Glycated protein / µg/10mg
1	0.70	0.30	4.0	6.6
2	0.75	0.25	5.0	3.7
3	0.70	0.30	4.0	8.0
4	0.75	0.25	3.0	8.0
5	0.60	0.40	4.0	6.5
6	0.70	0.30	4.0	5.0
7	0.65	0.35	3.0	5.0
8	0.60	0.40	6.0	8.0
9	0.80	0.20	6.0	9.0
10	0.70	0.30	6.0	4.0
11	0.60	0.40	2.0	5.0
12	0.70	0.30	2.0	7.0
13	0.70	0.30	4.0	2.9
14	0.80	0.20	4.0	6.0
15	0.80	0.20	2.0	10.0
16	0.60	0.40	2.0	4.0
17	0.80	0.20	6.0	8.0
18	0.60	0.40	6.0	10.0
19	0.70	0.30	4.0	4.3
20	0.70	0.30	4.0	5.6
21	0.70	0.30	4.0	4.9

Table 4.2.2: Experimental design (actual values) and responses for glycation of Bambara protein- cassava starch composites obtained by microwave heating

Runs	Independent variables			Dependent variable
	A: Bambara protein /g	B: Cassava starch /g	C:Microwave time /min	Glycated protein /µg/10mg
1	0.70	0.30	4.0	38.0
2	0.75	0.25	5.0	32.5
3	0.70	0.30	4.0	38.0
4	0.75	0.25	3.0	28.0
5	0.60	0.40	4.0	10.0
6	0.70	0.30	4.0	3.6.0
7	0.65	0.35	3.0	35.0
8	0.60	0.40	6.0	51.9
9	0.80	0.20	6.0	0.0
10	0.70	0.30	6.0	28.0
11	0.60	0.40	2.0	9.6
12	0.70	0.30	2.0	30.0
13	0.70	0.30	4.0	40.0
14	0.80	0.20	4.0	6.0
15	0.80	0.20	2.0	4.0
16	0.60	0.40	2.0	20.0
17	0.80	0.20	6.0	20.0
18	0.60	0.40	6.0	24.0
19	0.70	0.30	4.0	37.0
20	0.70	0.30	4.0	26.0
21	0.70	0.30	4.0	29.0

4.3 Effect of Bambara Protein-Rice Starch Composite and Microwave Time on the Formation of Soluble Glycated Proteins

The analysis of variance (ANOVA) is given in Table 4.4. The Fisher's F-value of 5.49 is significant implying the model is significant. Noise factors in an experiment refer to unexplained variations in the samples or factors that are impossible or too expensive to control (Steinberg and Burnsztyn, 1993). There is only 0.45 % chance that the model F-value is due to noise.

P- values were used to evaluate the significance of the coefficients, which where needed to understand the pattern of mutual interactions among the test variables. The smaller the magnitude of the P-values, the more significant the corresponding coefficient. Model terms are significant when their prob >F values are less than 0.05. However, values greater than 0.10 indicate that the model terms are not significant. From Table 4.4, it can be observed that the combine factor effect of AB, BC, ABC are all significant model terms as their corresponding values of prob >F are each less than 0.05.

The fit of a model was also expressed by the coefficient of regression (R-Squared). The R^2 value is always between 0 and 1 and the closer the R^2 is to 1.0, the stronger the model and the better it predicts the response (Haaland, 1989). From Table 4.3 it can be observed that the R^2 obtained from the quadratic × quadratic model regression analyses was 0.65 whiles the adjusted R^2 was 0.53 indicating that the model was fairly good in predicting variation of responses obtained. The adequate precision was 7.27. According to Montgomery and Myers (2002) adequate precision measures the signal to noise ratio and a ratio greater than 4 is desirable. The ratio 7.27 therefore confirms that there was adequate model discrimination.

Table 4.6 is the ANOVA of the response surface model for the glycation of Bambara protein-cassava starch composites. The model Fisher's F-value was 3.44 indicating that the model is significant with only 3.10 % chance of the model F-value is occurring due to noise. The p-value for the model term AB was less than 0.05 which makes it a significant model. However, the prob >F for BC, ABC were more than 0.05 which indicates that these model terms are not significant.

Tab The R^2 square was 0.55 as shown in Table 4.5 confirming that the model is good at predicting variations of the responses obtained. The adequacy of prediction was 5.06 which is greater than 4 indicating that there was adequate model discrimination.

Table 4.3: Summary of the statistics of the analysis of variance for the extent of glycation of Bambara protein-rice starch composites

Summary statistics	Std. Dev.	Mean	C.V. %	PRESS	R-Squared	Adj. R-Squared	Pred. R-Squared	Adeq. Precision
	1.43	6.26	22.91	69.74	0.65	0.53	0.20	7.27

Table 4.4: ANOVA for glycation of Bambara protein-rice starch composites

Source	Sum of squares	DF	Mean Square	F-value	prob >F	Significance
Model	56.47	5	11.29	5.49	0.0045	significant
Linear mixture	3.67	1	3.67	1.79	0.2014	
AB	22.98	1	22.98	11.17	0.0045	
AC	1.07	1	1.07	0.52	0.4825	
BC	21.67	1	21.67	10.53	0.0054	
ABC	12.35	1	12.35	6.00	0.0271	
Residual	30.86	15	2.057			
Lack of fit	11.79	6	1.96	0.93	0.52	Not significant
Pure Error	19.07	9	2.12			
Cor Total	87.33	20				

Table 4.5: Summary of the statistics of the analysis of variance for the extent of glycation of Bambara protein-cassava starch composites

Summary statistics	Std. Dev.	Mean	C.V. %	PRESS	R-Squared	Adj. R-Squared	Pred. R-Squared	Adeq. Precision
	10.18	22.93	44.41	2756.63	0.55	0.39	0.14	5.06

Table 4.6: ANOVA for glycation of Bambara protein-cassava starch composites

Source	Sum of Squares	DF	Mean Square	F-value	prob>F	Significance
Model	1783.08	5	356.62	3.44	0.0310	Significant
Linear Mixture	119.67	1	119.67	1.15	0.3009	
AB	1529.55	1	1529.55	14.75	0.0018	
AC	29.19	1	29.19	0.28	0.6041	
BC	30.17	1	30.17	0.29	0.5981	
ABC	33.39	1	33.39	0.322	0.59	
Residual	1452.24	14	103.73			
Lack of fit	207.56	6	34.59	0.22	0.95	Not Significant
Pure Error	1244.67	8	155.58			
Cor Total	3235	19				

4.4 Variation of Content of Glycated Protein Formed with Bambara Protein-Rice Starch Ratio and Microwave Time

Figure 4.1 shows the interaction among Bambara protein, rice starch and microwave time. It can be observed from the response surface plot that when protein content increased from 0.6 g to 0.8 g there was increase in glycated protein content. A similar trend was observed by Zhen-Chun *et al*. (2013) in a study conducted on the effects of protein to glucose ratio, substrate concentration, pH, reaction temperature, ultrasonication time and ultrasonic power on degree of grafting and

degree of browning. It was observed from their investigation that an increase in protein concentration led to an increase in degree of grafting in Mung bean protein-glucose substrate.

It can also be seen from the Figure 4.1 that, a decrease in microwave time resulted in an increase in glycation. This is contrary to observations made by Zhen-Chun *et al*. (2013) that an increase in ultrasonic time and temperature both led to an increase in degree of grafting. Observations made by Bourais *et al.* (2006) also revealed that an increase in incubation time led to an increase in the amount of glycated proteins. Achouri *et al*. (2005) observed the same trend, when they researched into the functional properties of glycated soy 11S-rich glycinin with glucose at varying incubation periods. A gradual increase in glycation from 34.8 % to 46.5 % was observed as incubation time increased from 6 to 48 h. It was reported by Kato *et al*. (1993) that glycation proceeds faster during the early stages because more ε-amino groups of lysine are accessible for glucose interaction.

Figure 4.2 shows the interaction among Bambara protein, cassava starch and microwave time in the form of a three dimensional response plot. It can be seen from the response surface plot that when protein content increased from 0.6 g to 0.8 g there was increase in glycated protein content and which is similar to the observations made in Figure 4.1. This trend is also similar to that observed by Zhen-Chun *et al.* (2013).

The response surface plot in Figure 4.2 also shows that, a decrease in microwave time resulted in an increase in glycation which is contrary to observations made by Zhen-Chun *et al.* (2013) that an increase in ultrasonic time and temperature both lead to an increase in degree of grafting.

Similarly, the findings of Bourais *et al.* (2006) revealed that an increase in incubation time lead to an increase in absorbance that was proportional to the amount of glycated proteins.

Maximum glycation of 40 (µg/10mg) of product was seen at the conditions of 0.7 g Bambara protein, 0.3 g rice starch and microwave time of 2.0 min. The lowest glycation content was however, observed at conditions of 0.8 g Bambara protein, 0.2 g rice starch and microwave time of 6.0 min.

The trends in glycation observed in Figures 4.1 and 4.2 indicate that with both graphs, glycation increased with increasing Bambara protein content. On the other hand, glycation increased with decreasing starch content and microwave time and accordingly decreased with increasing starch content and microwave time. Maximum glycation was however different for the two graphs as that it was 9 (µg/10mg) for Figure 4.1 and 33 (µg/10mg) for Figure 4.2.

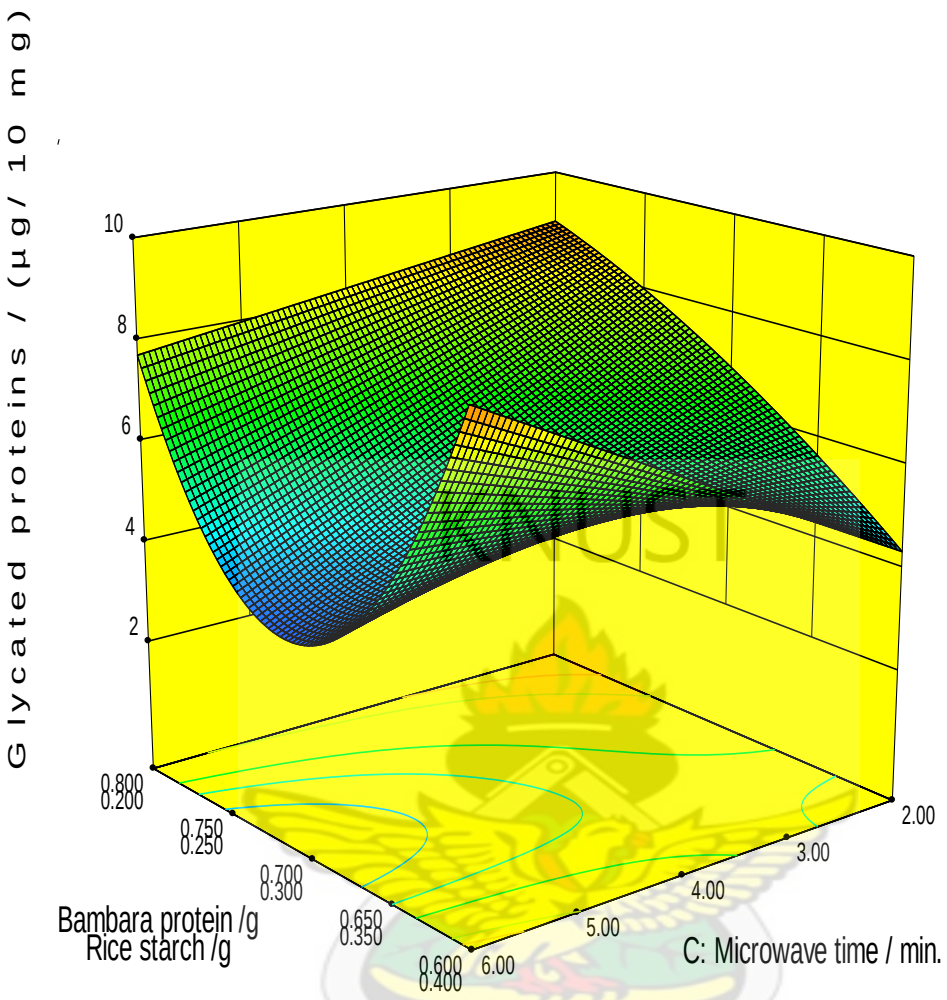

Figure 4.1: Response surface plot showing the variation of glycated protein content with Bambara protein-rice starch ratio and microwave time

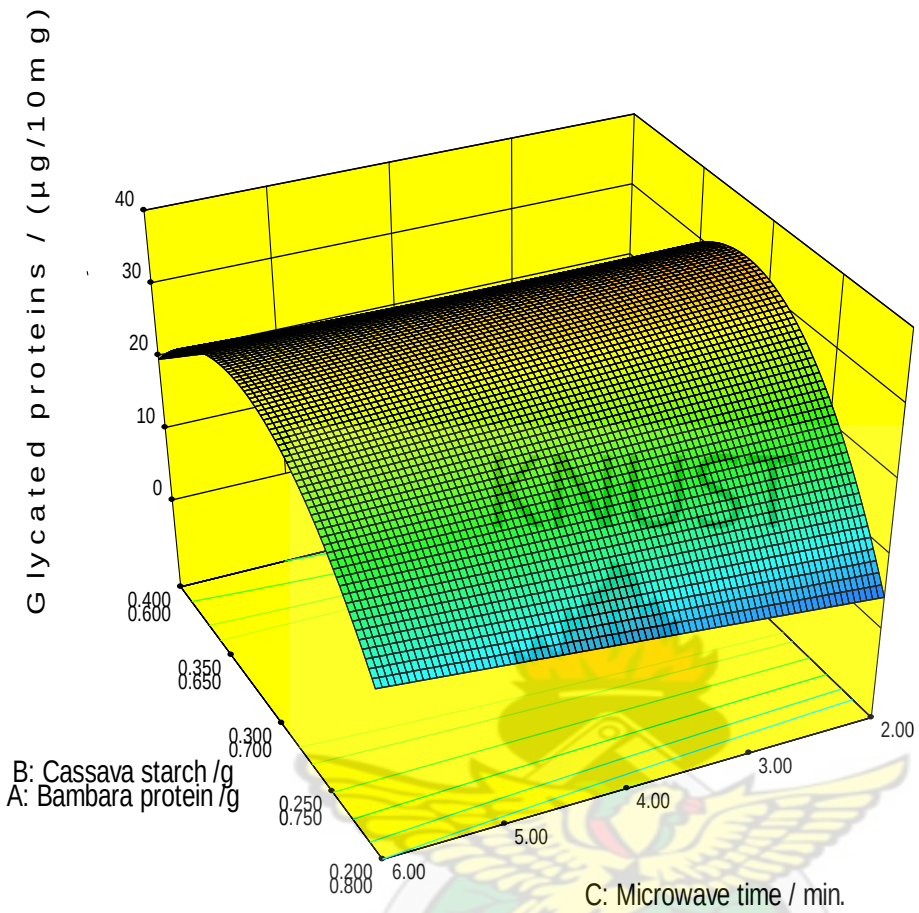

Figure 4.2: Response surface graph showing the variation of content of glycated product formed with Bambara protein-cassava starch ratio and microwave time

4.5 Effect of Different Bambara Protein–Rice Starch Combinations on the Extent of Glycation

Figure 4.3 is a contour plot showing the effect of Bambara protein–rice starch combinations on glycation. Each contour curve represents an infinite number of combinations of two least variables with the other maintained at zero level (Ghodke *et al*., 2009). It can be seen from the plot that for each contour, different microwave times and protein- starch combinations is required to achieve that particular glycated protein content.

The yellow region represents the area with maximum glycation whiles the blue represents the region with minimum glycation. It can be seen that the highest glycation value was recorded on the contour representing glycation of 8.47 (µg/10mg). Moving along that contour Bambara protein increased whiles rice starch content decreased. On the other hand microwave time required increased from 2.0 to 4.0 min.

"Dry" glycation has a lot of industrial benefits compared to "wet" glycation as according to Oliver *et al*. (2006), "dry" reactions are more desirable from an industrial perspective, based on the premise that they require less space and time to achieve the desired result than "wet" reaction conditions. Some "dry" Maillard reactions, however, particularly those involving formation of protein-polysaccharide conjugates derived from globular proteins, are performed over 2 to 3 weeks (Song *et al*., 2002). This effect can be reduced by using microwave heating as according to Guan *et al.* (2006) compared to the classical heating, the microwave heating speeded up the graft reactions of soy protein isolate with sugars.

From Figure 4.3 it can be seen that for maximum glycation (represented by the red region) of 8.47 (µg/10mg) to occur, microwave time below 4.0 min, Bambara protein content above 0.75 g and rice starch content below 0.25 g is required. It can also be observed that high glycation also occurred when Bambara protein–rice starch combination was 0.6 g protein and 0.4 g rice starch at microwave time of 4.0 min. This implies that high glycation can either occur at high Bambara protein content for shorter microwave duration or at low Bambara protein content for longer microwave duration.

The minimum glycation is represented by the blue region was found on the contour representing 4.3 (µg/10mg). On that contour the microwave time required ranged from 5.0 to 6.0 min. It can also be seen from Figure 4.3 that glycation generally increased as protein content increases from 0.6 g to 0.8 g. This trend was also observed by Zhen-Chun *et al*. (2013) where increases in protein concentration led to an increase in degree of grafting.

Figure 4.4 is a contour plot showing the effect of Bambara protein–cassava starch combinations on glycation. The yellow region represents the area with maximum glycation whiles the blue represents the region with minimum glycation as was the case of Figure 4.3. It can be seen that the highest glycation was recorded in between the 2 contours each representing glycation of 28.19 (µg/10mg).

It can be seen from Figure 4.4 that glycation increased steadily from 18.74 to 28.19 (µg/10mg) as protein content increased from 0.65 to 0.75 g after which it reached a peak of 28.19 (µg/10mg) at protein content of 0.75 g. This was immediately followed by a decline in glycation from 28.19 to

10.86 (µg/10mg) as protein content increased further from 0.75 to 0.80 (g). A similar observation was made by Zhen-Chun *et al*. (2013) when degree of grafting increased steadily with increasing protein content to a peak of 52.28 %. This was followed by a decline in glycation upon further increase in protein concentration. According to Staly (1987), theoretically, glucose concentration is the rate-limiting step in the glycation reaction. However, because the open-chain and cyclic forms of glucose exist in freely exchangeable equilibrium, it can be conjectured that, as carbonyl glucoses were removed by glycation, more glucose molecules would rapidly isomerize to the open-chain to maintain the equilibrium. If so, protein concentration would then also be an important factor in determining the glycation rate (Staly, 1987). This could account for the increase in glycation with increasing protein content.

From Figure 4.4, it can be seen that for maximum glycation of 28.19 (µg/10mg) to occur, protein content above 0.65 g but below 0.75 g is required. The minimum glycation represented by the blue region was seen at the combination 0.8 g and 0.2 g Bambara protein and cassava starch contents respectively within the microwave time range of 2.0 min to 5.0 min.

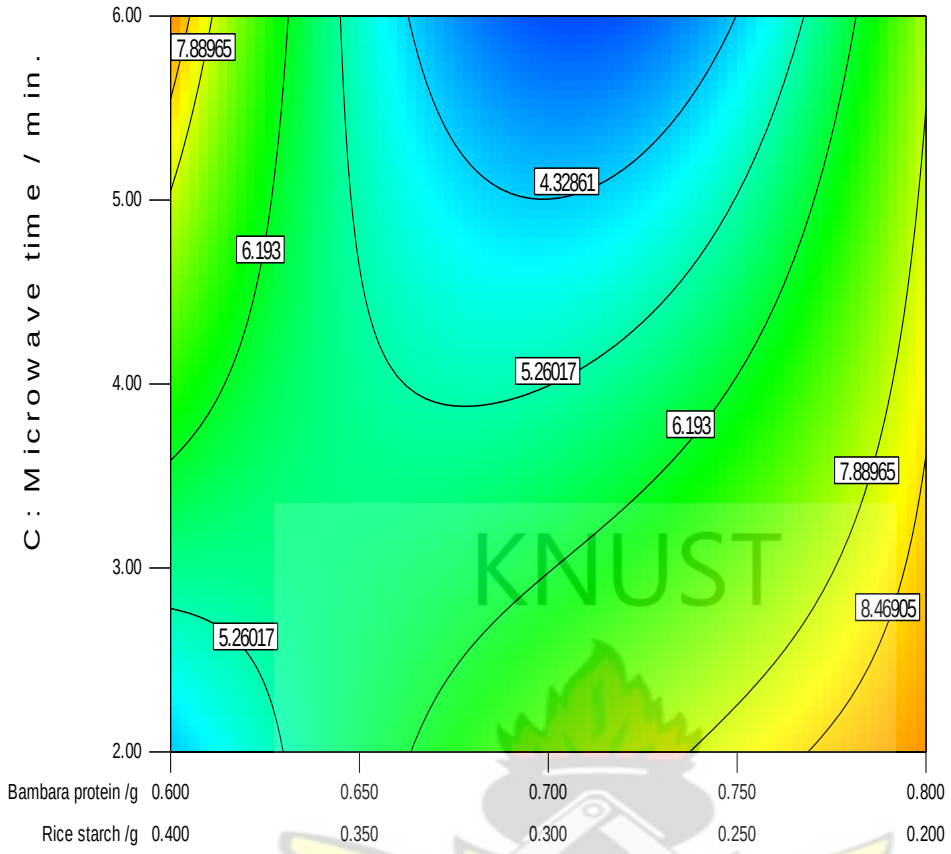

Figure 4.3: Response surface plot on the effect of Bambara protein-rice starch combinations and microwave time on glycation

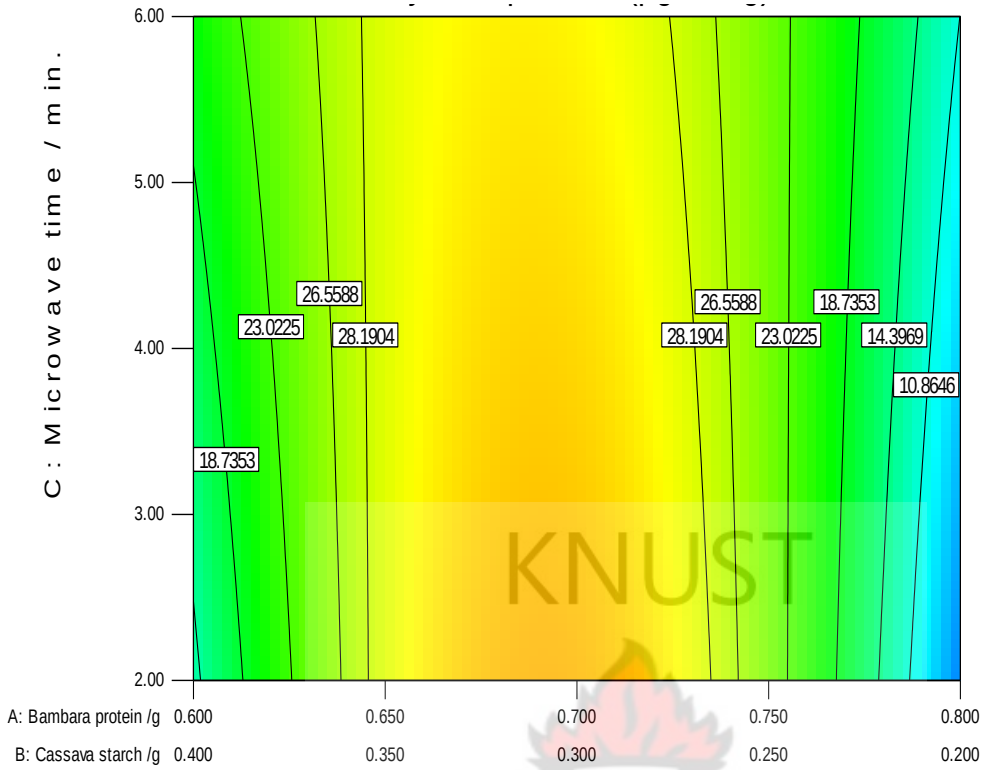

Figure 4.4: Response surface plot on the effect of Bambara protein-cassava starch combinations and microwave time on amount of glycation

4.6 Optimization of Conditions for Glycation of Bambara Protein-Rice Starch Composites

The model constraints were used to generate the optimum conditions for maximum glycation of Bambara protein-rice starch composites. After optimization, the optimum conditions for maximum glycation of were; 0.6 g of Bambara groundnut, 0.4 g of rice starch and microwave time of 6.0 min as shown in Table 4.7.1. The desirability of the optimization was 0.86 which is good as it is closer to 1. The closer the desirability is to 1, the better the optimization. A plot of the maximum glycation against Bambara protein and rice starch at the optimum microwave time of 6.0 min is shown in Figure 4.5. It can be seen from the graph that the glycated protein content decreased gradually as protein content increased until it reached minimum glycation value of 3.3 (µg/10mg) after which it started increasing gradually upon further increase in protein content. This is contrary to the trend to observed by Zhen-Chun *et al*. (2013) that glycation increased steadily with increasing protein concentration to a peak of 52.28 % and later followed by a decline in glycation upon further increase in protein concentration. It was revealed that the optimum conditions for maximum glycation of Bambara protein and rice starch, at time 6.0 min was 0.6 g Bambara protein and 0.4 g rice starch. The minimum response at microwave time of 6.0 min. was however observed when Bambara protein was 0.7 g and rice starch was 0.3 g.

Table 4.7.2 shows the constraints of the model and the selected conditions for optimum glycation of Bambara protein-cassava starch composite. It can be observed that the conditions of 0.7 g Bambara protein, 0.30 g cassava starch and microwave time of 2.0 min was selected for the optimum glycation of 32.92 (µg/10mg). The desirability of the optimization was 0.82 which is good as it is closer to 1. A plot of the maximum glycation against Bambara protein and cassava starch content at optimum microwave time of 2 min is shown in Figure 4.6. It was observed from

the graph that the glycated protein content increased gradually as protein content increased until it reached its peak at glycation value of 32.92 (µg/10mg) after which it started declining upon further increase in protein content. This is similar to observations made by Zhen-Chun *et al*. (2013) where degree of grafting increased steadily with increasing protein concentration to a peak of 52.28 % and later followed by a decline in glycation upon further increase in protein concentration. According to Kato *et al*. (1993), glycation proceeds faster during the early stages because more ε-amino groups of lysine are accessible for glucose interaction.

Table 4.7.1: Optimum conditions for glycation of Bambara protein-rice starch composites

Optimum conditions					
Bambara protein /g	**Rice starch /g**	**Microwave time /min**	**Max. glycation response /µg/10mg**	**Desirability**	**Rank of selection**
0.6	0.40	6.00	8.99	0.86	1st
0.8	0.20	2.00	8.96	0.85	2nd

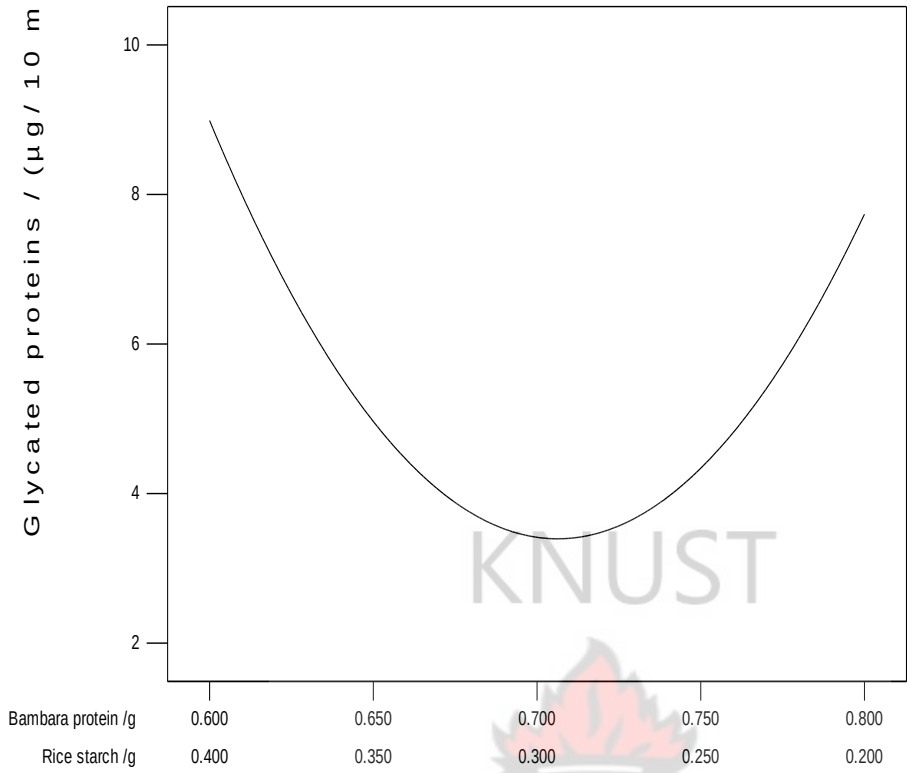

Figure 4.5: Response surface plot of maximum glycation of Bambara protein-rice starch composites at optimum time of 6.0 min

Table 4.7.2: Optimum condition for glycation of Bambara protein-cassava starch composites

Optimum conditions				
Bambara protein /g	**Cassava starch / g**	**Microwave time / min**	**Max. glycation response /µg/10mg**	**Desirability**
0.69	0.31	2.00	32.92	0.82

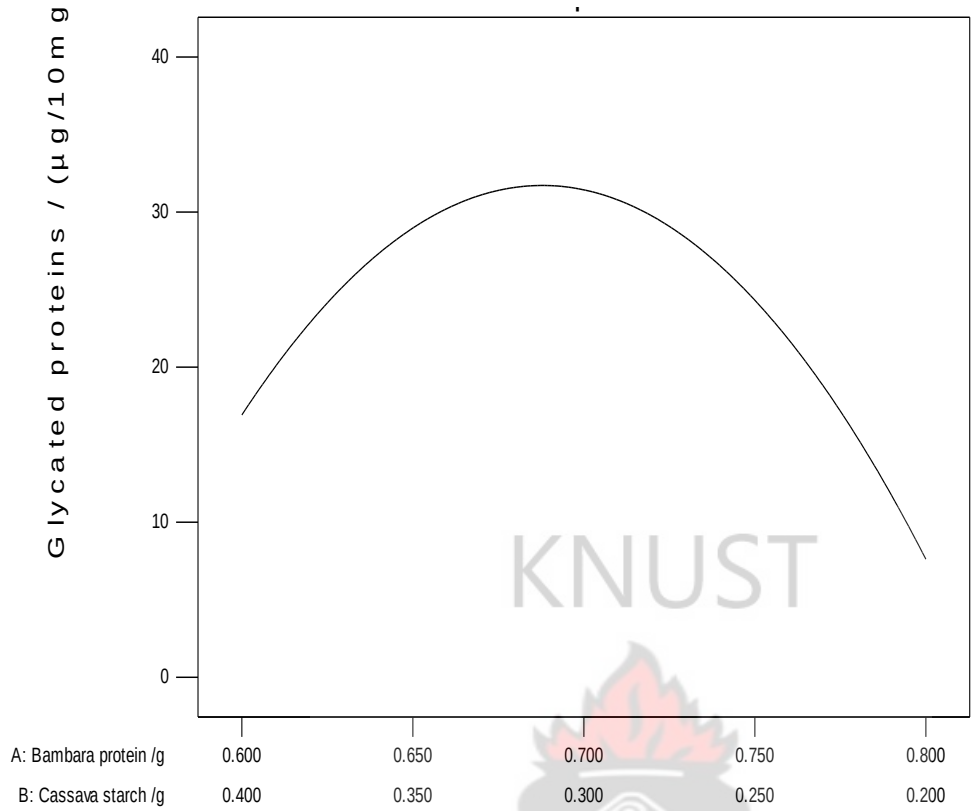

Figure 4.6: Response surface plot of maximum glycation of Bambara protein-cassava starch composites at optimum time of 2.0 min

4.7 Correlation Among Bambara Protein, Rice Starch and Microwave Time

Correlation analysis is used to quantify the degree of linear association between two variables. Correlation is strong when it is closer to 1 which can either be a positive correlation when the value is positive or a negative correlation when the value is negative. On the other hand, correlation is weak when it is closer to 0 which can also be either positive or negative. The direction of association however, does not affect the strength of association.

For Bambara protein-rice starch composite, it can be seen from Table 4.8.1 that a strong negative correlation exists between the combination of factors A and B and factor A only. This means that as the combined factor AB increases, factor A decreases and vice versa. Likewise a strong negative correlation exists between the combination of factors A and B and factor B only, implying that as the combined factor AB increases, factor B decreases and vice versa. A weak negative correlation is also observed between the combined factor ABC and AC.

With Bambara protein-cassava starch composites, it can be observed from Table 4.8.2 that a strong negative correlation exists between the combination of factors A and B and factor A only. This means that as the combined factor AB increases, factor B decreases and vice versa. Likewise a strong negative correlation exists between the combination of factors A and B and factor B only. A weak negative correlation is also observed between the combined factor ABC and AC as well as ABC and BC.

Table 4.8.1: Correlation matrix of regression coefficient for the glycation of Bambara protein-rice starch composites

	A	B	AB	AC	BC	ABC
A	1					
B	0.069828	1				
AB	-0.66194	-0.64832	1			
AC	0.013616	-0.01668	0.000295	1		
BC	-0.00819	0.023345	-0.00132	0.010841	1	
ABC	-0.02154	0.051329	-0.0026	-0.43479	-0.33208	1

Where: A=Bambara protein
B=Rice starch
C=Microwave time

Table 4.8.2: Correlation matrix of regression coefficient for the glycation of Bambara protein-cassava starch composites

	A	B	AB	AC	BC	ABC
A	1					
B	0.03531	1				
AB	-0.57572	-0.54692	1			
AC	-0.27303	-0.01453	0.153575	1		
BC	-0.00056	0.299874	-0.14343	0.009351	1	
ABC	0.099726	-0.07418	0.008999	-0.41737	-0.38401	1

Where: A=Bambara protein
B=Cassava starch
C=Microwave time

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The glycation between proteins from Bambara groundnut and starch from rice or cassava was optimized using composite design of the response surface methodology. The outputs of glycation of two types of composites (Bambara protein-rice starch and Bambara protein-cassava starch) were determined. The maximum glycation of Bambara protein-cassava starch was 51.9 (µg/10mg) whiles that for Bambara protein-rice starch was 10.0 (µg/10mg). This makes cassava the more preferred starch source in comparison to rice starch.

The best condition for optimum glycation of Bambara protein cassava starch composites was found to be 0.7 g protein, 0.3 g cassava starch and 2.0 min microwave time. On the other hand, the conditions that gave optimum glycation for Bambara protein-rice starch was 0.6 g protein, 0.4 g rice starch and microwave time of 6.0 min.

5.2 Recommendations

1. Further studies should be conducted to determine the characteristics of both rice and cassava that was responsible for the differences in their glycation yield.

2. Further studies should be conducted on the amino acid profile of the glycated protein that was produced so as to be able to determine its characteristics.

3. Since glycation of protein is known to improve the functional properties of food systems, the functional properties of the glycated proteins should be studied in a model food

system to verify if it can actually be used to improve the functional properties of that food system.

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APPENDICES

APPENDIX 1: CALCULATIONS 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 fat content

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

Appendix 1c: Calculation of crude protein content

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

Where:

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 1d: Calculation of ash content

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

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 1f: Calculation of carbohydrate content

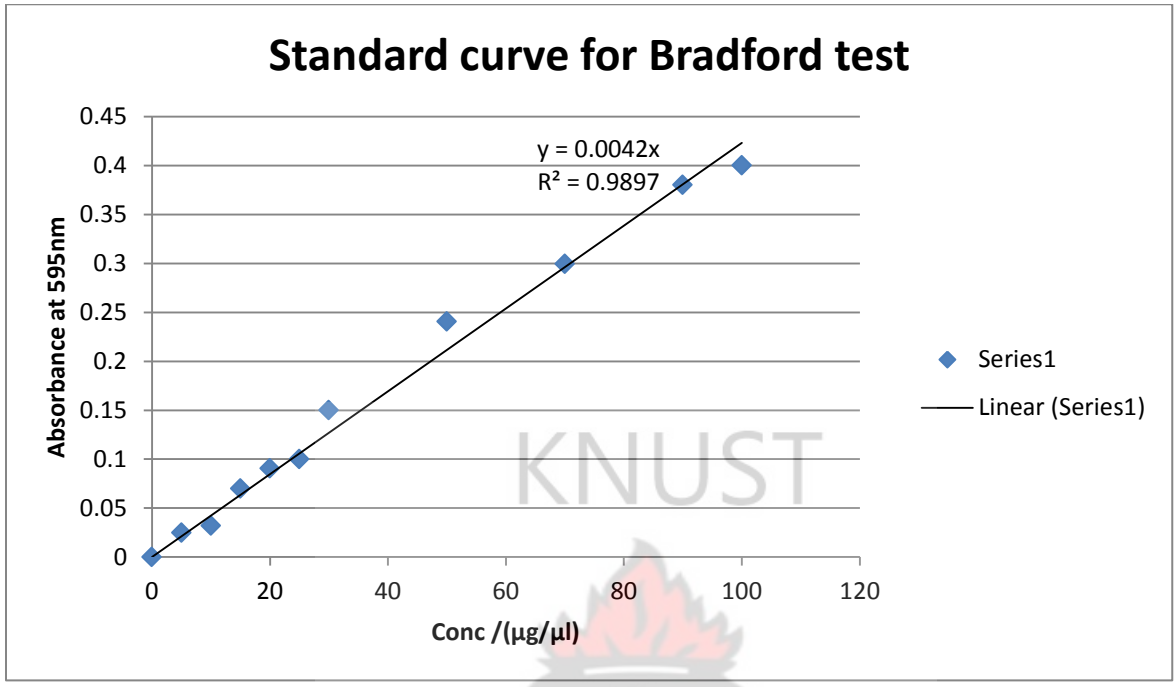
$$\% \text{ Carbohydrate} = 100 - (\%\text{moisture} + \%\text{crude fat} + \%\text{crude protein} + \%\text{ crude fiber} + \%\text{ ash})$$

APPENDIX 2: DATA AND STANDARD CURVES

2a: Data for preparation of standard curve for Bradford test for protein content at absorbance of 595 nm

Conc. /(µg/µl)	Absorbance /nm
5	0.025
10	0.032
15	0.070
20	0.091
25	0.100
30	0.15
50	0.241
70	0.299
90	0.380
100	0.400

2b: A graph of protein concentration in bovine serum albumin versus absorbance at 595nm.



2c: Data for preparation of standard curve for Bradford test for determination of soluble glycated proteins at an absorbance of 595 nm

Conc. /(µg/µl)	Absorbance /nm
20	0.001
40	0.005
80	0.009
160	0.019

2d: A graph of protein concentration in bovine serum albumin versus absorbance at 595nm