KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY,
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CHARACTERIZATION OF COPPER COMPLEXES FROM COPPER SALT AND CRUDE GARLIC EXTRACTS

A thesis submitted to the Department of Food Science and Technology, Kwame Nkrumah University of Science and Technology in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
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By

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Declaration

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

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Dedication

In loving memory of

Kwame Pepra-Ameyaw

(1988-2003).

Acknowledgement

I would also like to express my sincerest appreciation to the three women in my life; Mrs. Naa Angerley Pepra-Ameyaw for her love and support, Prof. Ibok Oduro for her guidance, wisdom and inspiration and Prof. Karen Duca for showing me that there is more to the universe than meets the eye. I would also like to thank the physical chemistry laboratory at KNUST for the lab space, Dr. Edward Carey for providing the freeze dryer used in the study and Dr. Friedrich Menges for the Spekwin32 software for spectral analysis.

Abstract

Two varieties of garlic; *Allium sativum var sativum* and *var ophioscorodon*, were studied to ascertain the feasibility of forming copper colloid suspensions with their crude extracts. They were extracted in borax, SDS-borax and urea-borax buffers gravimetrically at a pH of 10. By comparison, the extracts of *var ophioscorodon* which was a locally grown garlic variety contained significantly higher levels of protein (0.396-0.415 %) and phenols (23.00-29.46 au) than *var sativum* which ranged between 0.275-0.336 % for protein and 10.88-11.98 au for phenolic content. The buffers used for extraction did not have any apparent effect on differences in protein and phenolic content. The extracts were treated with equimolar cupric-nitrite solution. Uv/vis and IR spectroscopy were used to assess the relevant interaction of garlic extract with Cu (II) ions. Two uv/vis absorption bands observed for garlic extracts and their complexes band I (274-276 nm) and band II (307-365 nm). Band II was diminished upon complexation representing interaction of Cu ions with the garlic extracts. Multiple inflection points along the titration curves and their first derivatives indicated a stepwise formation of copper complexes. IR bands at 991.97-996.23 (C-H def), 1207.05 (C-O-C str), 1384.37-1384.97 (C-O str) and 1724.97 (C=O str) revealed a spectral pattern similar to that observed for the flavonols implicating such compounds in the chelation of Cu in the garlic extracts. In conclusion, garlic buffer extracts formed copper complex suspensions by chelating Cu(II) ions with flavonol-like compounds.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 **Background and statement of problem**

Allium Sativum which is also commonly referred to as garlic is an indispensable spice that is used in many cooked foods around the world and especially in Ghana. It is known to have some health and medicinal benefits beyond its regular nutritional benefits as food. These health benefits have encouraged the production of several garlic preparations available for different applications in the food and pharmaceutical industry. These garlic products rely on the diversity of compounds in garlic for their effectiveness, however the main active compounds and their mechanism of action remains elusive.

The use of nutritional substances and food for health purposes over the years has given rise to such terms as 'functional foods' and 'nutraceuticals'. Several studies have reported a dramatic increase in the use of nutraceuticals in the form of nutritional or dietary supplementation (Fugh-Berman, 2001). Colloidal metal suspensions are now being used and marketed as oral nutritional supplements (Fung and Bowen, 1996); this is because metals are very reactive and can act as reducing agents, bind proteins and denature enzymes. In topical formulations, they are also effective bactericides (Fung and Bowen, 1996) and are becoming popular in a lot of consumer products. Some of these metal colloids have been reported to have toxic side effects and they have been rebranded as "nano" compounds to generate interest in their use as nutritional supplements (Hillyer and Albrecht, 2001).

Researchers have begun studying these metal colloids known commonly as nanoparticles for their long term effects on food crops and health of consumers. Others have also examined novel ways of making these nanoparticles by using nontoxic and environmentally friendly materials (Katti *et al.*, 2008; McFarland *et al.*, 2004).

By employing known bioactive compounds such as those found in garlic, metal colloid suspensions can be produced that are safer and can have more useful applications in the food industry.

Garlic is among the earliest of cultivated food and spices, easily identified by its distinctive smell. It is a member of the well known and widely appreciated *Lilliceae* family and genus *allium* which comprises of more than 600 different species including onions and shallots. These *allium* species were valued by earlier civilizations not just as an important dietary constituent, but also for medicinal purposes in the treatment of many disorders (Callery, 1994).

Garlic has been used for thousands of years for a wide range of conditions. The Egyptians used them for medicinal purposes (Block, 1992). Even in Christian literature, the Bible records the use of garlic in the book of Genesis (Block, 1992). In Asia, Europe and the Middle-East, It is used as a traditional dietary supplement for diabetes. In France during the early 1700's gravediggers drank a concoction of crushed Garlic in wine which they believed would protect them from getting the plague that killed many people in Europe. It was also crushed and placed on wounds to stop them from turning septic and to prevent gangrene during both World Wars of the 20th century (GHS, 2009) It is evident now that the therapeutic properties of garlic have been exploited for at least

5000 years and its reputation by many as a “cure-all” has been the basis for a lot of scientific investigations. However it was not until the introduction of spectroscopic and chromatographic techniques that it became possible for scientists to determine the molecular basis for the odor, taste and biological activity of both fresh and processed garlic (Block, 1992).

Historically, garlic plants have been used to protect foods, raw and cooked meats, or boiled beans, from bacterial spoilage (Ariga and Seki, 2005). The preservative function of garlic has been replaced by more modern methods such as refrigeration, however scientists continue to novel food application for garlic. The most significant finding about garlic is that both quantity and quality of its active components change considerably upon processing (Ariga and Seki, 2005). Therefore, utilization of garlic and onion for functional foods is highly promising, considering these benefits.

Several studies have linked the health properties of garlic to certain bioactive compounds that it contains (Ichikawa *et al.,* 2003; Tepe *et al.,* 2005). Rodriguez Galdon *et al.,* (2008) associated the health benefits of garlic with three main chemical compounds, namely sulfur containing compounds such as thiosulphinates, non-structural and soluble carbohydrates such as fructooligosaccharides (FOS) and polysaccharides, and phenolic compounds such as flavonoids. Many of these compounds have been isolated and studied for their bioactivity (Block, 1992).

The compounds in garlic have been found to have metal coordinating properties which contribute to their health conferring properties (Torreggiani *et al.,* 2005).

The application of inorganic chemistry in industries and disciplines such as Food science, nutrition and medicine is a rapidly developing field and novel compounds and nanoparticles are now having an impact on food product and package development, food preservation technology, drug design and medical practice. Advances in bio-coordination chemistry are essential for improving the design of beneficial dietary and medicinal compounds to enhance their therapeutic properties, reduce their toxic side-effects and better understand their mechanisms of action.

1.2 **Purpose and Objectives of the Study**

The purpose of this study was to determine the capability of making copper colloidal suspensions with buffer extracts of garlic and to characterize the complexes formed.

The specific objectives of the study were to;

- Determine the total amounts of proteins and phenols in three buffer extracts of garlic; Borax, SDS-Borax and Urea-Borax buffer
- Establish the formation of Cu(II) complexes by titrimetric analysis and uv spectroscopy
- Characterize the complexes by FT-IR spectroscopy

1.3 **Scope of the study**

There are several methods available for extracting and isolating bioactive compounds from plant sources, and most of them generally involve extraction of the compounds into a suitable solvent and isolation by chromatographic techniques. These techniques

range from established processes such as steam distillation to emerging techniques like supercritical fluid technology.

In this study, the technique of metal complexation was employed and this involved treating crude garlic extracts with salts of a metal ion to form aqueous transition metal complexes. This approach avoids the isolation and purification of compounds from the samples before complexation or chelation. The formation of complexes will then be monitored by UV-visible spectroscopy and their identities characterized by FT-IR. This information about the new complexes will provide an alternative means of making metal colloidal suspensions for use in the food industry.

1.4 **Justification of Study**

The search for new beneficial compounds in vegetable materials in general has been given a lot of attention in the last decade and the demand for bioactive compounds continues to grow rapidly as researchers race to develop 'functional foods' and 'nutraceuticals'. Many research groups have put their focus on the extraction of such compounds from plants and agro-industrial byproducts, putting this topic at the forefront of academic and industrial research. As a result, the prevalence of a variety of fortified food products on the market and the use of plant extracts in food products such as yogurts and ice cream has been popularized.

A lot of food and nutrition companies are engaged in research on nanotechnology for food applications and a lot of materials manufacturers are also interested in selling their nanomaterials to food producers (Helmut Kaiser Consultancy, 2006). These compounds are important because their small size allows high oral bioavailability, accumulation

within the blood, and excretion through the kidneys (Hillyer and Albrecht, 2001). Considering the popularity of these compounds in food products, it has become necessary to find new ways of making these compounds with minimally processed and environmentally friendly materials.

Plants such as garlic are treasure chests to a variety of extremely beneficial compounds which could be used in the preparation of metal colloidal suspensions. This would reduce the number of synthetic and toxic materials that are used for the compounds and provide alternative ways for their application in the food industry. In this study, the capability of two garlic varieties; *Allium sativum var sativum and var ophioscorodon* to form copper colloidal suspensions and characterization of the complexes formed are investigated. The study will also redefine garlic as a value added crop for farmers.

CHAPTER TWO

2.0 REVIEW OF LITERATURE

2.1 **Metal colloid suspensions and their applications**

Metal colloid suspension more commonly referred to as nanoparticles are formed through the natural or human mediated disintegration of larger structures or by controlled assembly processes. The associated processes occur either in the gas phase, in a plasma, in a vacuum phase or in the liquid phase, eventually followed by the intentional or unintentional transfer into one or more relevant fluid media and then to an individual receptor in an exposure setting (SCENIHR, 2006).

New applications for metal colloids are continuously being developed in the food and agricultural industry. They are most likely to be found in dietary supplements. Manufacturers claim that because of their size, nanoparticles can be absorbed more quickly and easily. In the United States, there is a company that has developed a chocolate drink using the technology. They claim that by adding cocoa to 'nanoclusters' they can achieve a more intense chocolate flavour without the need for excess sugar (Anon., 2012).

For instance researchers are investigating the bactericidal effects of metal nanoparticles (Trapalis *et al.,* 2003). These metal nanoparticles are more effective against microorganisms than conventional materials due to their larger specific surface, which allows the same biocide activity using a lesser amount of metal (Pal *et al.,* 2007). Biocides of this nature have a large spectrum of applications in different fields such as

food packaging, medical instruments, medical implants, water treatment, food processing and agriculture.

2.2 **Garlic varieties under study**

Originally believed to originate from central Asia, garlic is a now a domesticated crop. Garlic is certainly one of the most frequently used spices in Ghanaian diet; however it is not commonly grown on a commercial basis. There are many different types of garlic which differ in size, colour, shape, and taste, number of cloves per bulb, pungency, and shelf-life. There are believed to be over 600 cultivated sub-varieties of garlic in the world, although many of them may have evolved from only a handful of basic types that have been grown widely and developed their own characteristics over several thousands of years as local growing conditions changed (Callery, 1994).

There are two main categories of garlic, the hardneck type '*Allium Sativum var ophioscorodon'* and softneck type '*Allium sativum var sativum'* (Anderson, 2007). Genetically there are 10 major varieties or types within these two categories. The climate of the region where garlic is grown can have a significant impact on both taste and scape production, and a variety considered as soft neck in one location may produce a flower in another. This has lead to the renaming of many strains that may instead be genetically the same plant. The hard-necked garlic was the original garlic and the soft-necked ones were selectively developed or cultivated over the years by farmers from the original hard-necks (Anderson, 2007).

Allium sativum var.sativum

The most common garlic variety found readily on the Ghanaian market is the soft-necked garlic shown in Figure 1, *Allium Sativum var sativum*. This variety of garlic is more commonly referred to on the Ghanaian market as 'white garlic or Chinese garlic'. Botanists often refer to this variety as arthichoke and in other parts of the world, they are often called red garlic or Italian garlic despite the fact that most of them are neither red nor were ever grown in Italy. In Ghana this type of garlic is mostly imported and commonly found in supermarkets. It is the generic garlic that most people imagine when they think of garlic (Anderson, 2007).

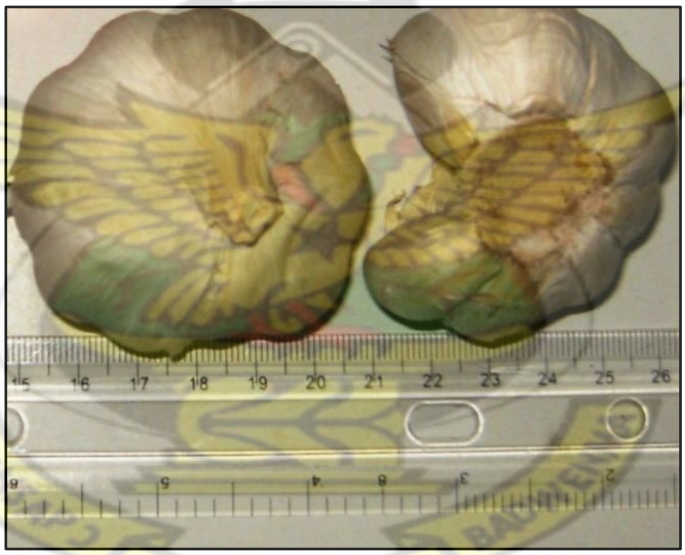


Figure 1 – *Allium Sativum var sativum* commonly known as white garlic or Chinese garlic.

Allium sativum var.ophioscorodon

The second variety of garlic commonly found on the Ghanaian market is quite unique and shares characteristics of one kind or another with all other kinds of garlic. It is usually grown in the Volta Region of Ghana and in some parts of the Northern Region, however, there is little information about this strain of garlic in Ghana. They are short storing garlic as most do not last more than 5 months at room temperature before sprouting (Anderson, 2007). On the Ghanaian market, they are commonly referred to as 'Alata garlic' or 'normal red garlic'. They are mostly patronized by local herbalists for use as traditional remedies.

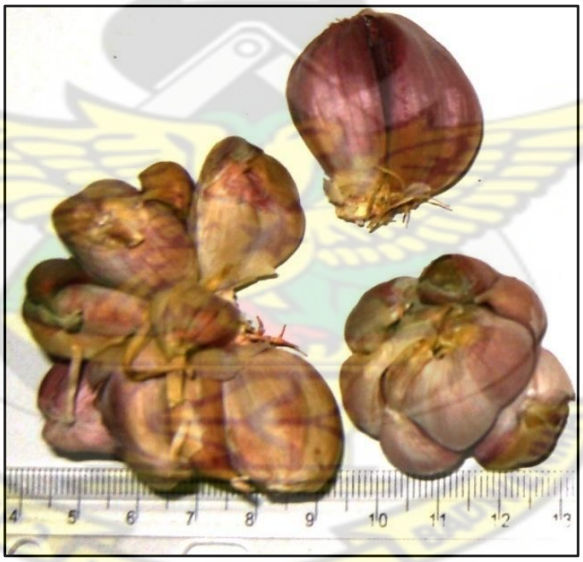


Figure 2–*Allium sativum var. ophioscorodon* commonly known as red garlic or Alata garlic.

2.3 **Brief history of the use of garlic**

The use of garlic as a spice is well documented in ancient Sumerian stone recipe tablets, in quotations from the Bible, the Talmud and Islamic writings. The medicinal uses of garlic are recorded in the earliest known medical writings, such as Papyrus Ebers Scrolls. Garlic was an important medicine to the ancient Egyptians listed in the medical text Codex Ebers also called the Ebers Papyrus; the oldest preserved medical document is dating from about 1552 B.C. The Codex Ebers identified garlic as a treatment for circulation disorders, malaise and insect and parasite infestations (Block, 1992).

Greek athletes ate garlic before they competed in Olympic Games and Hippocrates, the father of medicine, used garlic to treat maladies such as pulmonary disease and abdominal growths. The use of garlic is carried on with the Romans. They used garlic for strength building and endurance and they also gave it to soldiers and sailors (Lawson, 1998). There are thousands of journal publications about garlic and running a search for the "stinking rose" in Pubmed for instance, will generate over 2700 papers dealing with garlic. They include a range of topics including atherosclerosis, ischemia-reperfusion injury, fatigue, cancer, diabetes, metabolism, respiratory disorders, Alzheimer's disease; the list goes on and on (Rivlin, 2001). In ancient Chinese medicine, it was prescribed to aid respiration and digestion, most importantly diarrhea and worm infestation (Woodward, 1996). These ancient medical traditions in India, Tibb, Unani and Ayurveda made extensive use of garlic as a central part of the healing efficacy of plants (Moyers 1996). The leading Indian ancient medical text, Charak-Samhita recommends garlic for the treatment of heart disease and arthritis for over many centuries (Verma *et al.*, 2008). It is interesting to observe how various cultures all

around the world that never came into contact with one another, came to the same conclusions about the role of garlic in health and disease.

Scientific research on the activity of garlic however started in the second half of the 19th century with the work of Louis Pasteur, who in 1858 first noted antibacterial properties of garlic (Pasteur, 1858). In more recent scientific research, scientists have concentrated more on validating many of the properties of garlic, especially in terms of the identity of the active components, their mechanisms of action and exploring the potential benefits as food supplements.

2.4 **Use of garlic in food**

Garlic can be found in almost every market across the world and has a moisture content of about 65%. When stored in a refrigerator or in circulating air at ambient temperature it can be used for a few months. Depending on the processing or cooking methods used the composition and quality of garlic changes. In organic solvents or in cooking oils, the half-life of allicin is very short as compared to that in water, in which the half-life is estimated at up to 2 days (Lawson, 1996). Hence, the allicin may be recovered from garlic chopped in water. On the other hand, volatile and non-polar sulfides produced upon slicing may effectively be trapped with cooking oils. If the sliced garlic is dried up by blowing the air or with a microwave to prepare garlic tips, both allicin and sulfides may be lost completely (Block, 1992).

2.4.1 Domestic use of garlic

Heating raw garlic also changes the components of garlic. If the garlic bulb is not bruised or cut when heated, alliin stays unchanged inside the bulb, this can be a way to get rid of the pungent odor that garlic produces. However, if the heating is not sufficient to denature the alliinase, a large amount of alliin is transformed into allicin and sulfides while cooking or eating the bulb. When chopped garlic is heated, allicin and sulfides generated on the surfaces may disappear, and only a small amount of alliin may remain in the pieces. Heating the chopped garlic in cooking oils, allicin, sulfides as well as alliin must be present, in the oils or within the pieces. Hence, a lot of consumers prefer to cook garlic with cooking oils or meat that has a lot of fat (Heath, 1981).

Another form in which garlic is used is in pickled form using vinegar, alcohol or honey as a medium. Pickled garlic loses alliinase activity, depending on the period of its preservation. Acetic acid in vinegars (about 5%) and alcohol (20%) have been reported to be effective media in prevention of olfactory annoyance with garlic odor (Lawson, 1996; Pentz and Siegers, 1996). When the garlic clove is pickled after being sliced or powdered, it has different ingredients from that of the intact garlic clove.

2.4.2 Commercial garlic preparations

Many manufacturers have developed processing methods to produce specialized products. Garlic products can be found in markets or drug stores. There are many methods for preparing garlic products, and as a result different garlic products have different compositions. Industrially, garlic products are produced in forms that include aged garlic extracts (AGE), garlic oil and garlic powder. The aged garlic extracts (AGE)

have been produced by prolonged (about 6 months) soaking of chopped garlic into 20% alcohol (Nakagawa *et al.,* 1989; Hirao *et al.,* 1987; Amagase and Milner., 1993). Garlic oil is prepared by steam distillation of chopped garlic, and used for production of some sauces, pizza, cakes, ham and sausages.

Garlic powders are made in a number of different ways and studies have shown that garlic powders have no detectable amounts of alliin and sulfides (Heath, 1981). The easiest way in which manufacturers make garlic powder is by chopping garlic into fine pieces, followed by dehydration and pulverization. Although the ingredient of powder should be controlled to meet the formulation for a desired product, such as spiced sausages and sauces, the loss of sulfur compounds should be minimized (Lawson, 1996).

2.5 **Chemistry and Composition of Garlic**

Garlic (*Allium Sativum*) being a member of the onion family has a bulb which is made up of many individual cloves. The water content of garlic according to Verma *et al.,* 2008 is about 65%, which is rather low compared to most fruits and vegetables which contain about 80.90% of water. The bulk of the dry weight is composed of fructose-containing carbohydrate, followed by sulfur compounds, protein and free amino acids. Most literature on garlic's components have focused on sulfur compounds. The reasons for the interest are unusually high content of these compounds in garlic compared to other food plants, (Sulfur content is highest in garlic among common fruits and vegetables (Nielson *et al.*, 1991)).

When garlic is cut or crushed, an enzyme allinase is activated and converts allin (S-allyl-cysteine sulfoxide) to allicin (allyl-2-propenethiosulfinate). Allicin is responsible for the pungent odor of garlic and has been cited by many articles as having antibiotic activity, but it is very unstable and decomposes at room temperature within a few hours. This decomposition of allicin yields organosulfur byproducts which include diallyl sulfide (DAD), diallyl disulfide (DADS), diallyl trisulfide (DATS), ajoen and dithiines (Jones *et al.*, 2004).

The characteristic pungencies of different Allium bulbs depend on distinctive mixtures of organosulfur compounds that they produce (Jones *et al.*, 2004; Block, 1992). Figure 3 illustrates the reactions that take place in garlic, as described above.

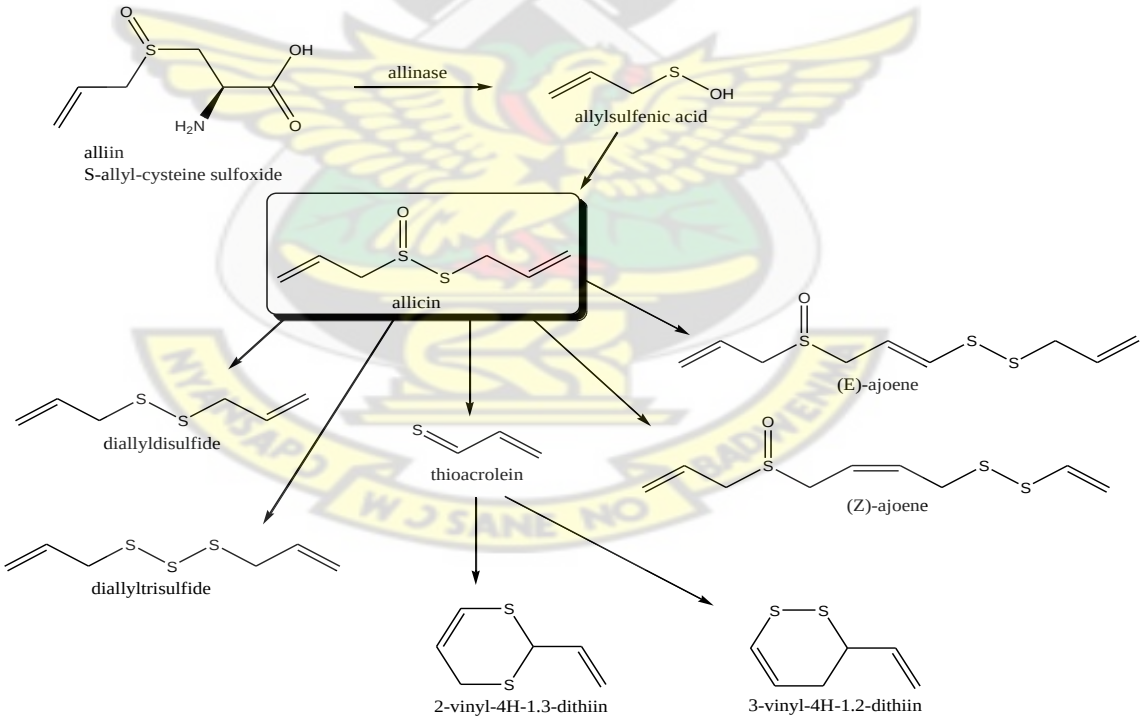


Figure 3 - Reaction of allinase on alliin in garlic (Source: Jones *et al., 2004*)

Many of the active compounds identified in garlic and other *Allium* plants, belong to one of three groups: dithiines, allyl sulfides, and ajoenes which all originate from thiosulfinates such as allicin, which in turn are produced by the action of the enzyme alliinase on cysteine derivatives as shown in Figure 3.

2.6 **Bioactivity of Garlic**

Scientific theories about the mechanism by which garlic confers its health benefits vary immensely, conceivably due to the wealth of compounds that it contains and the results of several studies seem to depend largely on experimental conditions. Many authors have made claims for a role of garlic and garlic preparations in antibacterial, antiviral, anti-inflammatory, and antineoplastic activities. There is also some evidence that seems to suggest that garlic may prevent colorectal tumor formation, reduce cholesterol, reduce blood pressure (BP) and provide anticoagulation (Alpers, 2009). Some authors have even reported that garlic is effective in regulating plasma lipid levels (Steiner and Li, 2001) and increases plasma anticoagulant activity (Apitz-Castro *et al*., 1992; Ackermann *et al*., 2001). The most studied and reported health promoting effect of garlic, however is its cardioprotective ability (Banerjee *et al*., 2002; Durak *et al*., 2002; Williams *et al*., 2005; Zahid *et al*., 2005). In the food industry, products such as garlic capsules as a food supplement and the use of aged garlic or garlic powder as a food preservative is evidence of its popularity. Some consumers however resort to simply consuming the whole garlic bulb.

As mentioned before, a myriad of pharmacological properties are attributed to garlic or its ingredients, ranging from blood lipid level and blood pressure lowering and inhibition of blood clotting to antiviral, antifungal, and antimicrobial activities and even cancerostatic effects (Siegel *et al.,* 1999; Block, 1992; Tsai *et al.*, 1985; Agarwal, 1996). Although these pharmacological effects are little understood, it is clear that most of them rely on the sulfur-containing components of garlic.

Among the compounds believed to give garlic its antioxidant ability are flavonoids. These compounds have been studied to some extent and their protective effects in biological systems are attributed to their capacity to transfer free radical electrons, activate antioxidant enzymes (Elliott *et al.,* 1992), reduce alpha-tocopherol radicals (Hirano *et al.,* 2001), inhibit oxidases (Cos *et al.,* 1998) and chelate metal ions (Ferrali *et al.,* 1997; Thompson *et al.,* 1976; Masataka and Murakami, 1998; Deng *et al.,* 1997; Brown *et al.,* 1998).

In fact, it has been shown that $O_2^{\cdot-}$, and H_2O_2 in the presence of transition metals such as iron and copper may be converted into highly reactive $^{\cdot}OH$ (Torreggiani *et al.,* 2005). In vivo most copper is 'tightly bound' to the plasma protein caeruloplasmin, they are also attached to albumin and to amino acids such as histidine that can catalyse free radical reactions (Marx and Chevion, 1995). Antioxidant compounds like flavonoids may sequester these metal ions by chelation and, preventing the metal-mediated generation of harmful oxidizing radicals, may protect the potential biological targets from oxidative stress (Halliwell and Gutteridge 1984; Minotti and Aust, 1989). Moreover, the participation of flavonoids and copper(II) in biochemical reactions has

become a topic of interest in recent years; in fact, enzymes such as Cu-containing quercetin-2,3 dioxygenase, occupy a special position among enzymes that catalyse redox processes involving flavonoids (Oka *et al.*, 1972).

There are however only a few studies on the use of flavonoids and organosulfur compounds for the treatment of acute tissue injury protection from chemical toxicity. The limited experimental studies on these compounds is partially due to their poor water solubility. Nonetheless, these dietary phytochemicals can form complexes with transition metal ions (Afanasév *et al.,* 1989) and these complexes are more hydrophilic and water-soluble than their corresponding ligands alone. More recently, quercetin, being among the most abundant flavonoids in nature and also found in garlic, has been shown by spectroscopic studies to chelate Cu (II) ions; however, on titrating out the bound Cu (II) ions, a new spectrum appeared, indicating that quercetin did not revert to its original form before Cu (II) interaction (Brown *et al*., 1998). Furthermore, the metal complexes possess higher scavenger potencies toward superoxide than the ligands alone and may act as superoxide dismutase mimics (Kostyuk *et al.,* 2004). Transition metals also enhance the anti-inflammatory activities of these compounds and their cytoprotective effects against oxidative injury in isolated cells (Afanasév *et al.,* 2001; Kostyuk *et al.,* 2001; Moridani *et al.,* 2003).

2.7 **Biological activity of organosulfur compounds**

The first alliin compound isolated from garlic in 1951 was (+)-S-(prop-2-enyl)-L-cysteine sulfoxide. This was one of the first naturally occurring sulfoxides to be identified. Later work showed the presence of analogous compounds. The

alkenylcysteine sulfoxides are the immediate precursors of the thiol sulfinate allicins, which are probably the major biologically active sulfur compounds in alliums. The allicins probably derive from the biological oxidation of the corresponding dialkenyl disulfides. Both symmetrical and unsymmetrical thiolsulfinates have been found, and several of their metabolites, e.g. the sulfoxide, have antithrombotic properties. Garlic may therefore be valuable in preventing heart disease; hence the increasing use of garlic capsules (Cremlyn, 1996).

2.7.1 Metal Binding ability of organosulfur compounds in Garlic

The thiolates in garlic are excellent ligands for a range of transition metal ions, most disulfides however scarcely coordinate to metal ions. The polysulfides, on the other hand, seem to form metal complexes, possibly due to their ability to coordinate with several sulfur atoms at a time and therefore act as poly-dentate ligands. For instance, Steudel *et al.,* (2005) calculated bond energies for a set of lithium-dimethylsulfide complexes; they found that binding energies increase from dimethylsulfide to dimethylpentasulfide. It is possible that similar metal binding events inside living cells may occur and lead to a disturbance of intracellular metal homeostasis or enzyme inhibition by means of 'adventitious' binding to active site metal ions (Steudel *et al.,* 2005).

2.8 **Chemistry and biological activity of flavonoids**

The study of flavonoids and their chemistry has emerged, like that of most natural products, from the search for new bioactive compounds with useful physiological properties. The term "flavonoid" is generally used to describe a broad collection of

natural products that include a C_6-C_3-C_6 carbon framework, or more specifically a phenylbenzopyran functionality. They are found in many plant tissues, where they are present inside the cells or on the surfaces of different plant organs.

Flavonoids differ in the saturation of the heteroatomic ring C, in the placement of the aromatic ring B at the positions C-2 or C-3 of ring C, and in the overall hydroxylation patterns shown in Figure 6. They may be modified by hydroxylation, methoxylation, or O-glycosylation of hydroxyl groups as well as C-glycosylation directly to carbon atom of the flavonoid skeleton. In addition, alkyl groups may be covalently attached to the flavonoid moieties, and sometimes additional rings are condensed to the basic skeleton of the flavonoid core. The last modification takes place most often in the case of isoflavonoids, where the B ring is condensed to the C-3 carbon atom of the skeleton. Flavonoid glycosides are frequently acylated with aliphatic or aromatic acid molecules. These derivatives are thermally labile and their isolation and further purification without partial degradation is difficult. There are now over 6000 different known flavonoid compounds due to the multiplicity of the possible modifications and this number continues to increase (Harborne and Williams, 2000).

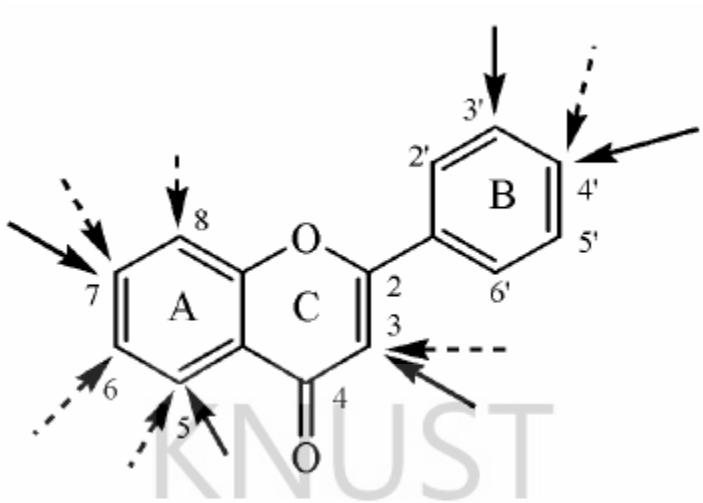


Figure 4. General flavonoid structure (Full arrows indicate most frequent hydroxylation sites and dashed arrows indicate most frequent C and/or O-glycosylation sites) (Harborne and Williams, 2000).

In the plant kingdom, different plant families have characteristic or distinctive patterns of flavonoids and their conjugates. All these compounds play important biochemical and physiological roles in the various parts of the plant (seed, root, green part, fruit) where they accumulate. Different classes of flavonoids and their conjugates have numerous functions during the interactions of plants with the environment, both in biotic and abiotic stress conditions (Dixon and Paiva, 1995; Shirley, 1996).

Furthermore, because of the common presence of flavonoid conjugates in most plants, they are important components of human and animal diet. Due to the different biological activities of plant secondary metabolites, their regular consumption may have serious consequences for health, both positive and negative (Beck *et al.,* 2003; Le March, 2002; Boue *et al.,* 2003; Fritz *et al.,* 2003; Nestel, 2003). For the mentioned reasons, methods

for the efficient and reproducible analysis of flavonoids play a crucial role in research conducted in different fields of the food and biological sciences.

2.9 **Extraction of useful compounds from garlic**

Different methods of isolation of the natural products may be applied, and the utilization of various strategies is dependent on the origin of the biological material from which the target natural products are to be extracted (plant or animal tissue or body fluids). In the case of polyphenolic compounds, it is important to initially determine whether the researchers are interested in the identification of individual components present in a mixture of target compounds or whether they would like to estimate the total amount of phenolic compounds in the biological material investigated. The second approach is most often preferred during the nutritional studies on different foods, mainly of plant origin (Robards, 2003).

The presence of carbohydrates and/or lipophylic substances may influence the profile of the qualitative and quantitative composition of flavonoids and their derivatives in the obtained extracts. The temperature conditions during the extraction procedures also have to be carefully adjusted because of the possibility of thermal degradation of the flavonoid derivatives. One has to consider all the above-mentioned selection of the methods for sample preparation and extraction, and in many cases additional cleaning based on solid-phase extraction (SPE) of the extracted samples is required (Naczk and Shahidi, 2004).

It can therefore be concluded that the choice of the extraction procedure for obtaining flavonoid conjugates from biological material is very important and depends on the aims

of the particular research. In some projects the evaluation of the spatial distribution of target compounds in the organ, tissue, cellular, or even subcellular level is of special interest. In these situations, the amount of biological material for the isolation of natural products may be extremely small, and the application of microextraction techniques is necessary (Vas and Veckey, 2004). In many cases, it is necessary to avoid the chemical and/or enzymatic degradation of the metabolites. This is of special importance in the profiling of flavonoid glycosides in research directed toward plant functional genomics or during physiological and biochemical studies that need information about all classes of flavonoid conjugates present, even the thermally labile acylated derivatives. On the other hand, in the phytochemical analysis of plant species or phytopharmaceutical studies of plant material, the repeatable isolation of all biologically active flavonoid aglycones with a good yield is more important. In these cases, more drastic extraction conditions are acceptable (Naczk and Shahidi, 2004).

2.10 **Nutraceuticals from plant sources**

The term "Nutraceutical" was coined in 1979 by Stephen DeFelice (DeFelice, 1992). It is defined "as a food or parts of food that provide medical or health benefits, including the prevention and treatment of disease." Subsequently, several other terms (medical food, functional food, and nutritional supplements) with similar descriptions were used. A nutraceutical is any nontoxic food extract supplement that has scientifically proven health benefits for both the treatment and prevention of disease (Dillard and German, 2000).

Nutraceuticals may range from isolated nutrients, dietary supplements, and diets to genetically engineered "designer" food, herbal products, and processed products, such as cereals, soups, and beverages. The increasing interest in nutraceuticals reflects the fact that consumers hear about epidemiological studies indicating that a specific diet or component of the diet is associated with a lower risk for a certain disease (Schreier, 2005).

The major active nutraceutical ingredients in plants are flavonoids (Kelm *et al.*, 2005). The flavonoids are a group of organic molecules ubiquitously distributed in many foods and beverages of plant origin, such as fruits, vegetables, tea, cocoa, and wine (Schreier, 2005). The flavonol quercetin is the most frequently occurring compound in foods. Also common are kaempferol, myricetin, and the flavones apigenin and luteolin. Tea, onions and garlic are the main dietary sources of flavonols and flavones. As is typical for phenolic compounds, they can act as potent antioxidants and metal chelators. They also appear to be effective at influencing the risk of cancer. Overall, several of these flavonoids appear to be effective anticancer promoters and cancer chemopreventive agents (Kelm *et al.*, 2005; Schreier, 2005)

Chemopreventive studies have demonstrated that the mechanisms of action of phytochemicals and nutraceuticals in the prevention of cancer go beyond the antioxidant activity scavenging of free radicals; regulation of gene expression in cell proliferation, oncogenes and tumor suppressor genes; induction of cell cycle arrest and apoptosis; modulation of enzyme activity in detoxification, oxidation, and reduction; stimulation of the immune system; and regulation of hormone metabolism. It is a general theme that the additive and synergistic effects of phytochemicals and nutraceuticals in fruits and

vegetables are responsive for their potent antioxidant and anticancer activities and that the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals and nutraceuticals present in whole foods (Liu, 2004). Recent development in the molecular mechanisms of garlic has provided a strong basis for performing the synergistic effects of phytochemicals and nutraceuticals in whole foods that have been ingested by the host. Along this aspect, it has been proposed that the cancer chemoprevention and anti-obesity effects of polyphenols might be accomplished through blocking the signal transduction pathways in target cells (Lin *et al.*, 1999; Lin, 2002).

2.11 **Complexation of plant flavonoid extracts**

The interaction of transition metals with organic plant extracts have been studied and plant compounds like flavonoids have been proven to form complexes with transition metals (Afanas'ev *et al.,* 1989). Kostyuk *et al*., (2007) demonstrated that the 'interaction of certain flavonoids with transition metals increased their water solubility and lead to the formaton of flavonoid-metal complexes which possessed effective free radical scavenger ability and had potent therapeutic benefits for the treatment of oxidative stress-related diseases and dysfunction.'

Quercetin, the most common flavonoid in foods including garlic was investigated in the presence of Cu (II) ions under basic conditions by Torreggiani *et al.,* (2005). This was to obtain some elucidation on the mechanism of its beneficial action against free radical-mediated damage. The study showed the capacity of quercetin to form complexes with Cu (II), rendering these ions inactive in generating radicals, and the Cu (II) complexes

were found to be effective radical scavengers. The study also showed that the structure of the Cu (II) complexes, depends on the M/L ratio and the catechol group on the B ring is the first site involved in the chelation under basic conditions.

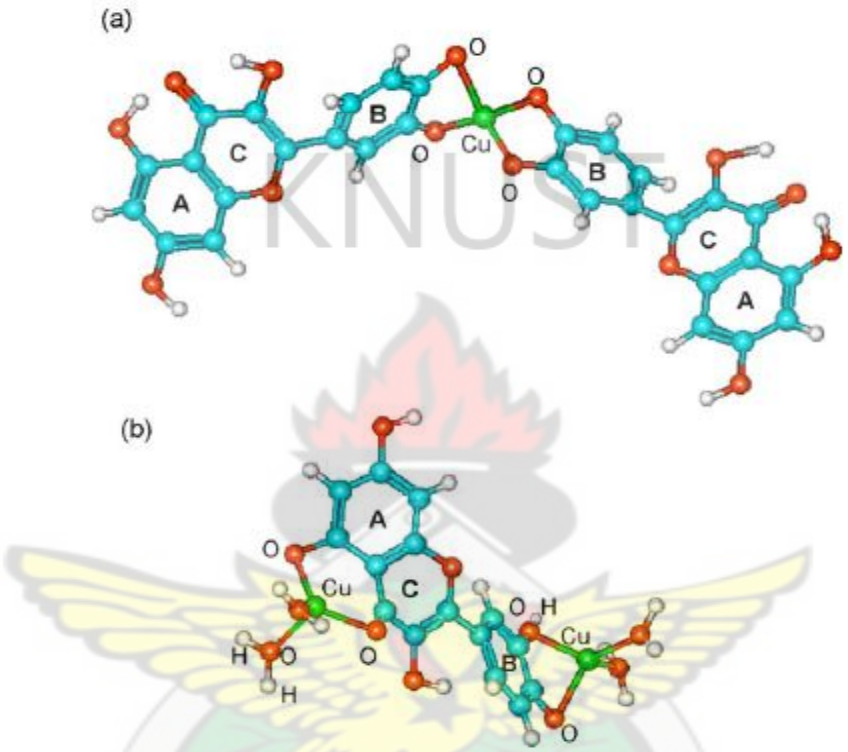


Figure 5. Proposed molecular structures of the Cu(II)–Querc complexes at pH 10 and M/L ratio 0.5 (a) and 2 (b) (Torreggiani *et al.* 2005).

Pereira *et al.,* (2007) also believed that the antioxidant activity of plant or dietary flavonoids increased when they were coordinated with transition metal ions, though the literature on this subject was contradictory and the outcome of such studies seemed to largely depend on experimental conditions. To understand the contribution of the metal coordination and the type of interaction between a flavonoid and the metal ion, a new metal complex of Cu (II) with naringin was synthesized and characterized by FT-IR,

UV-VIS, mass spectrometry (ESIMS/MS), elemental analysis and 1H-NMR (Torreggiani *et al.,* 2005). The study showed that Cu (II) ion coordinated via positions 4 and 5 of the flavonoid. The Naringin–Cu (II) complex also showed higher antioxidant, anti-inflammatory and tumor cell cytoxicity activities than free narigin without reducing cell viability.

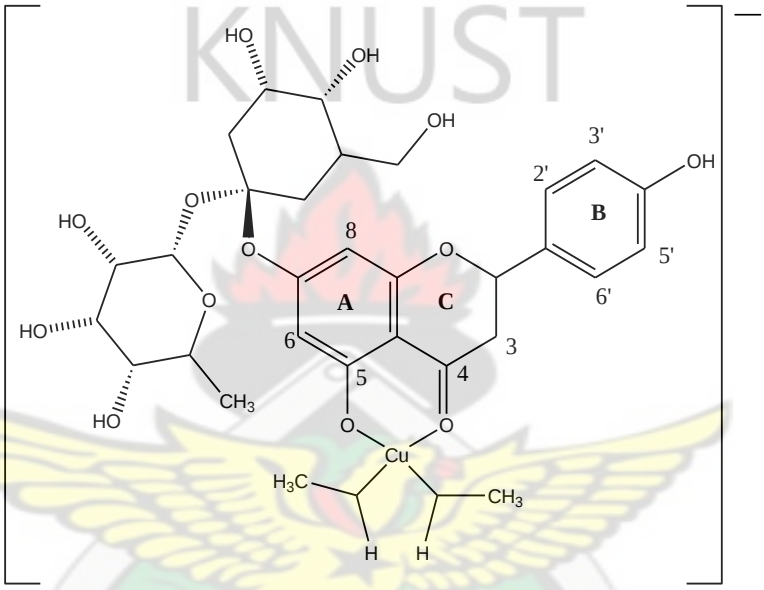


Figure 6. Proposed structure of Narigin-Cu (II) complex (Pereira 2007)

Priya *et al.,* (2008) also proposed this complexation technique as a new method of isolation of Isoflavones from Glycine max (soya beans). In that study, the technique of complexation was used to prepare isoflavones-enriched soya extracts and genistein was isolated from the soya bean extract. Their findings showed that isoflavones in extracts, form stable complexes with metals and suggested that this could be used as a novel method for isolation of flavonoids from plant sources.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 **Sample preparation**

Allium Sativum var. sativum and *var ophioscrodon*, the two main varieties of garlic commonly used in Ghana, were purchased from markets in Accra and Kumasi between the months of December 2010 and January 2011. The two varieties were separated into two groups of about 1000g each. The samples were carefully washed and removed to avoid bruising.

3.2 **Preparation of reagents**

3.2.1 Preparation of Sodium tetraborate (Borax) buffer

Exactly 9.5 g of borate was weighed and dissolved in an aliquot of distilled water in a beaker with a stirrer. The resulting solution was transferred into a 250 ml volumetric flask and topped up to the mark with distilled water to obtain 0.1 M sodium tetraborate. The pH of the solution was then adjusted to 10 with a 2 M NaOH solution.

3.2.2 Preparation SDS-borax buffer

Sodium dodecyl sulphate (25 g) was weighed into a beaker and dissolved in an aliquot of the sodium tetraborate buffer. The solution was transferred into a 250 ml volumetric flask and topped up to the mark to obtain a 10 % (w/v) SDS-borax buffer. The pH of the solution was then adjusted to 10 with a 2 M NaOH solution.

3.2.3 Preparation of urea-borax buffer

An amount of 90.1 g urea was weighed in a beaker and dissolved in an aliquot of the sodium tetraborate buffer previously prepared. The resulting solution was transferred into a 250 ml volumetric flask and topped up to the mark with more sodium tetraborate buffer. The pH of the solution was then adjusted to 10 with a 2 M NaOH solution.

3.2.4 Preparation of copper sulphate solution

In a beaker, 2.5 g of Copper sulphate was weighed and dissolved in an aliquot of 0.1 M HCl solution. The resulting solution was transferred into a 100 ml volumetric flask and topped up to the 100 ml mark with the HCl solution after shaking until all crystals were dissolved.

3.2.5 Preparation of sodium nitrite solution

To prepare 0.1 M $NaNO_2$, 1.73 g of sodium nitrite was carefully weighed and dissolved in an aliquot of 0.1 M HCl in a beaker with a stirrer. The resulting solution was transferred into a 250 ml volumetric flask. The solution was topped up to the 250 ml mark of the volumetric flask with the HCl solution.

3.3 Preparation of garlic extracts

For each of the two garlic varieties under study, 25 g of clean garlic bulbs were weighed. The weighed samples were then homogenized in a blender with 100 ml of the prepared buffer solutions. This was repeated for each of the buffer solutions previously prepared (0.1 M sodium tetraborate, 10% (w/v) sodium dodecyl sulfur in sodium

tetraborate and 6 M urea in sodium tetraborate). The pH of the solution was adjusted to 10, where deprotonation of hydroxyl groups favor the solubility of most polyphenols (Torregiani *et al.,* 2005). The resulting homogenate was vacuum filtered and the filtrate collected into labeled containers. The samples were kept cold at 8 ºC to avoid freezing until they were needed for further analysis.

3.4 **Determination of protein content**

The Biuret assay for determination of protein was used (Weaver and Daniel, 2005). This reaction involves the complexation of copper in alkaline solution with the peptide linkage of proteins and also with tyrosine residues.

3.4.1 Preparation of Biuret reagent

The Biuret reagent was prepared by weighing 1.5 g of $CuSO_4.5H_2O$ and 6.00 g of sodium potassium tartrate ($NaKC_4O_6.4H_2O$) with an electronic balance (OHAUS model AS260D) into a 1000 ml volumetric flask. The mixture was dissolved in 500 ml of distilled water after which 300 ml 10% (w/v) NaOH was added to the contents of the flask while stirring. The stirring bar was removed and the volume was made up to the mark with distilled water. The solution prepared was blue.

3.4.2 Determination of protein by Biuret reaction

The Biuret assay described by Connie and James (2003) was used to determine the total protein content of the extracts. The spectrophotometer was turned on and allowed to warm up while the samples were prepared. A protein standard (100mg/ml egg albumin) was obtained and pipette into 10 test tubes from 0.1 to 1.0ml respectively. The total

volume of the tubes was topped up to 1 ml with distilled water and up to 5 ml with the Biuret reagent. The mixture was shaken in a vortex mixer for five minutes to effect thorough mixing. The tubes were then incubated at 37^{o}C for 20 minutes.

The absorbance of each standard at a wavelength of 540 nm was measured with the spectrophotometer. This was done immediately after incubation as the colour observed was stable only for periods between one to two hours, but slowly increases over a period of several hours. A plot of absorbance against concentration was drawn to get a calibration curve. The unknown extracts were assayed in the same way and the protein content estimated from the standard curve.

Calculations:

$$A_{540nm} = A_{540nm}\text{Std} - A_{540nm}\ \text{Std Blank}$$

$$A_{540nm}\ \text{Sample} = A_{540nm}\ \text{Test} - A_{540nm}\ \text{Test Blank}$$

A standard curve was drawn by plotting the A_{540nm} of the Standards against mg of protein. The protein contents of the samples were determined from the standard curves follows:

mg Protein = (mg of protein from the Standard curve)(df)

$$\%\ \text{Protein} = \frac{(\text{mg Protein})(100)}{(\text{mg solid/ml buffer})}$$

3.5 **Determination of Total Phenolic Content**

The garlic extracts were transferred into a quartz cuvette and the absorbance measured at 280 nm in a spectrophotometer. The absorbance was measured in absorbance units (AU). Preliminary tests indicated that the observed absorbance was not within the acceptable precision of the spectrophotometer ie. A < 2 AU, as such the sample was diluted serially with excess buffer and measurements were repeated until an appropriate absorbance was observed. Using a convenient dilution factor of x50, the procedure was repeated for each garlic extract and distilled water was used as a blank (Cuvelier *et al*., 1996).

The absorbance of the blank was subtracted from the observed absorbance; this absorbance was then corrected to the original concentration and a 1-cm cuvette path length. A correction of 4 AU was subtracted to report the final value.

$$\text{Total phenolic content} = \left[A_{280} \times DF \times \left({}^{1cm}/_{b}\right)\right] - 4$$

Where DF is the dilution factor and b is the cell path length

3.6 **Quantitative formation of complex**

To monitor the quantitative formation of the copper–garlic complexes, the mole–ratio method (Harvey, 2000; Malesev and Kuntic, 2007) for determining the stoichiometry of complexes which involves preparing solutions containing different mole ratios of the reactants was employed. To two milligrams of 0.2M copper sulfate, two milligrams of

0.1M sodium nitrite was added and toped up to the 100ml mark with 0.1M HCl; this was then titrated against different volumes of the various garlic extracts ranging between 0.0 to 0.1ml. The mixtures were brought to an equal volume of 5ml with the respective extraction buffers. The mixtures were shaken for a minute and allowed to rest. A green precipitate readily soluble in the excess buffer was observed. The formation of the complex was monitored by UV-visible spectroscopy. A graph of the corrected absorbance at 281 nm was plotted against the percentage volume fraction of garlic extract to copper solution. The volume fraction was obtained by dividing the volume of garlic extract by the total amount of garlic extract and copper solution (Job, 1928; Harvey, 2000).

To determine the end point of the reaction of extracts in the three buffers, first derivative of the titration curves were drawn. The slope of a titration curve reaches its maximum value at the inflection point. The first derivative of a titration curve, therefore, would show a separate peak for each end point (Job, 1928; Harvey, 2000). The first derivative is approximated as $\Delta abs/\Delta V$, where Δabs is the change in absorbance between successive additions of titrant.

3.6.1 UV-Visible spectroscopy

UV–Visible Spectroscopy measurements of the copper-garlic complexes were performed at room temperature by means of a Shimadzu UVmini^{-1}240 spectrophotometer equipped with a 10 mm quartz cuvette. The absorbances were recorded at wavelengths between 200 and 900 nm. The data was transferred to a computer by means of a data logger and analyzed with the Spekwin32 software for

optical spectroscopy version 1.71.5 developed by Dr. Friedrich Menges from Germany in 2000. A plot of absorbance as a function of the volume of extract was drawn for each batch of copper-garlic complex at a selected wavelength of 281 nm.

3.7 **Lyophilization of copper–garlic complexes**

The prepared complexes were lyophilized with a vacuum freeze dryer type (TRUE TEN YK-118-50) at a pressure of 0.2 torr for 48 hours. The solid samples obtained after lyophilization, were all kept in a desiccator until further analysis.

3.8 **Infra–Red spectroscopy of copper-garlic complex**

The FT-infrared spectra of the garlic extracts and their corresponding complexes with Cu (II) were recorded with an INTERSPEC FT-IR spectrophotometer using the KBr-pellet technique. Approximately 1.00 mg of the sample was weighed, crushed and homogenized along with 200 mg of spectroscopic grade potassium bromide with a mortar and pestle. The homogenate was carefully packed and pressed with a spacedie disk pressing kit which comes with the FT-IR spectrophotometer. Care was taken to ensure that a clear film was produced for a reliable result. The spacedie was then transferred to the FT-IR spectrophotometer for analysis. The procedure was repeated for each of the samples and the spectral resolution was 4 cm^{-1} and the error in wave-number was about 0.4 cm^{-1}.

3.9 **Statistical analysis**

All the data collected were recorded as the mean ± standard deviations of triplicate determinations. The results for total phenolics were subjected to one-way ANOVA at a

significance level of $P < 0.05$ and the Tukey's HSD post test was used to determine which means were significantly different from each other. Statgraphics centurion XV (version 15.1.02), and GraphPad Prism 5 were used for statistical analysis and plotting of graphs.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 **Protein content of *Allium sativum var sativum* compared to *var ophioscorodon*.**

Garlic is not known for its protein content, however, the enzyme *allinase*, that it produces when bruised forms a great proportion of its overall protein content. The results of the percentage protein content obtained for the garlic extracts are shown graphically in Figure 10.

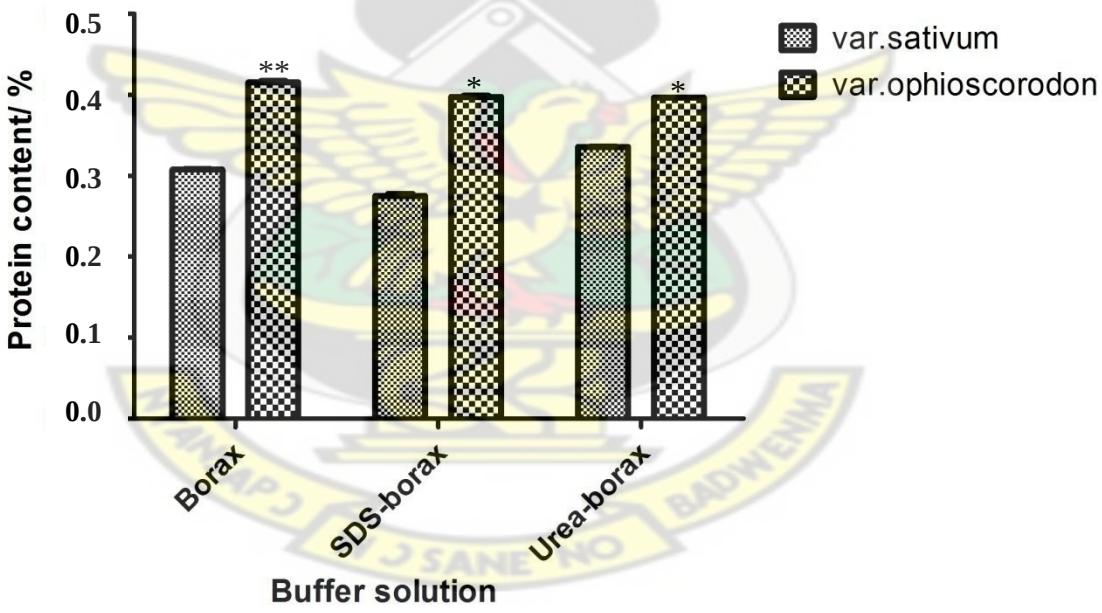


Figure 7. Percentage protein in three buffer extracts of two garlic varieties

The extracts of *var. ophioscorodon*, the locally grown garlic variety was found to contain significantly higher levels ($P<0.005$) of protein in all extracts as compared to the

imported variety. The extracts of *var. ophioscorodon* in borax buffer contained the highest protein levels ($0.415 \pm 1.95x10^{-5}$%). This was followed by the extracts in SDS-borax and urea-borax buffers which were found to contain $0.397 \pm 1.53x10^{-5}$ % and $0.396 \pm 4.12x10^{-6}$ % respectively.

The lowest percentage protein content was observed for *var. sativum* extracts in SDS-borax buffer at $0.275 \pm 2.52x10^{-5}$ %. This was followed by the extracts in borax buffer and urea-borax buffer at $0.308 \pm 8.93x10^{-6}$ % and $0.336 \pm 6.13x10^{-6}$ % respectively. These results were significantly different at a 95% confidence level.

4.2 **Total Phenolic Content of *Allium sativum var sativum* compared to *var ophioscorodon***

Phenolic substances can also be quantified by measuring absorbance at 280 nm. Again the extracts of *var. ophioscorodon* were found to contain significantly higher levels of total phenols than the extracts of *var. sativum* at $P<0.05$.

The *var. ophioscorodon* extracts in borax buffer showed the highest values for total phenols (29.46 ± 0.002 AU), this was followed by the extracts in urea-borax (25.11 ± 0.012) and SDS-borax (23.00 ± 0.013).

The lowest values for total phenols were recorded for *var. sativum* extracts in SDS-borax (11.98 ± 0.007) followed by extracts in urea-borax (11.91 ± 0.015) and borax buffer (10.88 ± 0.005) respectively.

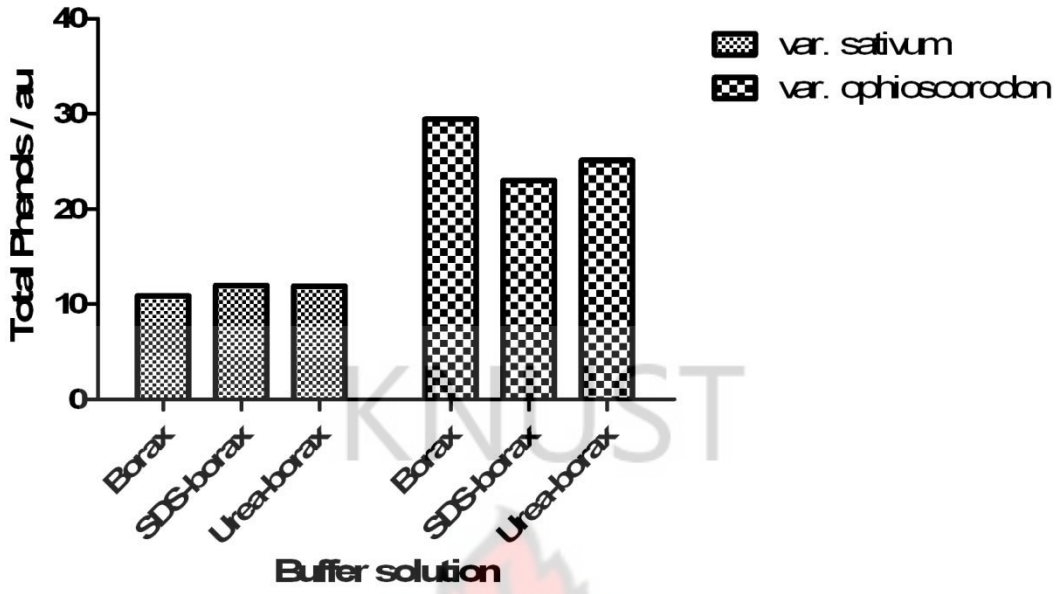


Figure 8. Total phenolic content of buffer extracts of the two garlic varieties in absorbance units (au)

The results show that the buffer extracts of the locally grown variety is a much richer source of bioactive compounds than its imported counterpart. This local variety is however not often patronized in Ghanaian kitchens due to its very pungent odor and small sizes which make it very difficult to prepare and include in cooked meals. The buffers used for extraction did not follow any discernable pattern with respect to total phenolic content.

4.3 **Titrations and absorbance measurements**

It was observed that the intensity of the absorption at 281 nm was a function of both the concentration of garlic extract and cupric-nitrite. Figure 9 shows the titration curves of

the Cu-Garlic extract complex formation for extracts of *Allium sativum var.sativum* (solid line) and *var. ophioscorodon* (dotted line) in borax buffer.

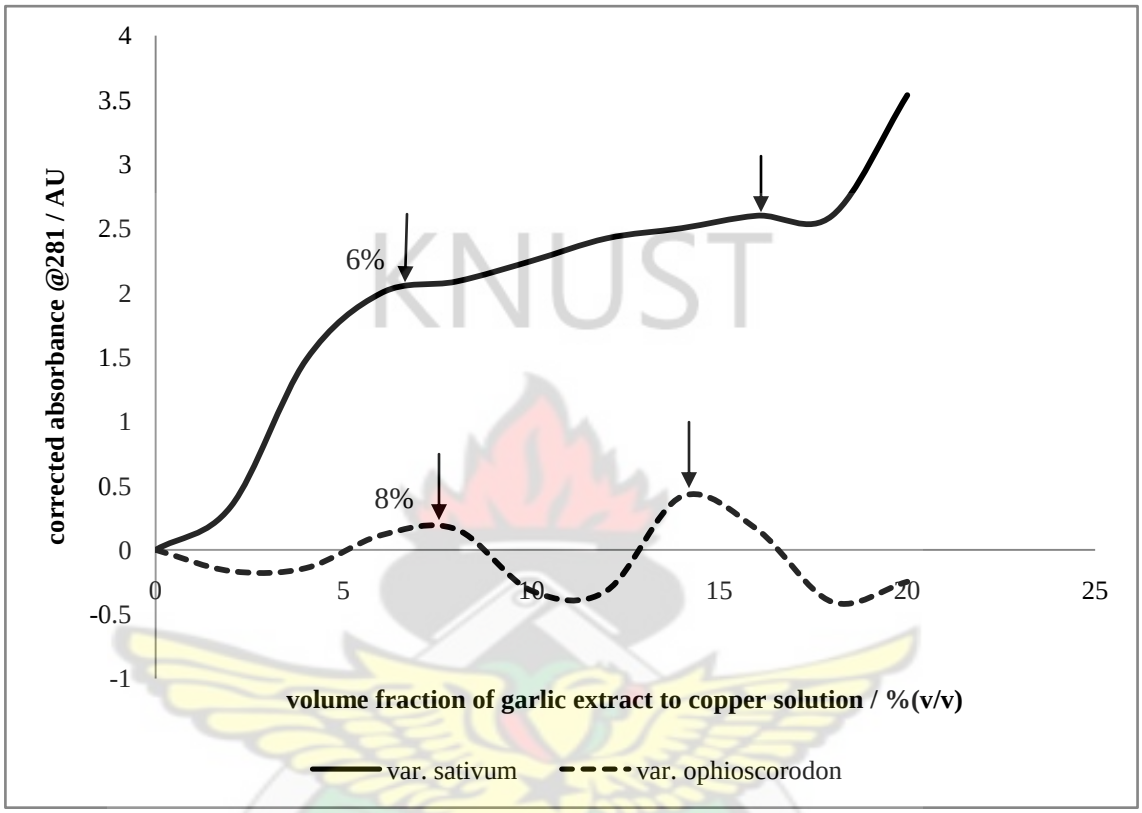


Figure 9. Titration curves of garlic extracts and Cu(II) in borax buffer.

The reaction curves showed a stepwise formation of copper complex with the garlic extracts. The crests along the curves were indicative of a complex reaction taking place (Harvey, 2000). The results showed that for *var. sativum*, the onset of complexation began at a volume fraction of 6 %(v/v). The curve for *var. ophioscorodon* on the other hand showed the onset of complex formation at a volume fraction of 8 %(v/v).

The first derivative curves of the reaction involving the garlic extracts and copper solution is shown in Figure 10. Derivative methods are particularly well suited for

locating end points in multiprotic and multicomponent systems, in which the use of separate visual indicators for each end point is impractical. The precision with which the end point may be located also makes derivative methods attractive for the analysis of samples with poorly defined normal titration curves (Job, 1928; Harvey, 2000). The first derivative curves for both extracts indicated multiple inflection points. This observation suggested multiple endpoints and a stepwise formation of complexes in the extracts.

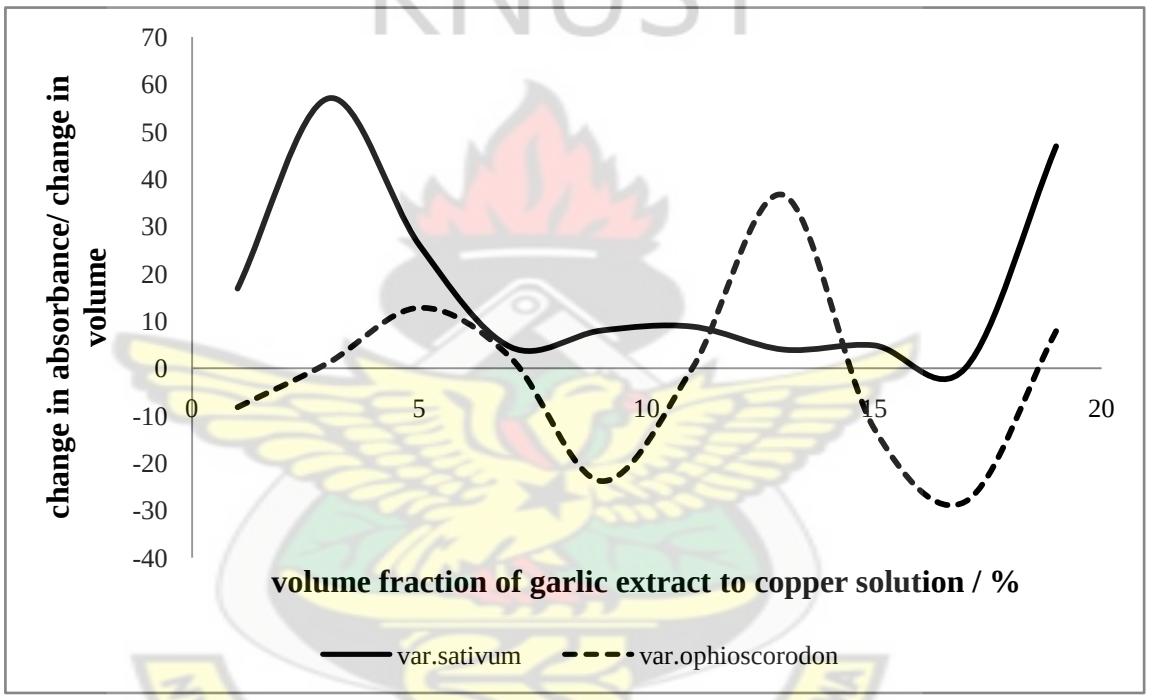


Figure 10. First derivative titration curves of Garlic extracts and Cu(II) in borax buffer.

The change in corrected absorbance of the Cu (II) complex versus an increasing volume fraction of garlic extract to copper solution in SDS-Borax buffer is shown in Figure 11. It was observed that the onset of complexation for both *var. sayivum* and *var. ophioscorodon* began at a volume fraction of 4% (v/v). The titration curves were also

found to be analogous, indicating a similar pattern in the stepwise reaction for both extracts in SDS-borax buffer. This similar pattern was evident in the first derivative plot for the reaction curves in SDS-borax buffer shown in Figure 11 (Harvey, 2000).

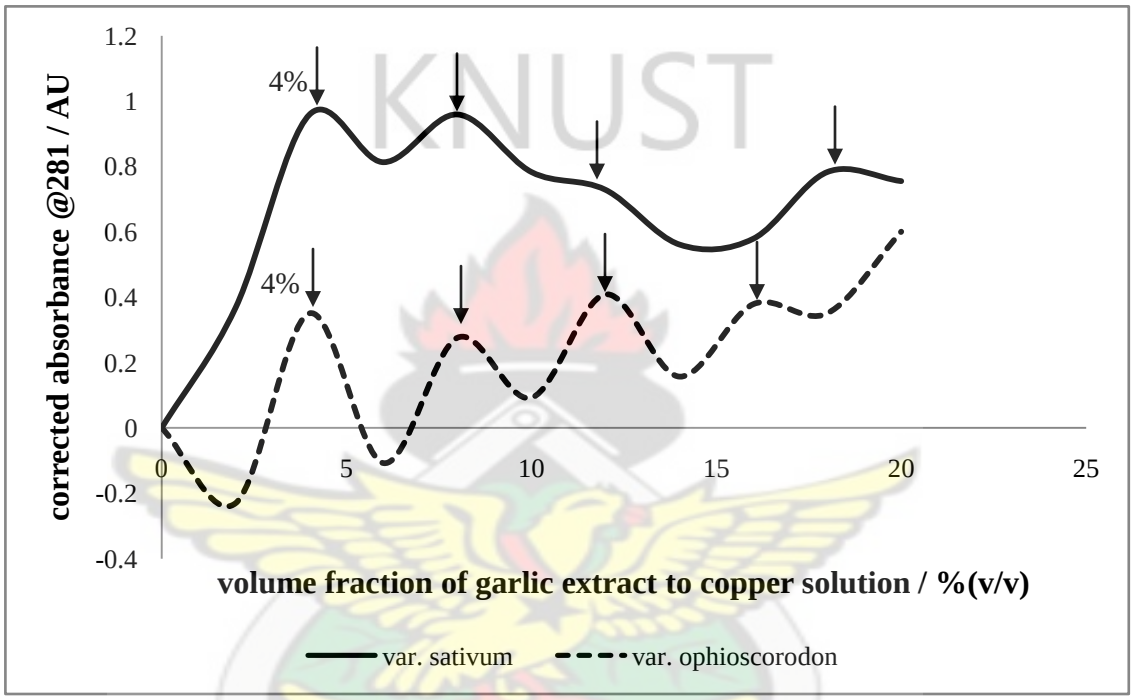


Figure 11. Titration curves of Garlic extract and Cu(II) in SDS-Borax buffer

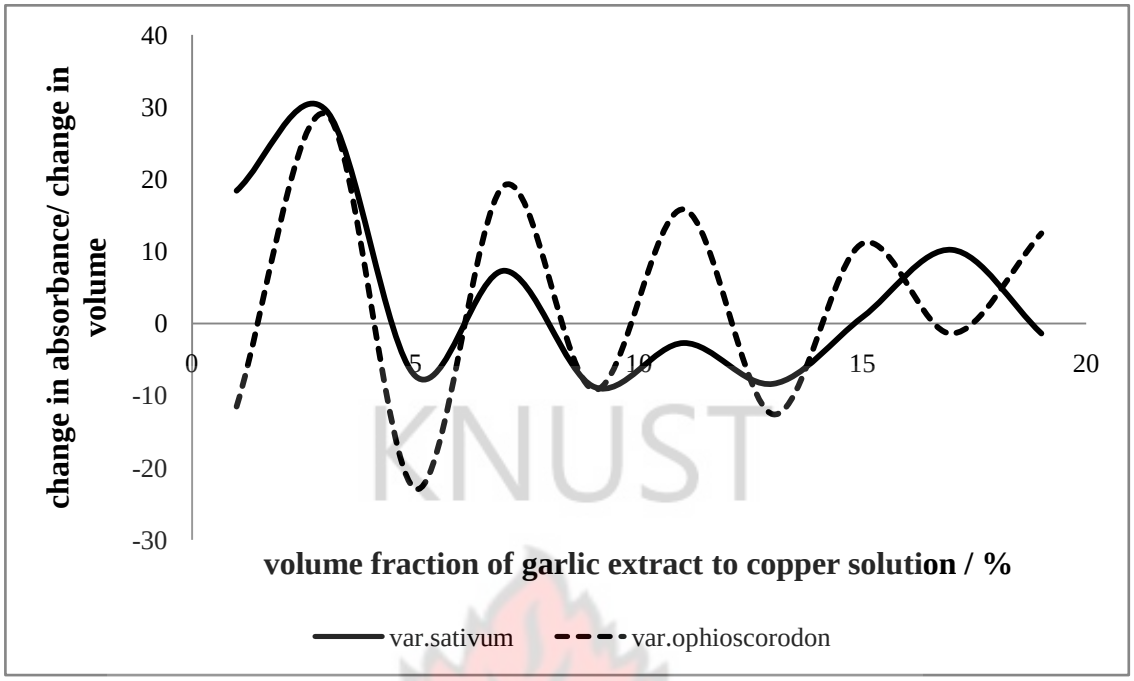


Figure 12. First derivative titration curves of garlic extracts and Cu(II) in SDS-borax buffer

In Figure 13, the titration curves showing the change in volume fraction of garlic extract to copper solution in urea-borax buffer against corrected absorbance showed two sigmoid curves with similar patterns for both garlic varieties. There was evidence of complexation at a volume ratio of 2% for both extracts. The first derivative plot for this reaction in Figure 13 also showed a similar pattern for complexation in both extracts in urea-borax buffer indicative of a comparable reaction mechanism (Harvey, 2000).

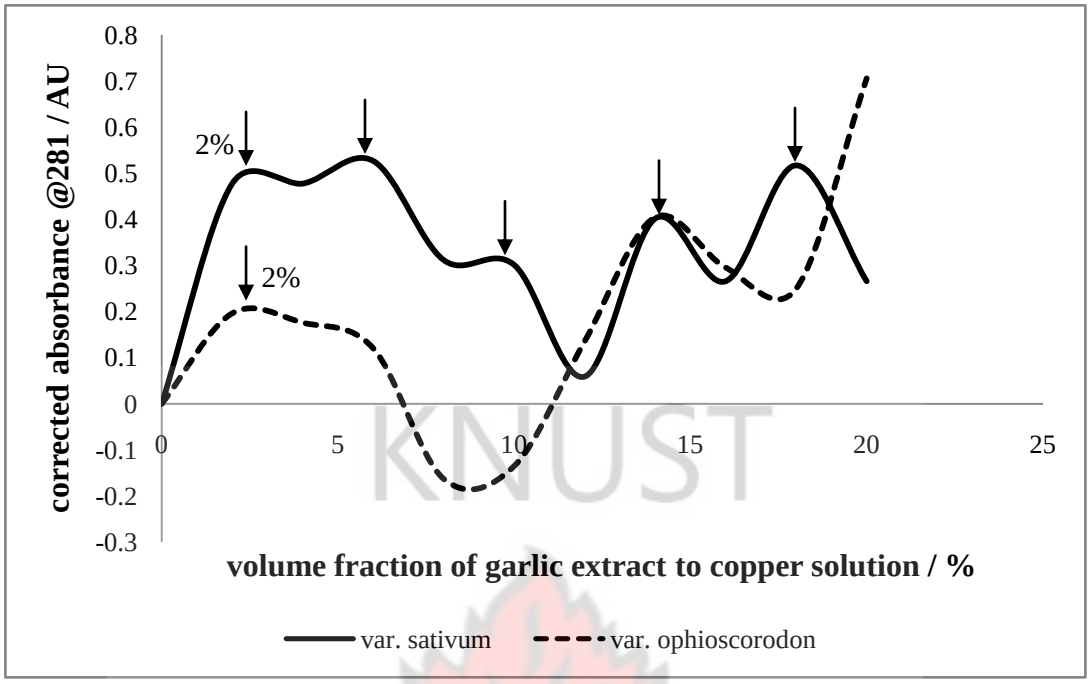


Figure 13. Titration curves of garlic extracts and Cu(II) in Urea-borax buffer.

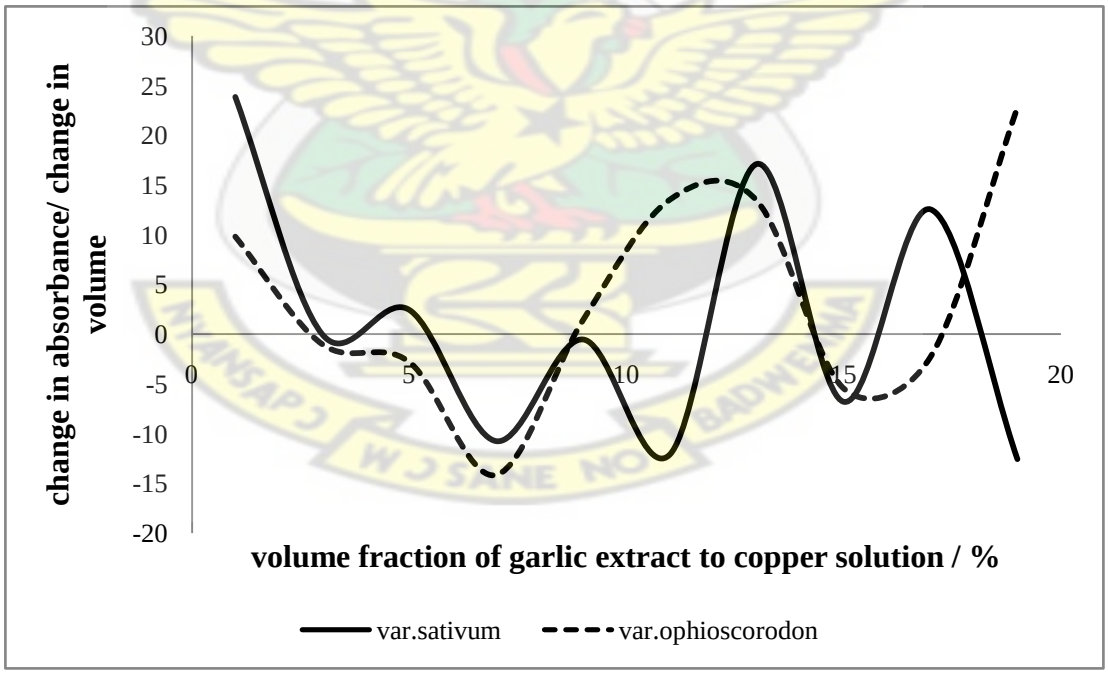
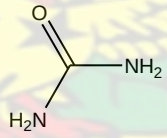


Figure 14. First derivative titration curves of garlic extracts and Cu(II) in Urea-borax buffer.

The titration curves obtained for the garlic extracts and copper solution were characteristic of the type of buffer solution used. The extracts in urea-borax buffer formed complexes at a much lower volume fraction (2% v/v), followed by extracts in SDS-borax buffer (4% v/v) and borax buffer (6-8% v/v). These results were consistent with data obtained by Sarfo *et al.,* (2012) who suggested possible interaction between SDS and urea shown in Figure 15 with the Cu(II) ions and garlic extracts.

Sodium dodecyl sulfate



urea

Figure 15. (a) Structure of Sodium Dodecyl Sulfate (SDS) and (b) Urea (ChemDraw 2005).

4.4 **UV-visible spectra of garlic extracts**

Previous studies have shown that pure flavonoids form stable complexes with metal ions (Torreggiani *et al.,* 2005). The formation of complexes of crude extracts of garlic with copper (II) ions were confirmed and monitored by UV-visible spectroscopy. A green

solution characteristic of many copper complexes was observed when the garlic extracts were treated with the prepared cupric–nitrite solution.

Most phenolic compounds such as flavones and flavonols, exhibit two major absorption bands in the ultraviolet – visible region, the first band is usually seen in the 300-500 nm range. In flavonoids, this represents the B ring absorption (cinnamoyl system). A second band usually observed below 280 nm is primarily due to a benzoyl system as seen in ring A of the flavonoid structure (Markham, 1989). The uv/vis spectra of all the garlic extracts showed similar bands suggesting the presence of flavones or flavonols.

Allium sativum var. sativum extracts in borax buffer showed two major absorption bands shown in Figure 16, a maximum absorption band at 274 nm (Band II) and a second weak band at 366 nm (Band I). The borax extract of *var. ophioscorodon* showed a single absorption maximum at 274 nm identical to the one observed for var. sativum but its intensity in absorbance was 38% greater.

In SDS-borax buffer, the absorption maxima were located at 276 nm and 366 nm for *var. sativum* and 276 nm for *var. ophioscorodon.* The extracts of *var. ophioscorodon* again showed 26 % increase in intensity over the extracts of *var. sativum*. Garlic extracts in urea-borax buffer also exhibited two absorption maxima for both *var. sativum* and *var.ophioscorodon*; Band I at 366 nm and Band II at 274 nm. The absorbances of the bands observed for *var.ophioscorodon* were 40 % (Band I) and 28 % (Band II) more intense than that of *var.sativum*.

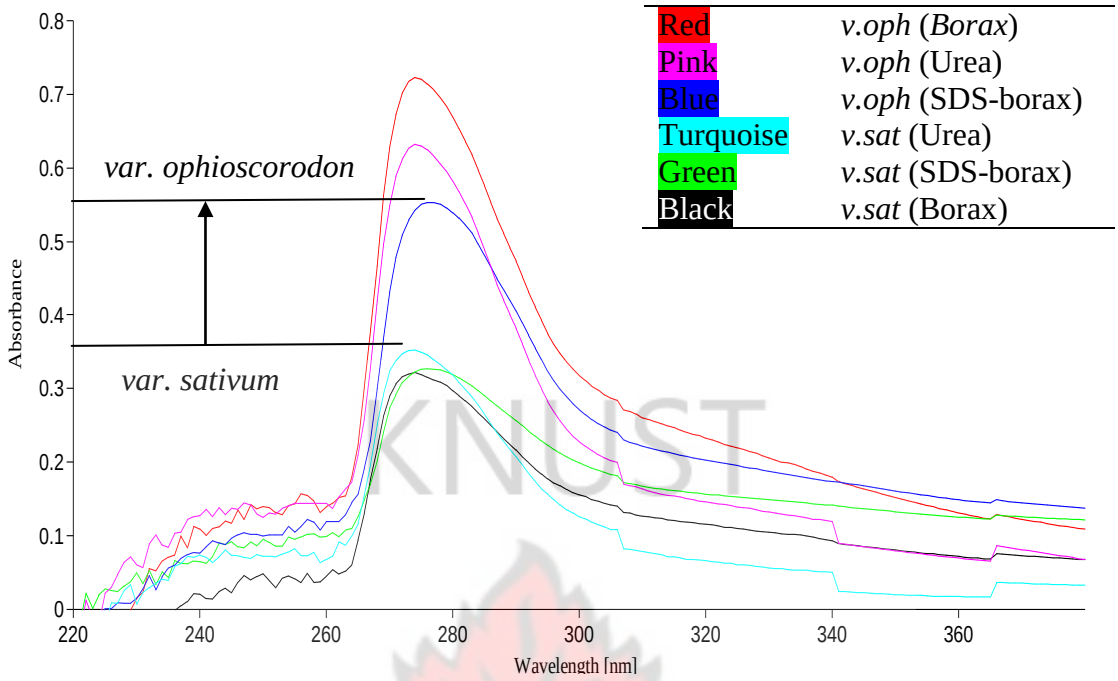


Figure 16. Uv-vis spectra of garlic extracts in buffer solutions

The general increase in intensities between the extracts in all three buffers suggested the presence of a greater amount of phenolic compounds in *var. ophioscorodon* than in *var. sativum* (Cuvelier *et al*., 1996).

4.5 **UV-visible spectra of copper complexes**

Two major absorption bands at 278 nm due to the copper component and 358 nm attributed to the nitrite group were observed. Another peak was detected at 811 nm characteristic of copper salt solutions.

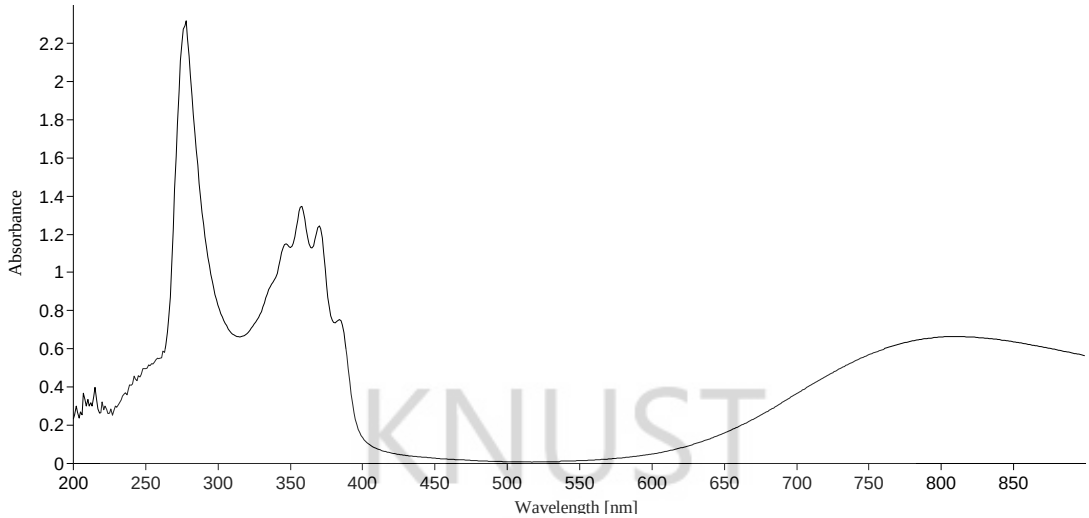


Figure 17. Uv-vis spectra of cupric-nitrite solution in acidic medium

When the cupric-nitrite solution was allowed to react with the garlic extracts, it resulted in the disappearance of Band I (366nm) for all the extracts. This suggested the interaction of Cu(II) with the hydroxyl groups at ring B as shown in Figure 18.

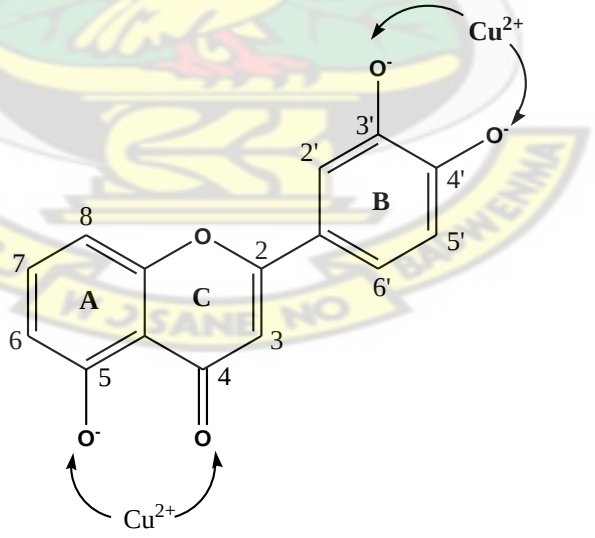


Figure 18. Interaction of Cu(II) with the functional groups on ring A and B of the general flavonoid structure

In contrast to the observation made with Band I for all the extracts, Band II on the other hand was broad. Upon addition of Cu(II), this band showed a remarkable increase in intensity. This was attributed to interaction of Cu(II) with benzoyl system of ring A as indicated in Figure 20 (Malesev and Kontic, 2007). These results were similar to the observations made by others (De Giovani and De Souza, 2005; Escandar and Sala, 1991.) suggesting that the band shift was caused by interaction of the Cu(II) ion with the condensed ring of the flavanone positions 4 and 5.

In borax buffer, band II shifted from 274 nm to 282 nm for *var. sativum* extracts and 274 nm to 281 nm for *var. ophioscorodon* (Figure 19).

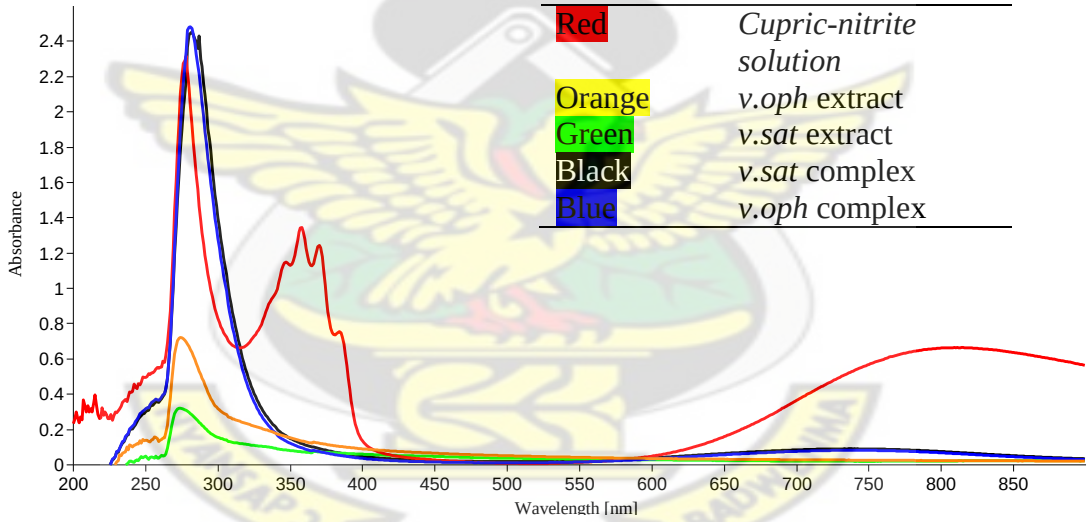


Figure 19. Uv-vis spectra of garlic extracts in borax buffer, cupric-nitrite solution and copper complexes

In SDS-borax buffer, band II shifted from 276 nm to 281 nm for var sativum and 276 nm to 280 nm for var. ohioscorodon (Figure 20)

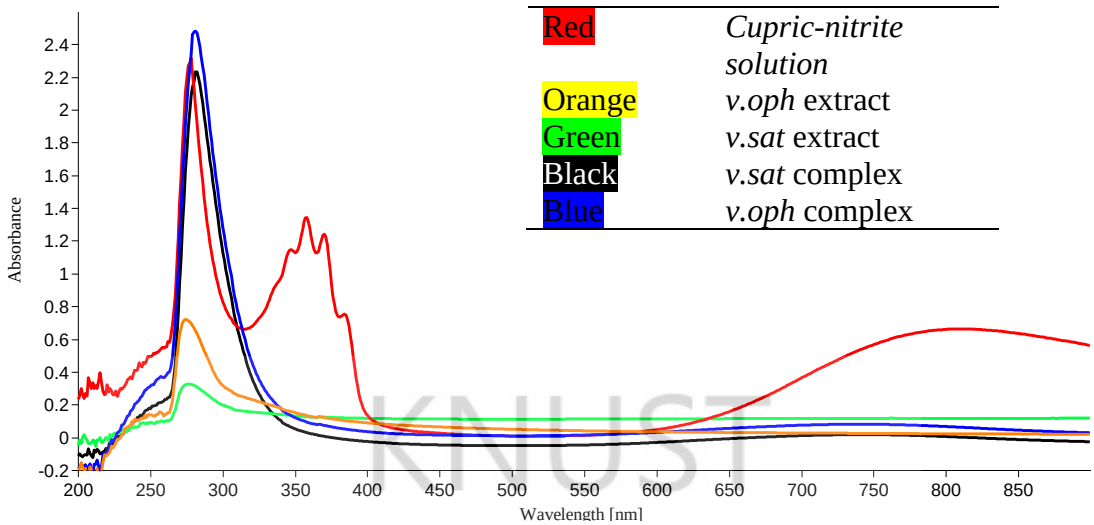


Figure 20. Uv-vis spectra of garlic extracts in SDS-borax buffer, cupric-nitrite solution and copper complexes

It was observed in Figure 23 that for both var. sativum and var. ophioscorodon extracts in urea-borax buffer, band II shifted from 274 nm in the extract to 279 nm in the metal complex. This was also due to interaction with Cu(II) ions (Sarfo *et al.,* 2012).

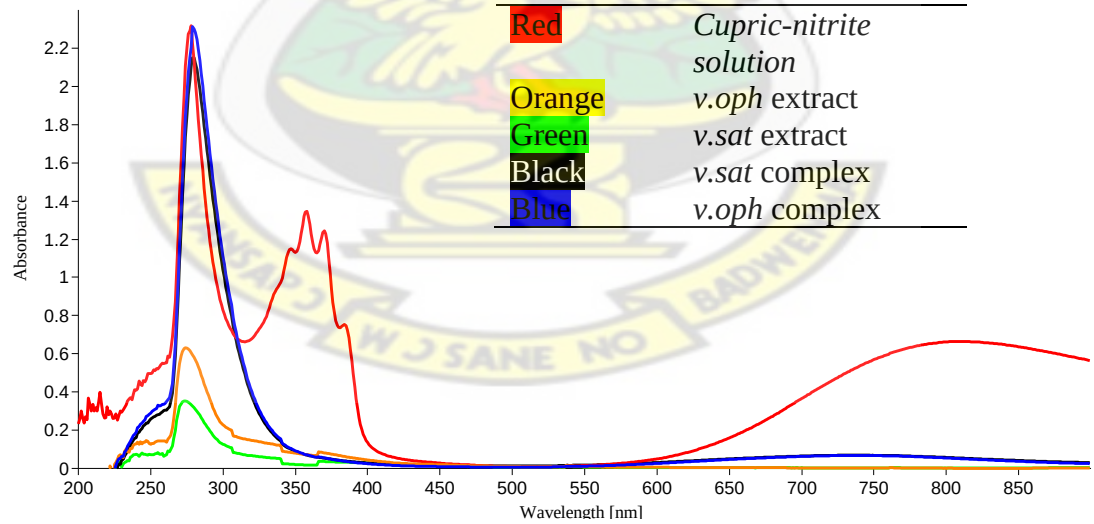


Figure 21. Uv-vis spectra of garlic extracts in urea-borax buffer, cupric-nitrite solution and copper complexes

4.6 **Infra Red characterization of garlic extracts and their complexes**

The spectra of both the extracts and the complexes were taken in order to determine the functional group transformations in the extracts as a result of bonding to the metal ion. This also confirmed the bonding of the extracts to the copper ions and suggested the possible bonding atoms of the extracts to the copper ions. Identical spectra were observed for all the extracts and their complexes irrespective of the garlic variety and the buffer used for extraction. For easy comparison the stretching frequencies in both spectra for the extracts and their complexes are indicated in the corresponding tables. The fingerprint region of the IR spectra ranging from 1500 cm^{-1} and below was compared to a spectral database and a match was found for the compound quercetin, which is a flavonoid. This result implicated the involvement of the flavonol-like compounds involved in the coordination of copper.

4.6.1 IR shifts of garlic extracts in borax buffer upon copper coordination

The extracts in borax buffer had more prominent, strong and sharp bands than the complex. The band at 3448.12 cm^{-1} was attributed to the O-H stretching vibrations of coordinated water. Another band at 1305.76 cm^{-1} for ν C-O in the extracts was shifted to 1384.37 cm^{-1} in the complex. This was attributed to the involvement of a carboxylate group coordinated with a Cu^{2+} ion (Zugle *et al., 2008*). A band at 1282.61 cm-1 for ν C-O-C in the extracts also shifted to 1207.05 cm^{-1}, this was attributed to coordination through an ester bond due to electrostatic attraction (Zhang *et al.,* 2009).

Table 1. Wave numbers (cm^{-1}) and assignments of the main IR bands of the Cu(II)–Garlic extract system in Borax buffer

Assignments	Extracts /cm^{-1}	Complex /cm^{-1}
ν C-OH	1116.48	
ν C-O-C	1282.61	1207.05
ν C-O	1305.76	1384.37
ν C=O		1724.97
ν OH	3448.12	

4.6.2 IR shifts of garlic extracts in SDS-borax buffer upon copper coordination

The extracts in SDS-borax buffer also had more prominent, strong and sharp bands than its complex with Copper. The band at 1395.94 cm^{-1} for ν C-O in the extracts was shifted to 1384.97 cm^{-1} in the complex. This was also attributed to the involvement of a carboxylate group coordinated with the Cu^{2+} ion (Zugle *et al., 2008*). The extracts showed two more band at 1246.66 cm^{-1} and 1634.79 cm^{-1}, which were diminished in the complex.

Table 2. Wavenumbers (cm^{-1}) and assignments of the main IR bands of the Cu(II)–Garlic extract systems in SDS-Borax buffer

Assignments	Extracts /cm^{-1}	Complex /cm^{-1}
ν C-O-C	1246.66	
ν C-O	1395.94	1384.97
ν C=O	1634.79	

4.6.3 IR shifts of garlic extracts in urea-borax buffer upon copper coordination

The IR spectra for extracts in urea-borax showed very broad bands due to coordination of water molecules. The extracts however, showed a prominent band at 1161.36 cm-1 for ν C-OH. This band shifted to 1118.70 cm^{-1} in the complex indicating an interaction of the hydroxyls groups in the extracts with the copper ions (Zugle *et al., 2008*).

Table 3. Wavenumbers (cm^{-1}) and assignments of the main IR bands of the Cu(II)–Garlic extract systems in Urea-Borax buffer

Assignments	Extracts /cm^{-1}	Complex /cm^{-1}
ν C-OH	1161.36	1118.70
ν C-O		1450.78

Metal colloid suspensions have unlimited application in the food industry, notably its importance in food organoleptic properties, food safety and human health. The results showed that the garlic extracts contained predominantly flavonol-like compounds which could be refined and incorporated into food formulations or preparations. The capability of these flavonol compounds to form complexes with Cu(II) ions could be employed in the preparation of metal colloid suspensions These flavonol compounds, also referred to as 'nutraceuticals' because of their health benefits (Tapas *et al.,* 2008) could enhance the application of metal colloid suspension.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 **Conclusion**

Allium sativum var. ophioscorodon, the locally grown variety of garlic was found to contain significantly higher total protein and total phenols than the predominantly imported variety at $P<0.05$. This was irrespective of the type of buffer used for extraction. The extracts of *var. ophioscorodon* contained total protein and phenols ranging between 0.396-0.415 % and 23.00-29.46 au respectively. The extracts of *var. sativum* on the other hand contained total protein and phenols of 0.275-0.336 % and 10.88-11.98 au.

The uv-visible spectra of the garlic extracts showed two absorption bands at 307-365 nm for band I and 274-276 nm for band II. Band I of the spectra for the extract was diminished after coordination with the copper ions. Treatment of garlic extracts with copper salt solution produced a green precipitate characteristic of copper complexes. The formation of complexes was found to occur in a stepwise manner as shown by multiple inflection points along the titration curves of copper salt solution against varying concentrations of garlic extracts.

The data showed that flavonol-like compounds in the garlic extracts were involved in complexation with the copper ions. The IR spectra showed interaction of copper ions with functional groups in the garlic extracts. Band shifts from 3448.12 cm^{-1} indicated the presence of coordinated water. Another band shift from 1305.76 cm-1 to 1384.94

cm-1 in borax buffer, 1395.94 cm-1 to 1384.97cm^{-1} in SDS-borax and 1450.78 cm^{-1} in urea-borax showed the involvement of a carboxylate group in coordinating copper ions. A hydroxyl group and an ester group were also shown to be involved in complexation by band shift between 1161.36 cm^{-1} to 1118.70 cm^{-1} for extracts in urea-borax buffer and 1282 cm^{-1} and 1207.05 cm^{-1} for extracts in borax buffer.

Chelation of crude garlic extract with copper ions presents an alternative means of isolating flavonoids from garlic with very minimal pre-processing. These results are important because flavonoids can be extracted from a range of food products with minimal processing.

Based on the data obtained a proposed structure for the complex formed from the garlic extracts and copper (II) ions is shown in Figure 22.

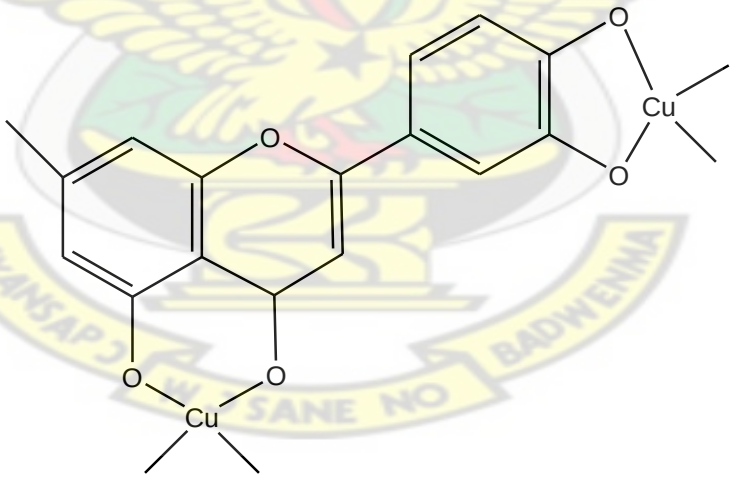


Figure 22. Proposed structure of the Cu (II) - garlic complex

5.2 **RECOMMENDATIONS**

Further studies could employ transmission electron microscopy to determine the relative sizes of the copper particles based on the reaction parameters. These particles could find useful applications in the manufacturing of food packages and bactericides. For such metal colloid suspensions to be incorporated directly into food products as food additives, toxicology studies must be carried out to determine any acute harmful effects that the metal suspensions may have on biological systems.

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APPENDICES

Appendix A: Absorbtion maxima of garlic extracts and change in intensities

	Alium sativum var. sativum		*Alium sativum var. ophioscorodon*		
Compound	**λ_{max} (nm)**	**Abs**	**λ_{max} (nm)**	**Abs**	**Δ Intensity**
Borax extract	274	0.3220	274	0.7231	+38 %
	366	0.0762			
SDS-borax extract	276	0.3274	276	0.5535	+26 %
	366	0.1284			
Urea-borax extract	274	0.353	274	0.6321	+28 %
	366	0.0372	366	0.0869	+40.1

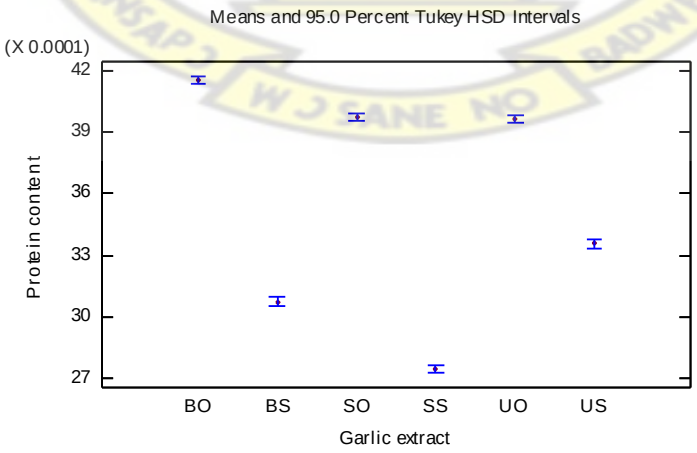
Appendix B: One-Way ANOVA - Protein content by Garlic extract

Dependent variable: Protein content

Factor: Garlic extract

Number of observations: 18

Number of levels: 6



ANOVA Table for Protein content by Garlic extract

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	0.00000484787	5	9.70×10^{-7}	4193.33	0.0000
Within groups	2.77462×10^{-9}	12	2.31×10^{-10}		
Total (Corr.)	0.00000485064	17			

Multiple Range Tests for Protein content by Garlic extract

Method: 95.0 percent Tukey HSD

Garlic extract	*Count*	*Mean*	*Homogeneous Groups*
SS	3	0.00274953	X
BS	3	0.0030755	X
US	3	0.00335829	X
UO	3	0.00396356	X
SO	3	0.00397354	X
BO	3	0.0041525	X

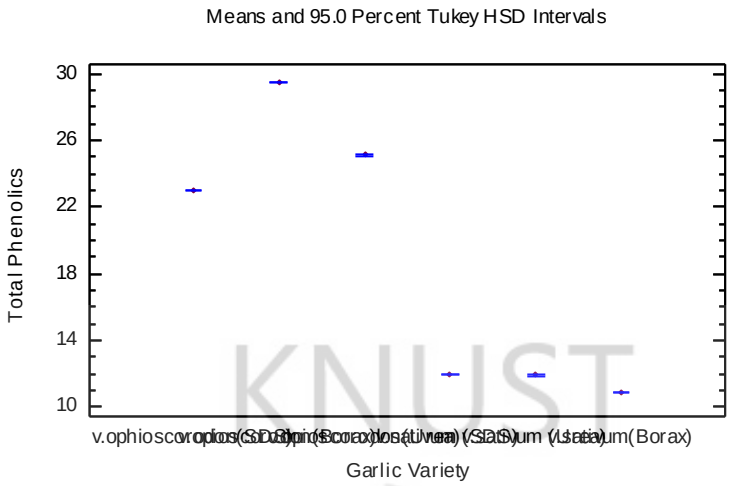
Contrast	*Sig.*	*Difference*	*+/- Limits*
BO - BS	*	0.001077	0.0000417084
BO - SO	*	0.000178959	0.0000417084
BO - SS	*	0.00140297	0.0000417084
BO - UO	*	0.000188943	0.0000417084
BO - US	*	0.000794209	0.0000417084
BS - SO	*	-0.000898041	0.0000417084
BS - SS	*	0.00032597	0.0000417084
BS - UO	*	-0.000888057	0.0000417084
BS - US	*	-0.00028279	0.0000417084
SO - SS	*	0.00122401	0.0000417084
SO - UO		0.00000998367	0.0000417084
SO - US	*	0.00061525	0.0000417084
SS - UO	*	-0.00121403	0.0000417084
SS - US	*	-0.000608761	0.0000417084
UO - US	*	0.000605267	0.0000417084

* denotes a statistically significant difference.

APPENDIX D: One-Way ANOVA - Total Phenolics by Garlic Variety

Dependent variable: Total Phenolics
Factor: Garlic Variety

Number of observations: 18
Number of levels: 6



ANOVA Table for Total Phenolics by Garlic Variety

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	983.35	5	196.67	1925973.92	0.0000
Within groups	0.00122538	12	0.000102115		
Total (Corr.)	983.351	17			

Multiple Range Tests for Total Phenolics by Garlic Variety

Method: 95.0 percent Tukey HSD

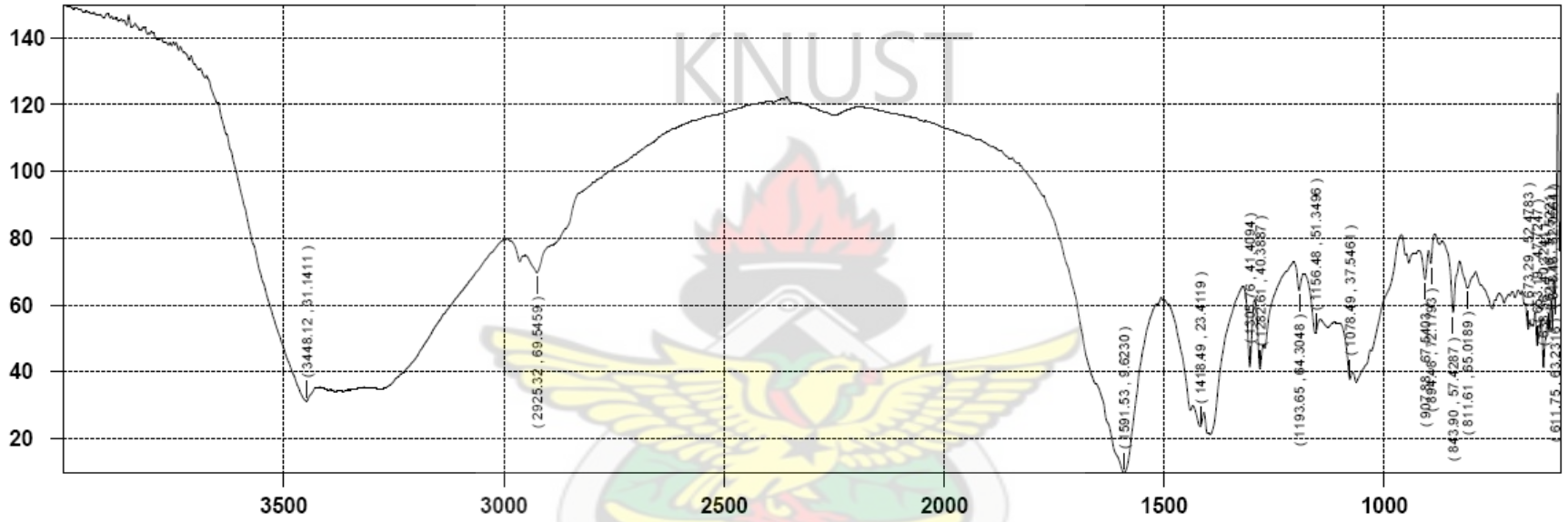
Garlic Variety	*Count*	*Mean*	*Homogeneous Groups*
v.sativum(Borax)	3	10.8795	X
v.sativum (Urea)	3	11.91	X
v.sativum (SDS)	3	11.9778	X
v.ophioscorodon(SDS)	3	22.9978	X
v.opioscorodon(Urea)	3	25.1072	X
v.opioscorodon(Borax)	3	29.4615	X

Contrast	*Sig.*	*Difference*	*+/- Limits*
v.ophioscorodon(SDS) - v.opioscorodon(Borax)	*	-6.46375	0.0277177
v.ophioscorodon(SDS) - v.opioscorodon(Urea)	*	-2.1095	0.0277177
v.ophioscorodon(SDS) - v.sativum (SDS)	*	11.02	0.0277177
v.ophioscorodon(SDS) - v.sativum (Urea)	*	11.0877	0.0277177
v.ophioscorodon(SDS) - v.sativum(Borax)	*	12.1183	0.0277177
v.opioscorodon(Borax) - v.opioscorodon(Urea)	*	4.35425	0.0277177
v.opioscorodon(Borax) - v.sativum (SDS)	*	17.4838	0.0277177
v.opioscorodon(Borax) - v.sativum (Urea)	*	17.5515	0.0277177
v.opioscorodon(Borax) - v.sativum(Borax)	*	18.582	0.0277177
v.opioscorodon(Urea) - v.sativum (SDS)	*	13.1295	0.0277177
v.opioscorodon(Urea) - v.sativum (Urea)	*	13.1972	0.0277177
v.opioscorodon(Urea) - v.sativum(Borax)	*	14.2277	0.0277177
v.sativum (SDS) - v.sativum (Urea)	*	0.06775	0.0277177
v.sativum (SDS) - v.sativum(Borax)	*	1.09825	0.0277177
v.sativum (Urea) - v.sativum(Borax)	*	1.0305	0.0277177

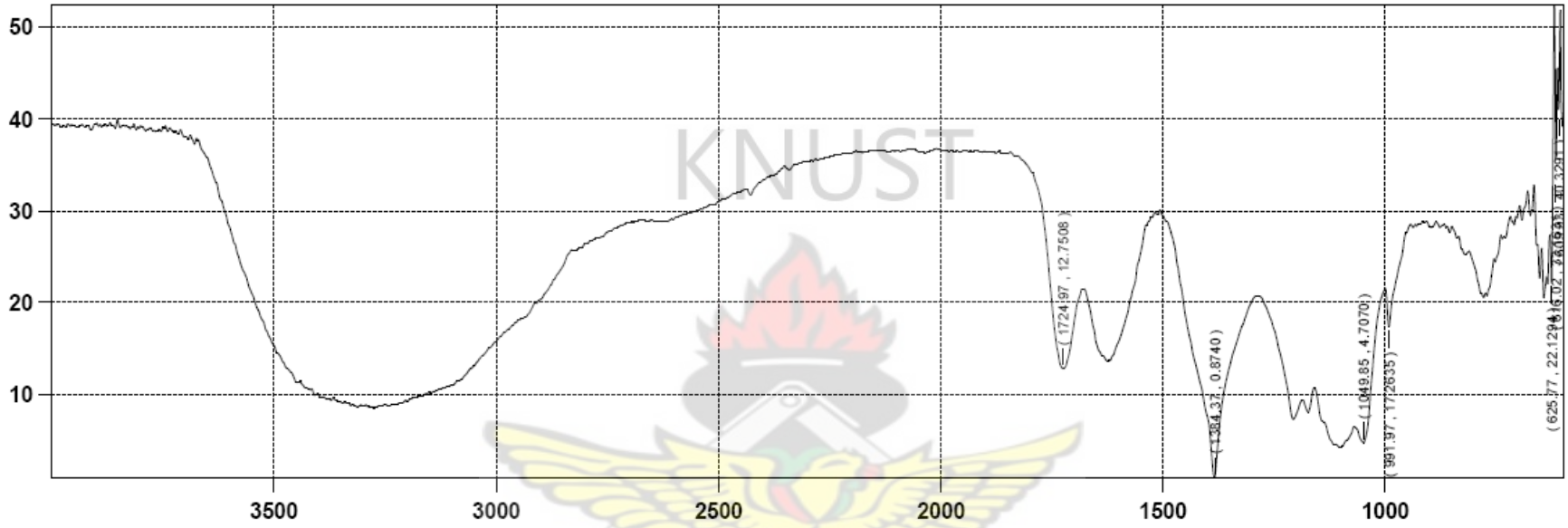
* denotes a statistically significant difference.

Appendix D: FT-IR spectral charts of garlic extracts and copper colloid suspensions

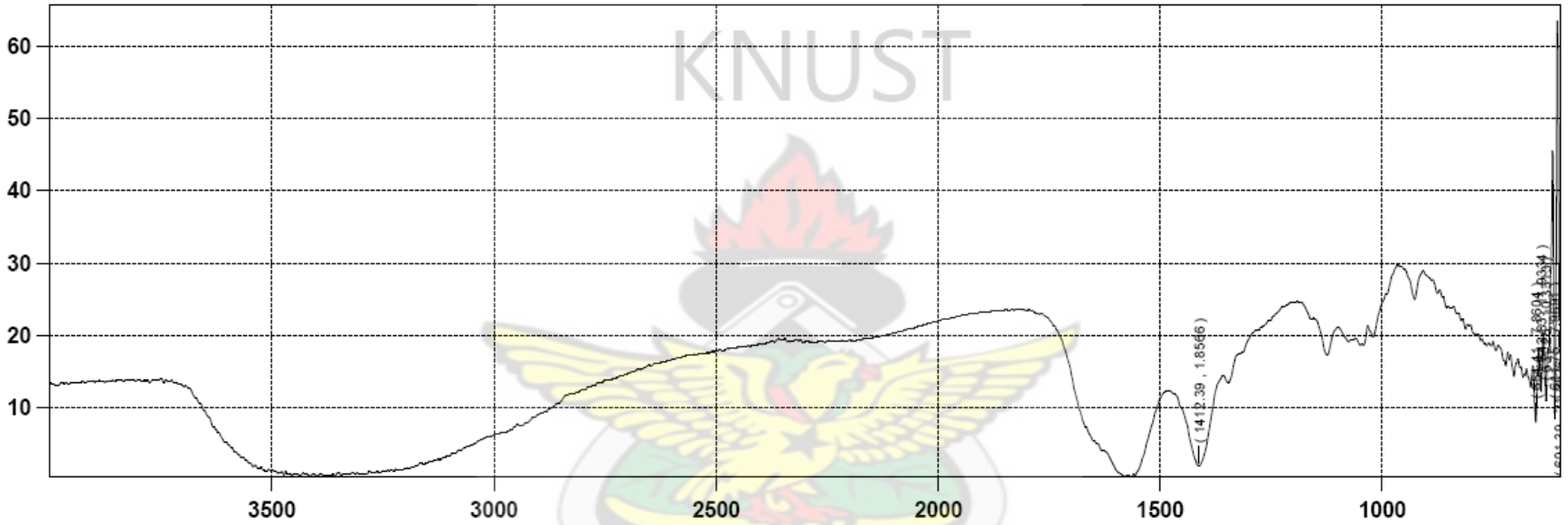
FT-IR spectral chart of garlic extract (*Allium sativum var. sativum)* in Borax buffer



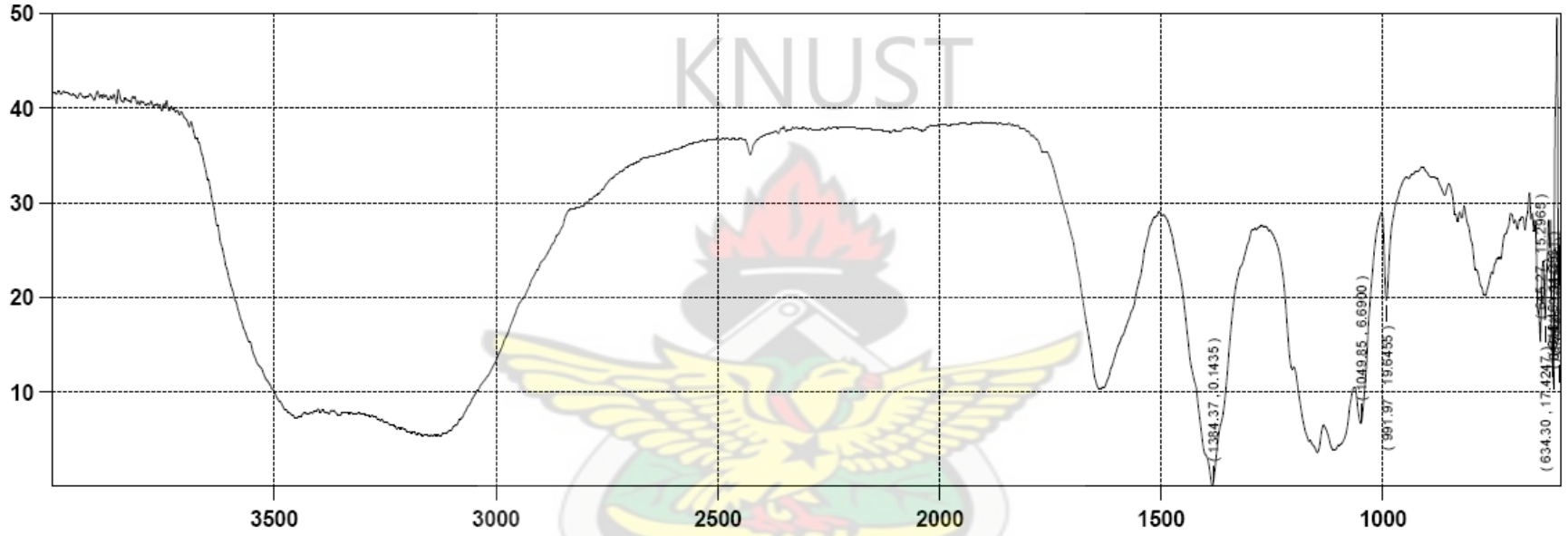
FT-IR spectral chart of Copper – Garlic (*Allium sativum var.sativum)* complexes in Borax buffer



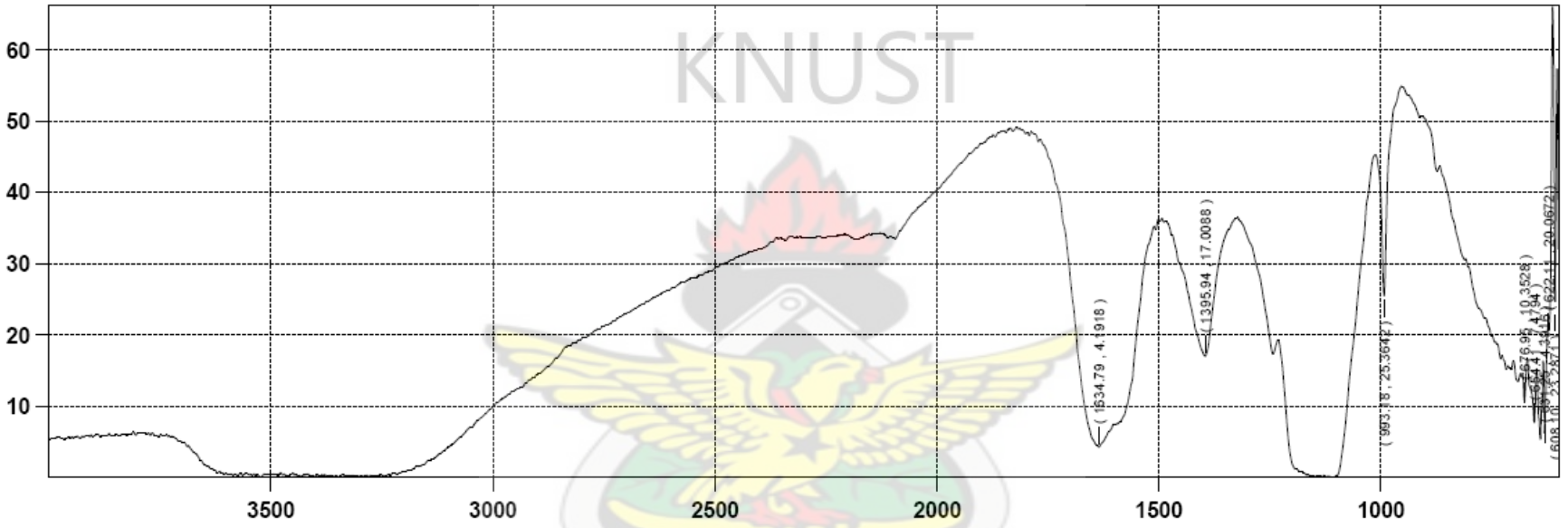
FT-IR spectral chart of garlic extracts (*Allium sativum var.ophioscorodon)* in Borax buffer.



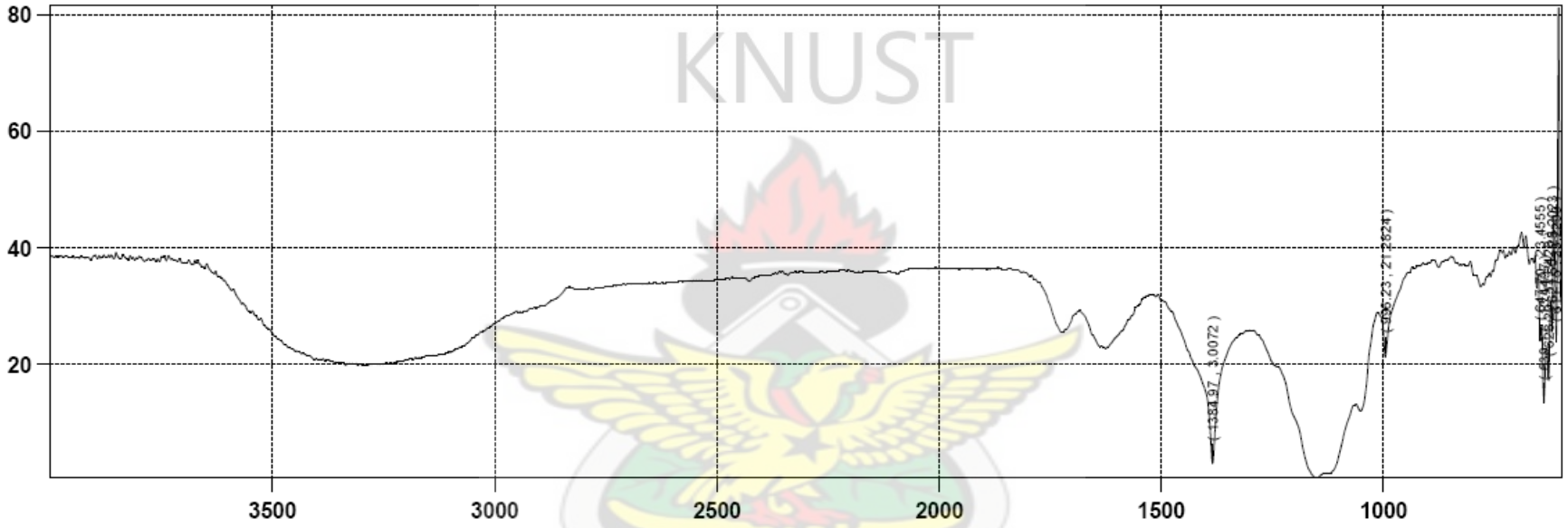
FT-IR spectral chart of copper – garlic (*Allium sativum var.ophioscorodon*) complex in Borax buffer.



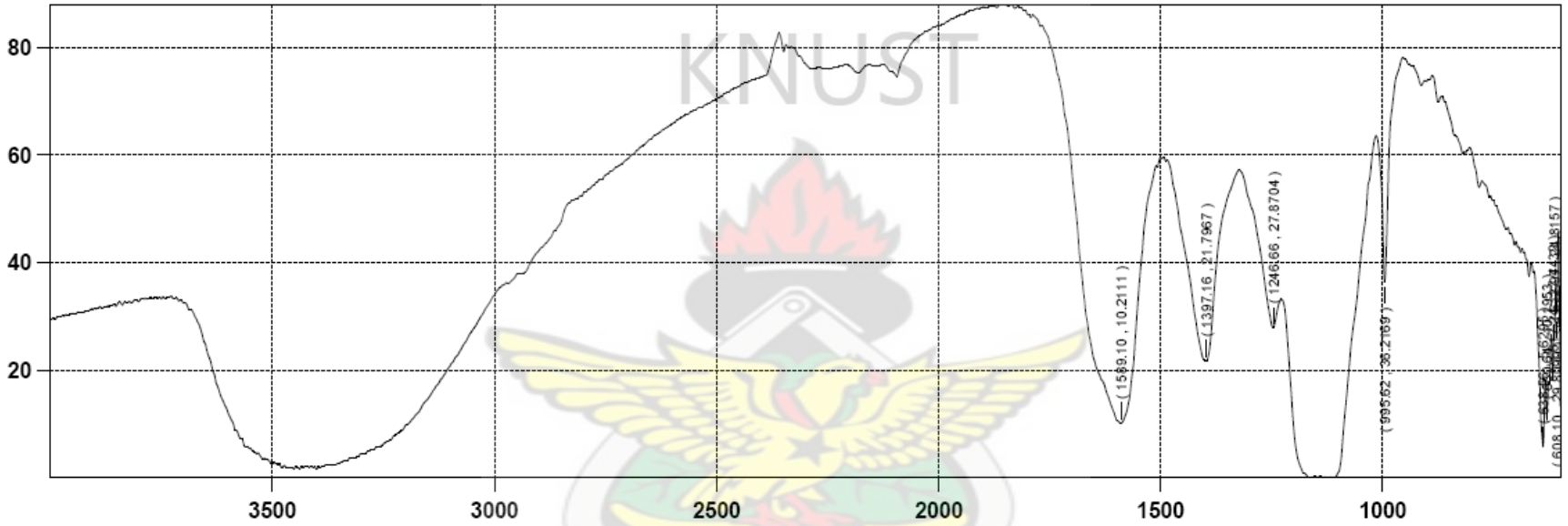
FT-IR spectral chart of garlic extract (*Allium sativum var.sativum*) in SDS-Borax buffer.



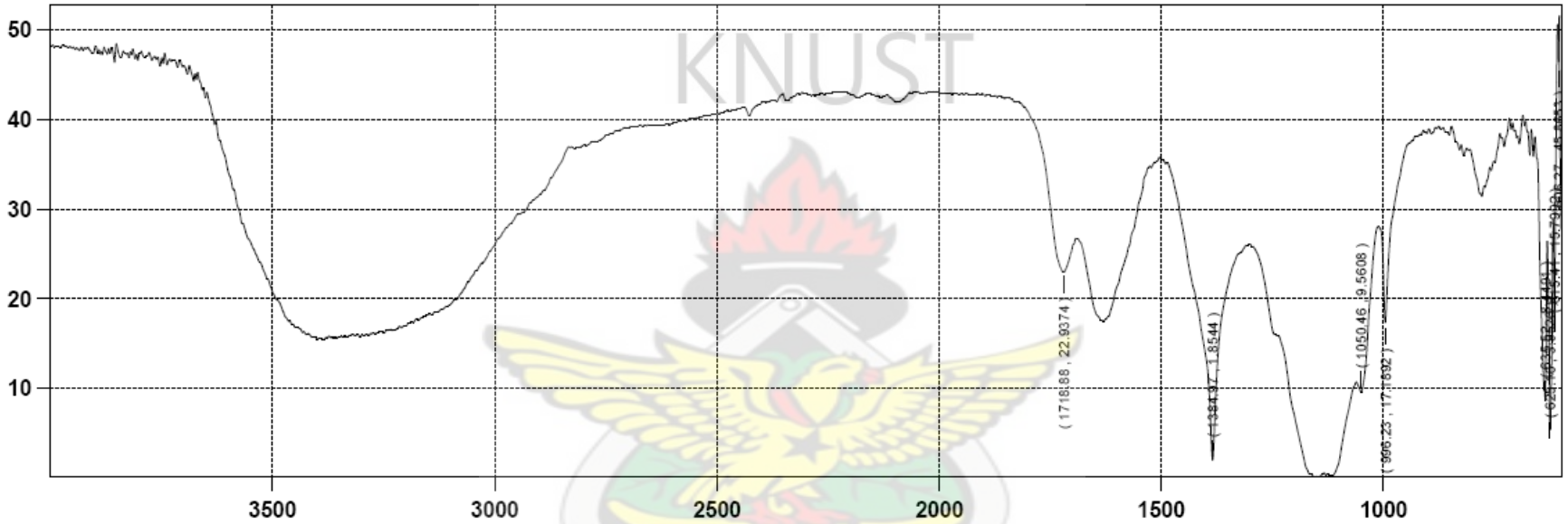
FT-IR spectral chart of copper – garlic (*Allium sativum var.sativum*) in SDS-Borax buffer



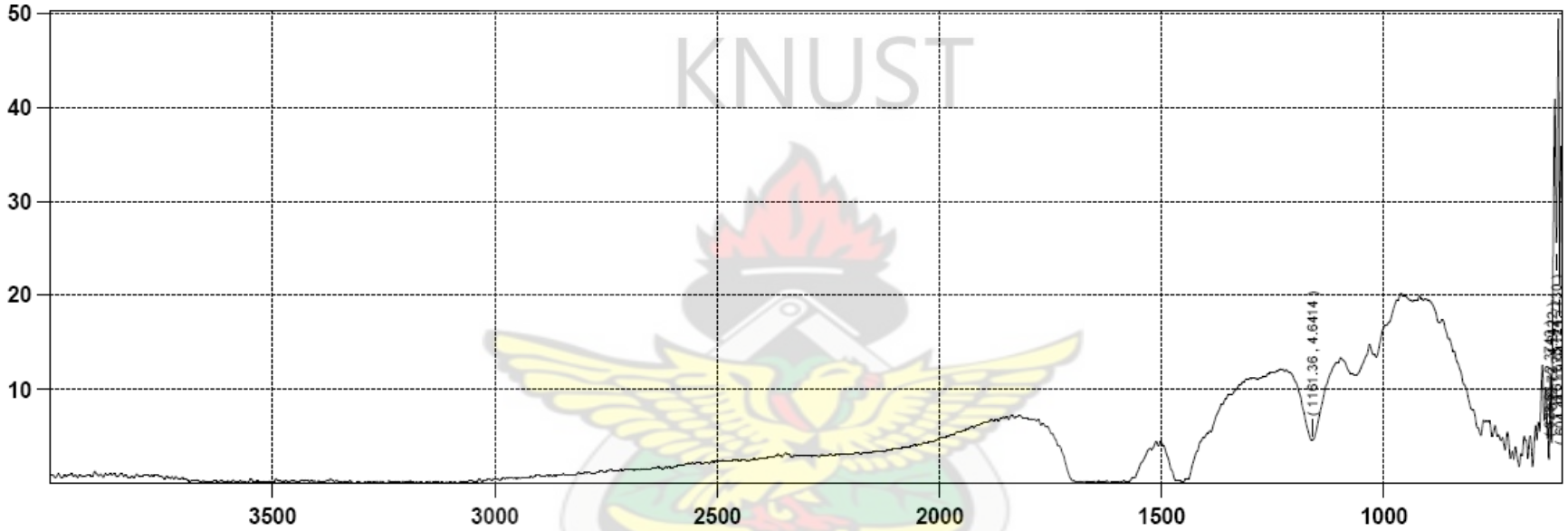
FT-IR spectral chart of garlic extract (*Allium sativum var.ophioscorodon*) in SDS-Borax extract



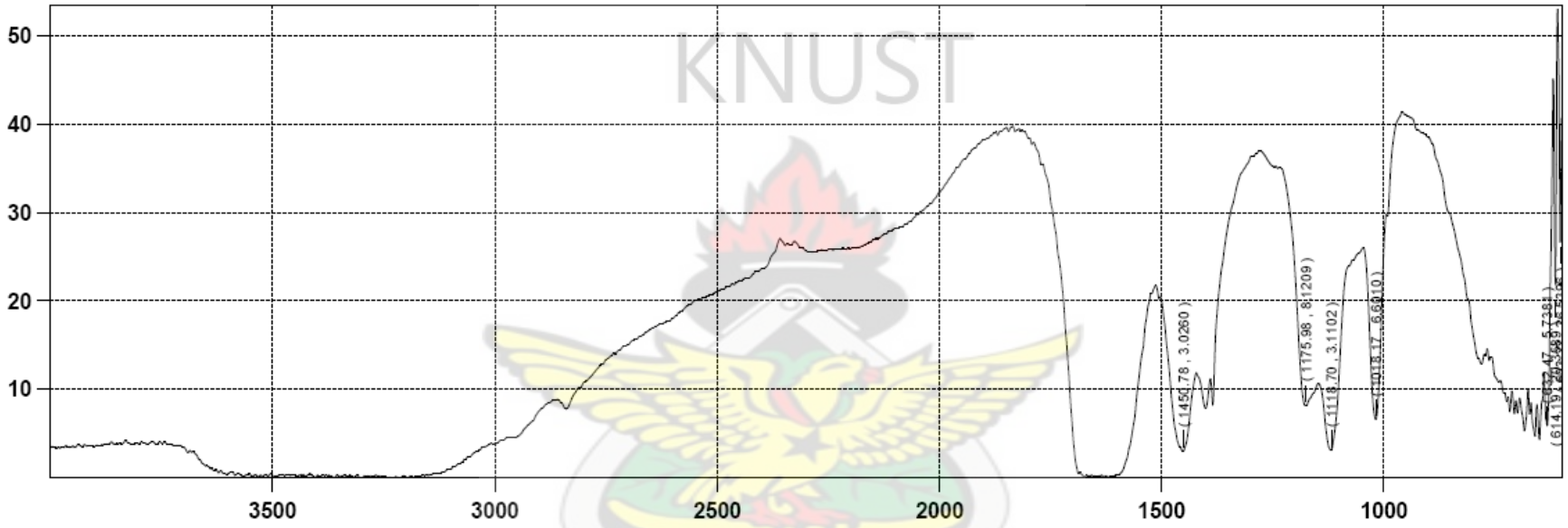
FT-IR spectral chart of copper – garlic (*Allium sativum var.ophioscorodon*) complex in SDS-Borax buffer



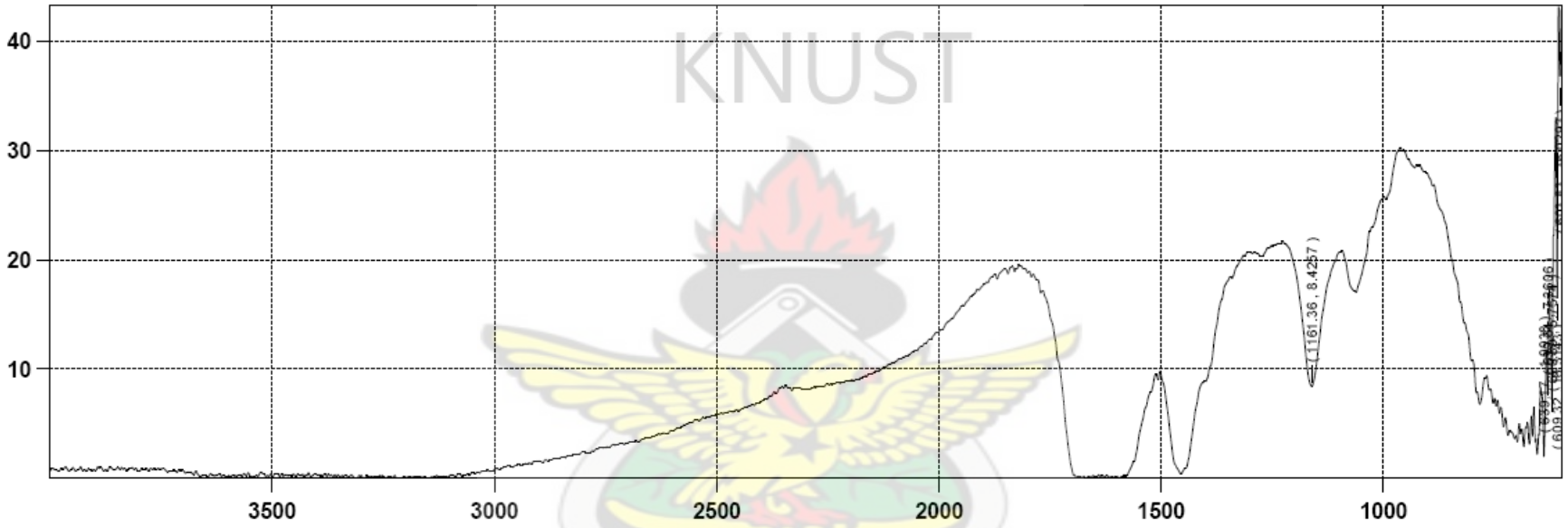
FT-IR spectral chart of garlic extract (*Allium sativum var.sativum*) in Urea-Borax buffer.



FT-IR spectral chart of copper – garlic (*Allium sativum var.sativum*) in Urea-Borax buffer.



FT-IR spectral chart of garlic extract (*Allium sativum var.ophioscorodon*) in Urea-Borax buffer.



FT-IR spectral chart of copper – garlic (*Allium sativum var.ophioscorodon*) complex in Urea – Borax buffer.