ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DETERMINATION OF IRON-, ZINC-, COPPER-, CALCIUM-, AND MAGNESIUM-BINDING BY PROCYANIDINS FROM BIRD-RESISTANT AND NON-BIRD-RESISTANT SORGHUM GRAIN

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TO MY GRANDMOTHER, DINEGHE CHEWAKA

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ABSTRACT

Determination of Iron-, Zinc-, Copper-, Calcium-,
and Magnesium - Binding by Procyanidins from Bird-
Resistant and Non-Bird-Resistant Sorghum Grain.

BY

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Dr. J.D. Reed.

Divalent -Fe, -Zn, -Cu, -Ca, and -Mg binding by Sorghum procyanidins (soluble and insoluble) has been investigated. Both the soluble and insoluble fractions of procyanidins bound Fe, Zn, and Cu, but not Ca and Mg. Binding of the cations by the insoluble procyanidins has been estimated as the difference in binding between the Bird-Resistant (BR) and Non-Bird-Resistant (NBR) NDF. Binding of Fe, Zn, and Cu by procyanidins was found to depend on p^H, cation concentration, amount of fiber, fiber particle size, and presence of competing cations. The Binding of NDF from twenty one lines of sorghum (16 BR and 5 NBR) was determined under optimal conditions.

A new procedure was proposed to study mineral binding by soluble sorghum procyanidins based on dialysis. Cation binding by the soluble procyanidins was determined based on the difference in cation concentration of the control and the cation solution equilibrated with the procyanidin solution (after dialysis). The trace element cations were bound by soluble procyanidins according to the order:
$Fe > Zn \ngtr Cu$.

A chelate structure consisting of two five-membered rings has been proposed for the cation (Fe, Zn, Cu) - procyanidin complexes.

1. INTRODUCTION

Sorghum is a cereal crop that grows in the tropics and subtropics in a zone of 40° on either side of the equator (1), and on altitude between sea level and 2700 m (2). It is a food as well as a feed crop that ranks fourth in acreage and production among the world's cereals including wheat, rice and maize. According to the 1979 FAO Plant Production and Protection Paper (3), world acreage and production are: Developed economies - 7 511 000, 24 292; Developing economies - 34 788 000, 28 484; Centrally Planned Economies - 162 000, 165 in hectars and metric tons respectively. In Ethiopia, it was grown on 1 250 000 hectars and the production was 1 100 metric tons in that year. In the developing countries, the largest proportion of the production goes for human consumption and a very small fraction is used in animal feeds, whereas in the developed countries, it is mostly used for animal feed.

1.1. Origin of Sorghum

A West African origin for sorghum has been suggested. However current evidence is in favor of the view that cultivated sorghum was developed in Abyssinia. Doggett states that "... It seems likely that the Agau people who were the Cushites occupying the Northern and Central part of the Ethiopian plateau played a major role in the development of the African crop plants including sorghum"(2).

1.2. Classification of Sorghum Crop

Sorghum belongs to the tribe "Andropogonea", and is one of the genera under the subtribe "Sorghastae". "*Sorghum Vulgare*" has been used as a specific name for sorghum, but because this includes some of the wild sorghums such as the Sudangrass, the name suggested by Clayton "Sorghum bicolor (Linn.) Moench" has been regarded as the correct specific name for cultivated Sorghum (1,2).

There are about 17 000 distinct and maintained cultivars in the world collection of sorghum(2). Herban and Dewitt (4) identified five basic and hybrid races among these.

1.3. Sorghum Grain

Sorghum usually matures in 100 - 140 days (3) after planting and the time taken by the grain to mature is in the range 25-55 days (2), from blooming. The anatomical parts of the mature sorghum grain is shown in Fig. 1.

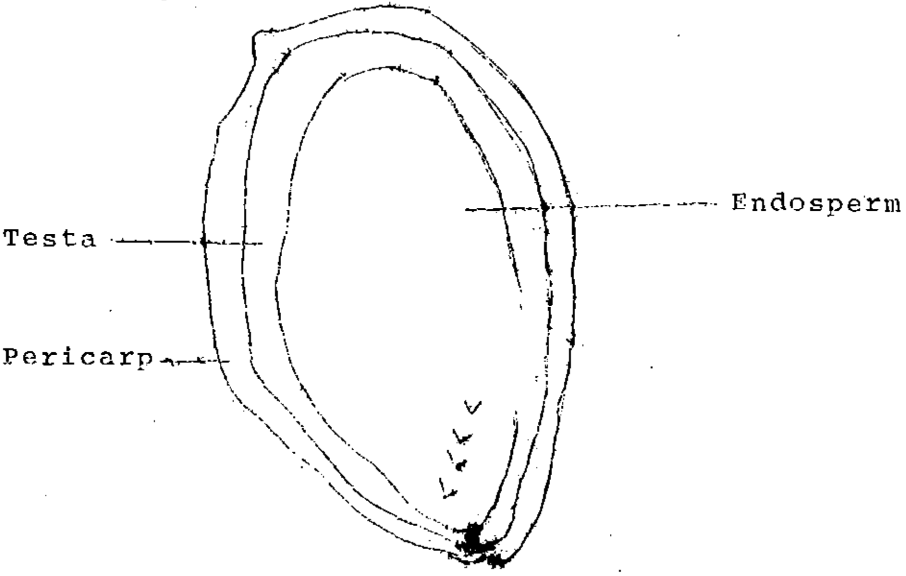


Fig. 1. Structure of the Sorghum Grain Caryopsis (5)

Grain color varies from white transluscent to a deep reddish brown with gradations of pink, red, yellow, brown, and intermediates (3). Grain color is determined by pigmentation in the pericarp, testa and endosperm. There are specific genes for color of each of these parts (3,5).

1.4. Chemical Composition of Sorghum Grain

The chemical composition of the grain is affected by several factors such as age, genetic variability, and the soil and climatic conditions under which it was grown. The endosperm is rich in starch which accounts for over 70% of the total chemical content of the grain (2,6). The protein content is about 10%, and the seed coat contains cellulose, hemicellulose and lignin as its major constiuents. Sorghum varieties with brown seeds are found to contain significant amounts of polyphenols in their paricarps or tests, though color classification is not a good criterion for polyphenolic content (6). The polyphenols in mature sorghum grains are predominantly condensed tannins (or procyanidins) (7).

1.5. The Aim and Scope of the Present Investigation

The procyanidins (or tannins) in the grain of the brown-seeded varieties of sorghum are important in their agronomy and nutritive value. They render the grain resistance to predatory birds and insects (8-10). They are also associated with resistance to molding (11) and preharvest germination (12). Tannins also prevent bloat in ruminants (13). However, these beneficial aspects come at the expense of some nutritional disadvantages. The presence of high levels of procyanidins in the grain lowers nutritive value, by precipitating dietary proteins (14-16) and inhibiting digestive enzymes (17-19)·gorghum varieties with the above mentioned charcterstics are termed Bird-Resistant (BR), whereas those which lack these properties are termed Non-Bird-Resistant (NBR).

Tannins react with divalent cations and render them unavailable for absorption (20). However, a systematic study of procyanidins on the bioavailability of mineral nutrients has not been reported. Therefore, the purpose of this project is to study the interactions (binding) of sorghum procyanidins with the mineral nutrients, iron, zinc, copper, calcium, and magnesium. The results of the investigation will be used to evaluate the nutritional quality of some varieties of sorghum grown in the various regions of Ethiopia with respect to mineral availability. The data will also be used to test the validity of the recently postulated "polyphenol hypothesis" for the p^H dependance of mineral binding by dietary fiber(21).

2. THEORETICAL BACKGROUND

2.1. Sorghum Polyphenols, and their Properties

2.1.1. Classification of Sorghum Polyphenols

Plant polyphenols are structurally diverse and widely distributed in the plant kingdom (21). In some plants, they are found in surprisingly high concentrations (e.g. tea leaf, up to 40%) (22).

Tannins are a group of polyphenols found in the vegetative tissues and seeds of many plants. It is difficult to give a concise definition for the word "tannin". Historically, the name is associated with the process of leather making (tanning) from animal skins and hides.(23). This led White (24) to suggest that any definition of tannins must include the fact that they convert animal skins to leather. Based on this suggestion, Bate-Smith and Swaine (25) defined vegetable tannins as "water-soluble phenolic compounds having molecular weights between 500 and 3000; and in addition to the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins . This is the most widely accepted definition.

Tannins complex with dietary proteins and render them unavailable for digestion, which decreases the nutritive value of the grains (14-16). Hydrogen bonding, covalent bonding, and hydrophobic interaction are involved in the formation of these complexes(26). The process may be reversible or irreversible depending on the type of force(s) involved. If the predominant forces are H-bonding and/or hydrophobic, the process will be reversible, whereas covalent forces lead to irreversible processes (27).

When tannins interact with salivary proteins, they give rise to a characterstic astringent taste (25). Astringency is a property which renders the plant tissue unpalatable by precipitation of salivary proteins. Many workers tried to explain bird resistance of "high tannin" sorghum varieties in terms of these properties.

Freudenberg (28) classified tannins into two groups based on structural types. These are the hydrolyzable and the non-hydrolyzable or condensed tannins. The hydrolyzable tannins split into sugar and phenolic carboxylic acids by simple treatment with acids or alkalis and in some cases by hydrolytic enzymes such as tannase (29). Condensed tannins do not readily break down in this way, nor do sugars contribute to their structure. However, condensed tannins covalently bonded to polysaccharides have recently been reported (29). Condensed tannins contain the three ring C_{15} flavanoid nucleus as the basic unit (Fig.2) (30).

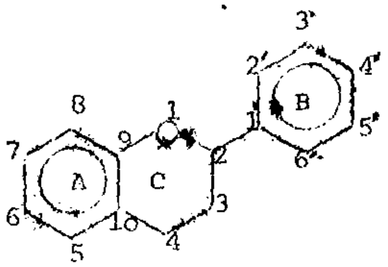
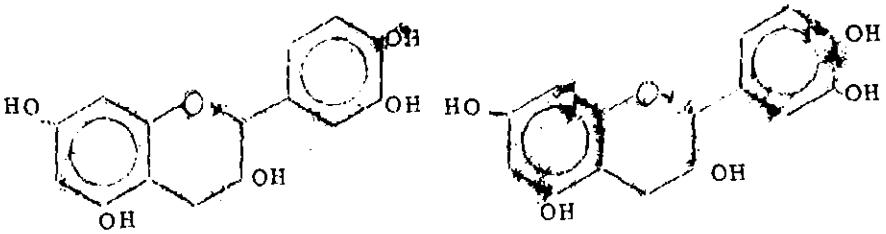


Fig. 2. The basic unit of flavanoids.

Condensed tannins form red pigments called anthocyanidins when heated in strong acid solutions. They are also named Proanthocyanidins, based on the type of anthocyanidin produced in the reaction. There are two major classes of proanthocyanidins: procyanidins and prodelphinidins (31). Procyanidins are cyanidin-producing condensed tannins, whereas Prodelphinidins are delphinidin-producing condensed tannins. Procyanidins are the most widely distributed in the plant kingdom (21). Usually, proanthocyanidins occur with one or both of the flavon - 3 - ols;(+)- catechin and (-) - epicatechin (Fig. 3).



(a) (b)

Fig. 3. The Structure of (a) (+)- catechin (b) (-) - epicatechin.

The low molecular weight procyanidins (upto 3000) are soluble in water, whereas higher MW procyanidins are difficult to extract in water and these may represent the major fraction in plant tissues.

Procyanidins undergo acid catalyzed fission to form flavan-3-ols and a carbocation as exemplified by a procyanidin dimer shown in Fig. 4.

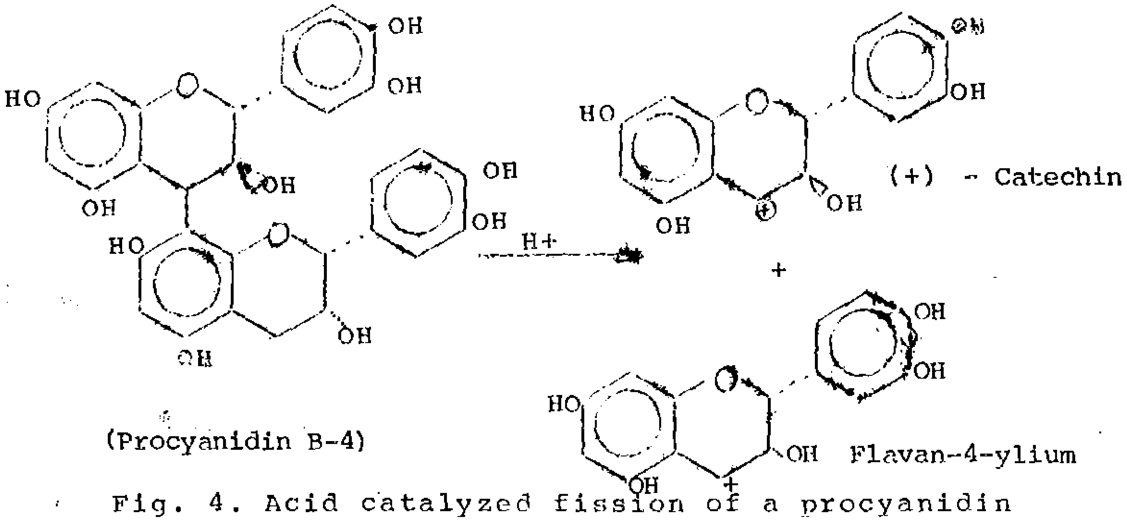


Fig. 4. Acid catalyzed fission of a procyanidin dimer B-4.

The Carbocation is a reactive intermediate, and unless trapped by a nucleophile, it decays by loss of a proton and a hydride ion to form the cyanidin pigment (Fig. 5).

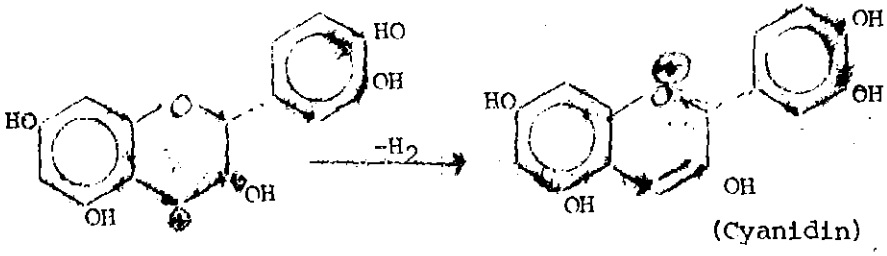


Fig. 5. Formation of the cyanidin pigment.

If trapped by a nucleophile such as toluene α- thiol, the carbocation forms a thioether (Fig.6). This reaction may be used to determine the structure of polymeric procyanidins (section 2.1.3.).

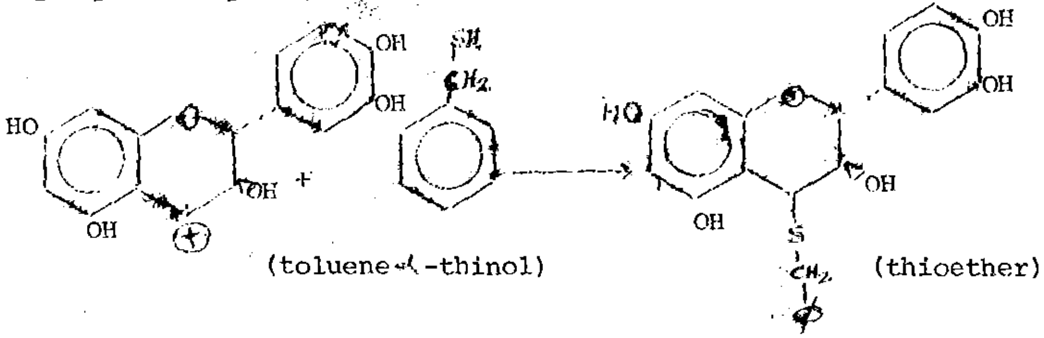


Fig. 6. Reaction of the flavanylium ion with toluene α - thiol.

2.1.2. Isolation of Sorghum Procyanidins

The chemical nature of procyanidins make them difficult to isolate and purify (32). Several solvents and solvent-mixtures were tried, including methanol, ethanol, and acetone (24-28). Acidified methanol is a stronger solvent for some procyanidins than pure methanol (33). Aqueous acetone (70% V/V) is superior mainly because of its ability to break procyanidin - protein complexes (34).

Thompson (21) et al. used successive extraction of the ground grain with methanol, light petroleum (bp 60 - 80^{O}), water, and ethyl acetate followed by chromatography on polyamide-woelm column and finally purifying on sephadex LH-20 and eluting with ethanol to isolate polymeric procyanidins. Gupta and Haslam (7) used a modification of this procedure. They extracted the finely ground grain successively with methanol, light petroleum, triturate with ethanol, and applying the resulting suspension onto sephadex LH-2o column with double elution with ethanol and methanol. Using this procedure, they obtained the brown granular powder of polymeric procyanidins with 0.75% yield.

The procyanidins obtained by any of the conventional methods contain considerable amount of protein. Reduction of the protein content has been made possible by preliminary extraction with ethanol (35).

Hagerman and Bulter (36) developed a method which is currently receiving a wide range application. Although the method is lengthy, it gives purified tannins (or procyanidins) that can be used as standards in spectrophotometric determinations involving tannins.

Separation by column chromatography is of limited help for sorghum procyanidins mainly because of their interaction with chromatographic supports (37), and consequently loss of much of the applied material. When polyamide or polyvinyl pyrolidone is used there is a loss of more than 70% of the applied material (38). Using, HPLC on sepharol - GB with H-bond breaking solvents such as DMF led to a decreased loss. However this enabled the separation of the low molecular weight procyanidins (upto trimers). Silica gel with anhydrous organic solvents also enabled the separation of procyanidins upto trimers (39). The isolation of a pentameric procyanidin has been reported very recently (40).

2.1.3. Structure and Biosynthesis of Polymeric Procyanidins of Sorghum Grain

Determination of the chemical structure of procyanidins awaited the development of spectroscopic techniques. Using the two fundamental reactions of procyanidins (Figs. 4 and 6) and ^{1}H.n.m.r. spectroscopy, Gupta and Haslam (7) were able to establish the structure of polymeric procyanidins as a polytetrahydroxy flavan -3-ol. They treated the polymer with an acid in the presence of excess tolune α- thiol. The sole products of the reaction were: (+) - catechin and the thio ether (Fig.7). The ratio of the two products was 1:5-6, giving an average chain length of 6-7 flavan 3-ol units in the polymer.

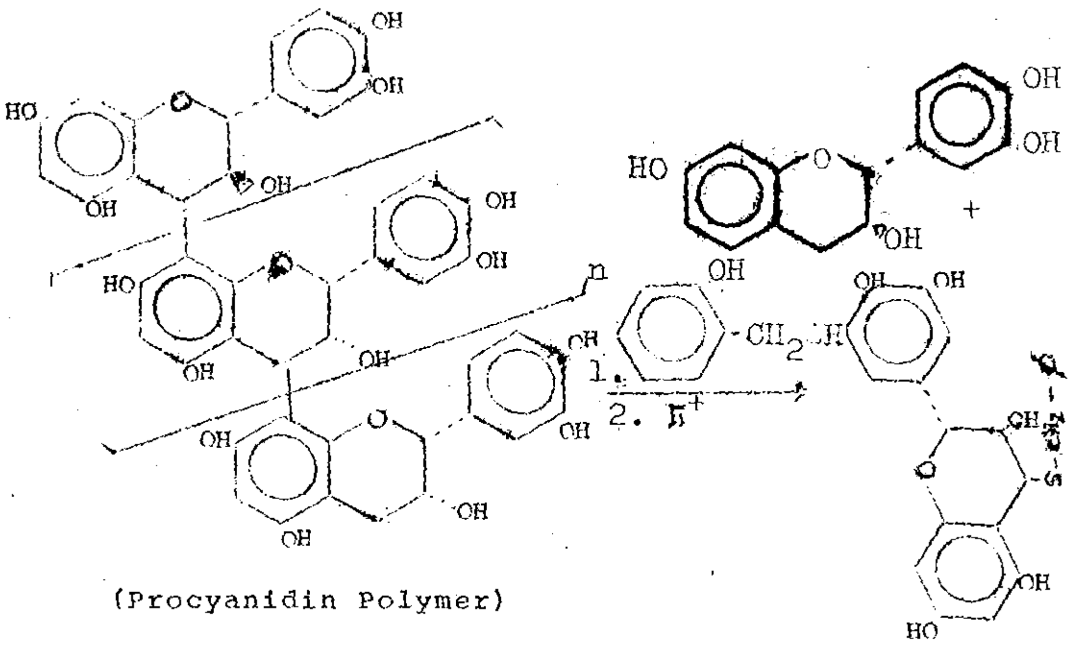


Fig.7. Acid catalyzed degradation and nucleophilic reaction of the sorghum procyanidin polymer.

A linear structure, with a central core composed of rings A and B of the flavan repeating unit, and ring C projecting helically from the central core, has been suggested for this procyanidin (22). However, evidence is being accumulated to disfavor the linear structure (41-43). The opposing views mainly emanate from the presence of heterogenous interflavanoid bonds which will lead to a less regular structure.

Based on the evidence from isotopic tracer studies, and enzymic work in tissue cultures, it has been found that sorghum procyanidin biosynthesis follows the general flavanoid bio-synthesis pathway, in which ring A of the flavanoid carbon skeleton is derived from acetates (or malonates) and ring C and the three carbon atoms of the heterocyclic ring originate from cinnamates (21,7). The synthesis is initiated in the seed-coat as soon as chlorophyll is formed. At the early stages of seed development, only (+) - catechin and the dimer

{B-1 (+) - catechin bonded to (-) - epicatechin through C-4 to C-8} were detected. When the seed ripens to a red pink appearance, the level of the mono-and di-meric flavan-3 - ols decline repidly leaving the polymeric procyanidin, the principal and in many cases the sole, procyanidin of the seed coat.

2.1.4. Quantitative Determination of Sorghum Procyanidins

The widely used vanillin/HCl (44) method for determination of polyphenols is not convenient for shorghum procyanidins since it does not discirminate between procyanidins, dihydrochalcones, and other flavanoids (45). The prussian blue method (46) is also not suitable because of its lack of specificity (47). The n-butanol/HCl (48,49), which is based on the measurement of the absorbance of the cyanidin pigment produced by the acid catalyzed fission of the interflavan bond is prefered because of its simplicity and relatively higher specificity. This method gives the relative procyanidin content without the need to use a standard for the calibration curve, and it is this method which has been used in the present investigations.

The n-butanol/HCl solution may be directly assayed on the neutral detergent fiber (NDF) to give the amount of the insoluble or fiber bound procyanidin (49). If assayed on the extract in solution, it gives the amount of soluble procyanidin. The major disadvantage of the n-butanol/HCl method lies in the fact that during acid catalyzed fission, the intermediate species of the reaction, the carbocation (Fig. 4), may interact with the neutral polymer to give a precipitate of highly polymerized products with indefinite composition (phlobaphenes) (23). However, the extent of phlobaphene formation can be minimized by carrying out the reaction under reduced pressure and in the absence of oxygen (50). Shortening the reaction time will also decrease philobaphene formation.

2.2. Mineral Nutrients

Twenty six elements of the periodic table have been found to be essential for animal life(51). These are divided into two groups: major elements including C, H, O, N, S, Ca, P, K, Na, Cl and Mg and minor elements including Fe, Zn, Cu, Ni, Co, Mo, Se, Cr, I, F, Sn, V and As. Several criteria were set to classify an element as essential or non-essential. According to Cotzias (51), an element will be considered essential if it is present in all living tissue and its concentration does not vary from one animal to the other (same species), In Mertz's (51) classification, an element will be essential if its difficiency or consistency results in impairement of a biological function from optimal to suboptimal, In this context, elements such as Al, Sb, Cd, Ge, etc. occur in animal tissues, though found in finite amounts, were regarded as enviormental contaminants because they could not fulfil one or more of the criteria.

The action of the trace mineral nutrients is primarily as catalysts in enzyme systems in the cell. Their role ranges from weak ionic effects to highly specific associations known as metallo - enzymes. The tissue levels of many metalloenzymes have now been related to the manifestation of defficiency or toxicity state in the animal. However, many physiological disorders as a result of their excesses cannot yet be explained in bio-chemical or enzymic terms (51).

Both the minimum requirements for normal bodily function and the toxicity levels are related to the age, health, sex, etc. of the animal. The total amount of minerals ingested in the diet as determined by the analytical methods cannot be equated to availability since the latter is influenced by such factors as the chemical form by which it is ingested, the level of other mineral nutrients, etc.

Components such as phytates, cellulose, uronic acids etc. have been reported to reduce the availability of many mineral nutrients, whereas some others such as ascorbates, amino acids citrates, etc, were found to enhance mineral availability.

2.2.1. The Role of Iron in Nutrition

The total iron content of the animal body varies with age, sex, nutrition, state of health, and species (55). The normal adultman is estimated to contain 4-5g of Fe or 6o-7o ppm of the total body weight of a 70kg individual. Body organs such as liver and spleen usually carry the highest concentrations of Fe, followed by kidney, skeletal muscles and brain (51).

In monogastric species Fe absorption takes place mainly in the duodenum and in the ferrous state (53). Inorganic forms of Fe and Fe-protein complexes need to be reduced to the ferrous state and released from conjugation for effective absorption, whereas the Fe in the heme compounds is absorved as the heme complex into the mucosal cells of the intestine. The reduction and release from conjugation are accompanied by the gustric juice and other digestivesecretions. Inorganic Fe is able to form complexes with normal gustric juices at low p^H which remains soluble even when the P^H is raised to neutrality and enable the Fe to be available in a suitable state for absorption. Among the food components studied for their effects on Fe absorption the following are worth-noting:

1. Promoters
    - a) Carboxylic acids: ascorbic, citric, pyruvic, succinic acids ;
    - b) Amino acids: histidine, lysin, cystein;
    - c) Carbohydrates according to the order: Glucose > sucrose > lactose .
2. Inhibitors
    - a) Amine: desferroxamine;
    - b) Inorganic Compound: phosphates or phytates (except mono-ferric phytates);
    - c) Inorganic cations: high concentrations of Co, Zn, Cd, Cu, Mn.

Generally, Fe is absorbed better from foods of animal origin than foods of plant origin (57).

Unabsorbed Fe is usually excreted through the feces. Other ways of Fe excretion include urinary, sweat, and menstrual flow (in females).

Food samples vary in their iron content depending on the soil and climatic conditions under which the food material was grown and depending on varietal differences (2). Usually the richest sources of total Fe are: organ meats (liver & kidney), egg yolk, dried legumes, cacao, cane molasses and pursley;

poor sources: milk & milk products, white sugars, white flour and bread, polished rice, soys, potatoes, and most fresh fruits; intermediate sources: muscle meats, fish, poultry, nuts, green vegetables, and whole meal bread. The Ethiopian teff is also reported to contain very high concentration of total iron (51).

Iron defficiency manifests itself in children and adults in different ways. In children Fe defficiency is mainly manifested by anorexia, depressed growth and decreased resistance to infection. In adults it is manifested by listlessness and fatigue, palpitation on exertion, sore toungue, angular stomatotis, disphagia, and koilonchia. Health abnormalities as a result of Fe defficiency has become so large. For example, iron-defficiency anemia of the hypochromic microcylic type brings about the following health disorders: reduced hemosiderin, ferritin and transferrin in the blood accompanied by decreased lipid metabolism, red cell survival, protein metabolism, muscle myoglobin levels, activity of the heme enzymes etc. Fe defficiency is probably the most prevalent defficiency state affecting the human population. It is much more common in women than in men because of the additional losses in women through menstrual flow, lactation and pregnancy (51).

2.2.2. The Role of Zinc in Nutrition

Zinc in the body of an adult man is estimated to be 1.4 - 2.3 g. It is distributed in the skin, blood, eye, glands, bones. liver, pancrease, kidney, etc. In the blood Zn is found in plasma, erythrorytes & platlets. In plasma Zn is bound to α - macroglobulins and albumins. Almost all the Zn in erythrocytes occurs as carbonic analydrase, together with a small fraction association with other Zinc enzymes(51).

Zinc is probably the 2nd most important mineral nutrient next to iron among the heavy metals (excluding Na+, Ca++, Mg++). In the past 15-20 years, more than 25 Zn - containing proteins have been identified, most of them enzymes (52).

The Zn^{2+} enzymes play significant roles in carbohydrate, lipid and protein metabolism in virtually all organisms (57).

The absorption of Zn may take place from the duodenum, illium and jejunum, stomach or colon, the small intestine depending on the species of the animal. The extent of Zn - absorption varies with the level of Zn in the body, and the range of other dietary components. Inhibitors of Zn absorption includes inorganic phosphates, high intakes of Cu andCa, cellulose. The major organ involved in Zn - metabolism is the liver. The Zn absorbed is taken to the liver by the plasma transferrin. The liver cytosal contains binding components of different MW and lability, the amounts and proportions of which vary on the Zn status of the animal. In case of need this Zn is transported to various tissues(54).

Zn is mainly excreted from the body through feces. Fecal Zn contains mainly unabsorbed Zinc with a small amount of endoegenous origin secreted in the small intestine. Very small amounts of Zn leave the body through urine. In contrast to iron, menstrual loss of Zn is very small.

A large number of diseases were reported to arise from Zn difficiency. Acrodermatitis entropetica, a hereditary disease appearing in early infancy is characterized by postular and aczematoid skin leission, allopecia,diarrhea are due to aberrant Zn metabolism. Without treatment,a relentless progression of severe mulnutrition, poor growth and development, intercurrent infection, and deaŧh in 3 days is usual. Spermatogenesis and secondary sex organs in the male and all phases of reproduction in the female from estrus to partirition and lactation, can be adversely affected by Zn defficiency. Zn is required in wound healing therapy hypogonadism with suppression of the secondary sex characterstics are conspicous features of Zn defficiency. Zn is involved in nucleic acid synthesis and hence cell replication. The earliest known metabolic defect of Zn-defficiency is a decreased activity of thymidine kinase, the enzyme leading to thymidine triphosphate formation which is a prerequisite for DNA synthesis and thereby replication. Zn defficiency decreases the activities of other Zn enzymes and Zn-dependent enzymes (51,54,55).

The following are common food sources of Zn. Richest sources: Oysters and other sea foods, muscle meats and nuts; poorest sources: White sugar, pome and citrus fruits, non-leavy vegetables and tubers; intermediate: cereal grains.

2.2.3. The Role of Copper in Nutrition

The healthy adultman contains about 80 mg of total copper, with higher concentrations in new born babies than in adults. Cu is distributed in the liver, blood, glands, ovary, testies, pancreas, skin, muscles, heart, hair, etc. Exceptionally high concentrations of Cu occur in the pigmented parts of the eye. Most of the Cu in blood is bound to albumin. The latter form of Cu is in a rapid equilibrium with Cu in the tissues and is considered to be the transport form of Cu in the blood (51).

A number of Cu- protein complexes have been isolated from plant and animal tissues, several of which are enzymes with oxidative functions. Tryrosinase, locease, uricase, cytochrome oxidase, ascorbic acid oxidase and others are believed to be Cu-compounds. Cu associates with Fe in the formation of hemoglobin. It has been shown that although the body could similate Fe without Cu, it would not utilize Fe and regenerate hemoglobin unless Cu is present (52).

Cu is absorbed from the stomach and all portions of the intestine, particularly the upper small intestine. The extent of absorption is influenced by the amounts and chemical forms of the Cu ingested, by the dietary level of several other metals and organic substances, and by the age of the animal. Little evidence is available that an absorption from the intestine is regulated in accordance with bodily needs (54).

Copper may be transported through the intestinal mucosal both as ionic and in the form of complexes. The affinity of Cu ions for inorganic and organic ligands can reduce the rate of absorption depending on the size and stability of the complexes. For example, phytates form very stable complexes with Cu and reduce its absorption. High levels of ascorbic acid also significantly depress Cu absorption. Mineral nutrients such as Cd, Zn, Fe, S, etc. also reduce Cu absorption. The mechnism of Cu absorption is not clearly understood, however, the involvement of metal binding components has been suggested. Cu entering the blood plasma becomes loosely bound to serum albumin and amino acids, in which form it is widely distributed to the tissues and can pass readily to the erythrocytes (54).

Similar to Fe and Zn, a high proportion of Cu is excreted in feces, with small amounts in urine , sweat, menstruation and lactation (53).

Cu defficiency manifests itself depending on age and species of the animal and with severity and duration of the difficiency (51). Anemia is a common expression of Cu defficiency in all species where the defficiency is severe or prolonged. The following diseases arise from copper defficiency: bone disorders in rabbits, chicks, pigs, dogs, and mice; nervous disorders in lambs and goats; acromotrichia in rats, rabbits, guinea pigs, cats, dogs, cattle, sheep; impaired keratinization in rats and rabits; kinky hair in human children; infertility in female, rats, guinea pigs, hens and cattle, cardiovasculal disorders and diarrhea in cattle; decreased fatty acid metabolism in rats.

Copper occurs in many foods; crustaceans and shellfish, organ meats (lamb & beef liver), nuts, dried legumes, dried vine, cocao (richest sources), white sugar (poorest), non leavy vegetables, fruits, cereals (good sources).

2.2.4. The Role of Ca and Mg in Nutrition (55-57)

These alkaline earth elements are not as important as the trace elements in the sense that they are not involved in as many functions as the trace elements. However, it should be born in mind that the task of these elements cannot be covered by those highly important trace elements.

Calcium occurs in the body in the greatest amounts of all metals, whereas Mg is about one-fifth as abundant as Ca. Almost all of Ca and about 50% of Mg are found in the bone. Other organs containing Ca & Mg are the muscle, liver, and spleen. Both Ca & Mg are found in blood serum. In blood serum Ca is found in two forms: the ionized (or diffusable) and the non-ionized protein complex (or non-diffusable). About 35% of serum Mg is bound to proteins.

Calcium is essential for clotting reactions, being required for the conversion of protothrombin to thrombin, a process which must be preceded by reduction of plasma Ca level. Ca is also involved in protection against other ions especially Na & K and cell permeability. Mg is involved in many enzyme activities including phosphatases, redox enzymes, synthesizing enzymes, phosphorylating enzymes, etc.

Both Ca & Mg are found in large quantities in both plant and animal tissues. The absorption of these elements is inhibited by several food components including phytates and cell wall materials.

Defficiencies of Ca lead to: reduced muscle contraction including heart, kidney disease, infantile-titany, etc. whereas decreased Serum Mg is reported to cause chronic nephititis, acute pancreatitis and alcoholic cirrhosis. Elevated Mg level is also defective because it causes renal insufficency, hypertension and diabets.

In contrast to the trace elements, the highest proportions of Ca & Mg are excreted through urine.

2.3. Mineral Binding by Food Components

In the preceeding discussions it has been noted that the availability of minerals for absorption is affected by the chemical form in which the mineral is ingested. Several food components such as amino acids, simple sugars and ascorbic acid were found to enhance mineral absorption in most cases, by forming readily absorbable soluble complexes or chelates. In contrast, other food components such as phytates, cellulose and other cell wall constituents reduce mineral bioavailability. Reinhold et al. (60), from their studies on wheat, came up with the conclusion that among the food components, starch, protein, and fiber had significant binding capabilities for minerals and can interfere with mineral bioavailability. They further noted that dietary fiber can interfere more than the other wheat solids because it remains undigested in the small intestine. Thereafter, scientists pooled their attentions around dietary fiber, and finally, its inhibitory effect became a universally accepted truth than being a simple chemical phenomenon. Several workers went even deeper to find if there is a particular component in the fiber to play the role. Therefore, it becomes an object of the following two sections to reveal some of the important works in this regard.

2.3.1. Classification of Food Components and the Detergent System of Fractionation

Plant materials are divided into three major classes (58):
(1) total availability, (2) incomplete availability, and (3) total unavailability. Compounds constituting the total availability class include soluble carbohydrates, starch, organic acids, proteins and pectins. For this class of substances, actual extent of digestion is determined by the competition between rates of digestion and passage through the gastrointestinal tract. Compounds of the total unavailability class are lignin, cutin, silica, tannins,

essential oils and other polyphenols. These compounds are resistant to the action of enzymes and are removed unutilized through the feces. Compounds with incomplete availability are the strucural carbohydrates, cellulose and hemicellulose. The availability of these compounds varies widely among plant sources and are made unavailable by the presence of a refractory entity due to enzymatically unhydrolyzable bonds with the available portions. Compounds of this class remained to be problematic in that they cannot be resorted into digestibility classes based on chemical analytical procedures. However, Van Soest (58), and his coworkers at Cornell University brought the problem to an end by introducing the detergent system of analysis. In this procedure protein, the principal problem cuasing substance in contaminating the cell wall materials is removed by detergent solutions.

The detergent system of analysis was developed to fractionate food of plant origin in a manner consistent with the nutritive availability of fiber. It is based on the fact that detergents form strong protein complexes which are soluble under proper conditions. It involves extraction of the plant material either with a neutral solution of sodiumlauryl sulfate or with an acidic solution of cetyltrimethylammonium bromide (usually in H_2SO_4). If the extraction is under neutral conditions, the recoverable components are the major cell wall constituents lignin, cellulose and hemicellulose. The residue also contains minor cell wall components including pectin, some proteins and bound tannins. Common contaminants are starch and mineral elements. Starch is removed by adding α-amylase and mineral elements are removed by EDTA. The fiber prepared according to this procedure is termed neutral detergent fiber (NDF).

2.3.2. Mineral Binding by Dietary Fiber and its Components

Studies on the binding of cations by fiber was initiated by McConnell et al. (59) in Scotland. McConnell and co-workers studied the cation exchange behavior of fibers from vegetable sources. They observed the facts that different fibers show different functionalities for cations. However they didn't try to correlate these cation exchange behavior of fibers to mineral availability. Therefore a systematic study of mineral binding by dietary fiber awaited the discovery by the group of scientists in Iran (Reinhold and his collaborators) (60).

From the studies in some villages of the Middle East it has been revealed that despite the fact that the villagers eat breads and diets high in Fe & Zn, they were found with Zn- & Fe- defficiency diseases such as hypogonadal dwarfism (77). Several workers attributed this discrepancy to high phytate contents, which form complexes that are less available for absorption. However foods with low phytate content were found with similar effect (59,60). This fact when added to the experimental observations of James & Branch (61) destroyed the phytate hypothesis.

The failure of the phytate hypothesis led Reinhold et al. (60) to examine other constituents of wheat as possible metal binding agents. They found that wheat starch, protein and fiber bound minerals, with fiber being the most important. Latter work by the same group on dietary fiber (NDF & ADF) from wheat and maize has shown that Fe(II) was firmly bound by NDF by an amount that accounts for almost all of the iron binding capability of the cereals (63). Fractionation of the fiber into its components has shown cellulose to be the principal binder. Binding by cellulose was more pronounced by heat treatment (65).

The fact that hemicellulose is a binder of minerals was demonstrated using alkali - and water-soluble fractions of rice hemi-cellulose and minerals such as Cu, Fe, and Zn (62). Subsequent studies have shown that alginates (66), and pectin (78) also bind minerals.

Mineral binding by dietary fiber is effected by the P^H of the media (20,63,66) the concentration of the minerals in equilbruim with the fiber (20), the fiber concentration and fiber particile size (20), presence of competing cations (68) and temperature (65). Binding capacities of fibers increase until the saturation point is reached. But, increasing either the fiber concentration or fiber particle size decrease the mineral binding capacity of the fiber. Though the effects could not be satisfactorily explained, it has been suggested to be either physicochemical or compositional (20). In many cases the presence of one or more competing cations in the equilibrating mineral-fiber system decreases the binding capacity of the fiber for the particular metal. This was attributed to the competition between the various mineral cations for the active sites of the fiber.

Mineral binding by dietary fiber is strongly affected by P^H within a certain P^H region (usually 4.5 - 7.0). At low P^H the binding is also low. As the P^H increase from around 4 there is a gradual increase in binding. As the P^H is raised further to around neutrality, there is a dramatic rise in binding. This experimental phenomenon could not yet be satisfactorily explained and remained to be the point of departure among the scientists.

3. EXPERIMENTAL

3.1. Materials and Methods

3.1.1. Equipment

NDF was prepared by the method of Goering and Van Soest (73). UV-Visible absorbance was measured using the Pye Unicam Sp 6-550 UV/Vis Spectrophotometer. Grain samples were ground to pass a 1-mm screen in a Cross Beat Grinder. Grain mineral content measureemt and binding capacity studies were done using the Perkin Erlmer Model 2380 Atomic Absorption Spectrophotometer with air/acetylene flame. Grain sample digestion was done using the Technican BD-40 heating unit. PW 9410 Digital p^H Meter was used for p^H measurements. Dialysis was done using the spectra Por Dialysis Membrane (Specta/por 7, MWCO 1000).

3.1.2. Materials

Glassware was washed with hot 50% (v/v) solution of concentrated hydrochloric acid. Glass distilled and double deionized water (ddDw) was used except in NDF preparation, where simple glass distilled water was used.

Neutral Detergent Solution (NDS): The composition of NDS was:

a. Distilled water	20 l
b. Sodium lauryl sulfate (NF/FCC, Fisher)	600 g
c. EDTA - acid (Cert. A.C.S., Fisher)	330 g
d. Sodium hydroxide (Baker, Anal. Reag., Baker)	90.20 g
e. Sodium borate decahydrate (GPR, BDH)	136.20 g
f. Disodium hydrogenphosphate (Cert. A.C.S., Fisher)	91.20 g
g. 2-Ethoxy ethanol (purified, Fisher)	200 ml

The solution was prepared by dissolving the weighed quantities of EDTA, sodium hydroxide and borax and the phosphate in separate beakers containing about 400 ml of distilled water over heat. Sodium luaryl sulfate and ethoxy ethanol were dissolved separatly in 400 ml of distilled water and the solutions were mixed with the remaining volume of distilled water and kept over night before use.

Saturated Amylase Solution: The solution was prepared by dissolving 2.5 g α-amylase (Sigma) in 1oo ml of phosphate buffer of p^H 7.0 {phosphate buffer was prepared by mixing 60.8 ml of o.1 M Na_2HPO_4 (cert. A.CS, Fisher) and 39.2 ml of o.3 M KH_2PO_4 (cert. A.C.S., Fisher)] and filterting through a whatman No. 40 ashless paper after 20 min dissolution time.

HCl/n-Butanol, (5% v/v): The solution was prepared by mixing 5 ml of concentrated hydrochloric acid (Reag. A.C.S., Fisher) and 95 ml of n-butanol (AR. BDH).

Atomic Absorption Senectrophotometer Standards: Three standard solutions were prepared for each cation by diluting the respective AAS stock solutions (spectrosol, BDH) containing Fe (III), Zn (II), Cu (II), Ca(II), and Mg(II) in the form of nitrates. The concentrations of the standard solutions are given in Table 1.

Table 1. Atomic Absorption Standard
Solutions, Concentration (ppm)

Standard	Fe	Zn	Cu	Ca	Mg
S_1	5	1	1	5	5
S_2	15	3	3	15	15
S_3	3o	6	6	3o	3o

Test Cations Stock Solutions: All salts were of analytical Reagent (BDH) and HCl was of Reagent A.C.S. (Fisher) grades.

a. Fe(II), 625 ppm: 2.1493 g $(NH_4)_2 Fe(SO_4)_2 6H_2O$ in 500 ml of dddw containing 10 ml of concentrated HCl.

b. Zn(II), 625 ppm: 2.7492 g $Zn SO_4.7H_2O$ in 1 l dddw containing 10 ml of concentrated HCl.

c. Cu(II), 2500 ppm: 2.4575 g $CuSO_4.5H_2O$ in 250 ml of dddw containing 10 ml of concentrated HCl.

d. Ca(II), 1000 ppm: 0.9186 g $CaCl_2 \cdot 2H_2O$ in 250 ml of dddw water.

e. Mg(II), 1000 ppm: 2.5674 g $MgSO_4.7H_2O$ in 250 ml of dddw water.

Glucose Saline Solution (GSS): The solution was composed of the following substance in 2 l dddw:

a.	KCl (AR, BDH)	0.6 g
b.	NaCl (AR, BDH)	15.0 g
c.	Diglucose monphydrate (Monk's Lab. Chem., Monk)	10.0 g

Buffer Solutions:

a. p^H 7 standard: prepared by dissolving the given quantity of p^H 7 scatchets (GPR, BDH) in 200 ml distilled water.

b. p^H 4 standard: prepared by dissolving the given quantity of $p^H{}_4$ scatchets (GPR, BDH) is 100 ml distilled water.

c. Triethanol amine (TEA)/HCl buffers:

a series of buffer solutions of p^H 2.5, 3.o, 3.5, 4.o, 4.5, 5.5, 6.o, and 6.5 were prepared by mixing equal volumes of o.1 M TEA (A^R, BDH) and HCl (Reag. A.C.S., Fisher) and making to the required value using the p^H meter and o.ol M TEA or HCl.

Working Cation Solutions (i): required concentrations and volumes of solutions were prepared from the test cation stock solutions in GSS. In the case of iron, 2 ml per litre of 1% (w/v) ascorbic acid (cert. A. C.S., fisher) was added to keep iron in the ferrous state.

Working Cation Solutions (ii) : required concnetrations and volumes of solutions were prepared from the test cation solutions in TEA/HCl buffer of p^H 6.5.

Grain Samples: The samples were prepared by grinding the grain to pass a 1-mm sieve.

NDF: NDF samples were prepared according to the Amylase procedure of Robertson and Van Soest (74). Ground sample (1 g) was refluxed in a 600 ml beaker with 100 ml of NDS for 40 min, 2 ml of the amylase solution was added and refluxing was continued for another 20 minutes. Additional 2 ml of the amylase solution was added and the solution was filtered through a Gooch cruicible. The residue was washed twice with hot distilled water and twice with acetone. The washed fiber sample was dried overnight at room temperature.

3.2. Analytical Procedure

3.2.1. Determination of Relative Procyanidin Content

Although the determination of procyanidins by the modified n-butanol/HCl method (49) does not give quantitative results (confer section 2.1.4), one can get sufficient information about the relative procyanidin content in sorghum grain NDF. The procedure used in our experiment is as follows: about 10 mg NDF samples were weighed into a 20 ml test-tube and treated with 10 ml of the 5% HCl/n - butanol reagent at 95°C for 1 hr, with stirring at 5 min intervals on a spin mix. Testtubes were covered with glass beads to avoid atmospheric oxygen, and loss due to evaporation. Then, the mix was removed, air cooled, and absorbance was measured at 550 nm after 15 minutes against a reagent blank. Analyses were done in duplicate and results are expressed in A550/g NDF (Table 2)

3.2.2. Determination of Endogenous Levels of Minerals (Fe, Zn, Cu, Ca, and Mg).

Mineral content of both the whole grain and NDF were determined by the procedure of AOAC (75) with minor modifications. About 500 mg of the sample was weighed into 75 ml digestion tubes and treated with 4 ml H_2SO_4 and 2 ml H_2O_2 and digested at 270°C for 30 min , followed by dropwise addition of H_2O_2. The addition of H_2O_2 was continued at intervals until complete oxidation of the organic matter was indicated by disappearance of the dark color of the solution. The tube was then removed, cooled to room temperature and made to volume with distilled and double deionized water. The tube was rapped with parafilm and was kept over night. Fe, Zn, and Cu content of the solution were directly determined by AAS while Ca, and Mg were determined after diluting the solution (1:20) with 1% $LaCl_3$ solution. Results are expressed in mg element/g grain or NDF (Tables 3& 4).

3.2.3. Determination of Analytical Ranges of Concentrations

The range of concentrations within which Beer-Lambert law was obeyed were assessed in the given solvent and buffer systems. A series of solutions were prepared by diluting suitable aliquots (2-15 ml) of the standard solutions containing 12.5 ppm Fe(II), 6.25 ppm Zn(II), and 25 ppm Cu(II), respectively, with TEA/HCl (p^H 6.5) in 25 ml volumetric flasks. The solutions were allowed to stand for 2 hours and the concentrations were measured at the respective wavelengths using AAS. Results are given in Fig.8.

3.2.4. Mineral Binding by Insoluble Procyanidins (NDF).

Mineral bound by NDF were measured as described by Lee and Garcia-Lopez (21) with slight modifications. The NDF samples were weighed into 50 ml centrifuge tubes and washed twice with 10 ml GSS for 20 min. After washing, the samples were centrifuged at 3000 rpm for 10 min and the supernatant discarded. Aliquots (10 ml) of the cation solution (i) were added to the fiber residues. The mixtures were agitated for 20 min using a spin mix, then the p^H was adjusted using TEA/HCl buffer. Mixing was continued for 40 min and the tubes were centrifuged again for 10 min. Aliquots of the supernantant were placed in 20 ml dilution tubes for determination of mineral concentrations by AAS. In the case of Ca & Mg, the supernatant was diluted (1:20) with 1% $LaCl_3$ solution. Differences in mineral concentration between the unexposed control and that exposed to fiber (corrected for dilution for Ca & Mg) equaled mineral bound by the fiber.

The effects of p^H, cation concentration, amount of fiber, fiber particle size, and competing minerals were studied by varying the experimental parameters: p^H - 2.5 to 6.5, cation concentration - o.50 to 30.00 ppm, amount of fiber - 12.5 to 100,0 mg, fiber particle size - 150 to 1000 µm. During the study of the effect of one parameter, all other parameters were kept constant.

The effects of phytates was also studied by treating the sample with 1.1% HCl for 5 hours and comparing the mineral binding after removing phytates to that of the control. The control contained the same fiber material except that it was not exposed to the HCl solution. It was washed for the same length of time with distilled & deionized water. Binding by the grain NDF was measured after determining optimal experimental conditions.

3.2.5. Mineral Binding by Soluble Procyanidins

3.2.5.1. Extraction and Recovery of Soluble Procyanidins

Isolation of procyanidins was done as described by Reed et al (76). 300.0 mg of NDF was extracted with 70% aqueous acetone (v/v) (5 X,3 ml), filtered through a Gooch cruicible into a 50 ml centrifuge tube. 2.o ml of o.1 M Yb $(C_2H_3O_2)_3 \cdot 4H_2O_{(aq)}$ and 15 ml of o.1 M TEA in 70% acetone were added to the filterate and kept in cold room maintained at 4° over night. The Yb-phenolate precipitate was separated after centirifuging at 3000 rpm for 10 min and washed twice with aqueous acetone and twice with dddw. The supernatant solutions after centrifuging at each stage were discarded.

The washed residue was treated with 2.0 ml of 0.15 M $C_2O_2H_2 \cdot 2H_2O$ (aq) and 2.0 ml buffer (p^H 6.50). The phenolic (procyanidin) solution was filtered into a 5 ml volumetric flask through a Gooch crucible and made to volume with additional buffer.

3.2.5.2. Dialysis and Binding Studies

A 10 cm segment of the dialysis tube was carefully cut with stainless steel scissors and washed with 300 ml dddw 5 times. The bottom of the tube was closed with about 1 cm overhanging and the procyanidin solution (5 ml) was transferred to the tubes. The top of the tube was also closed and soaked with about 300 ml dddw for 1 hr. Soaking was repeated two more times with dddw and two times with the same volume of buffer (p^H 6.50). The tube with its contents was placed in 300 ml of 5.0 ppm Fe(II) solution prepared in buffer of p^H 6.50, for 24 hrs. Binding capacity was calculated from the difference between the concentration of the blank and that of the supernatant as determined using AAS. The same procedure was repeated for the other elements using the same volumes of solutions and the following concentrations: Zn-3.0; Cu-17.5; Ca-100.0; and Mg-100.0 ppm.

3.2.5.3. Solubility Behavior of the Fe(II) - Procyanidin Complex

The Fe(II) - procyanidin complex which adhered to the wall of the dialysis tube was examined for its solubility in the following solvents: water, dil. HCl (0.1 M), 70% aqueous acetone, pure acetone, and carbon tetrachloride. In each case 10 ml of the solvent was used.

4. RESULTS AND DISCUSSIONS

4.1. Relative Procyanidin Content and Classification into Bird-Resistant and Non-Bird Resistant Classes

The results of the n-butanol/HCl method enabled classification of the 21 lines of sorghum used in the present investigation into Bird-Resistance and Non-Bird-Resistance classes.

Absorbance (A_{550}) ranged from 2 to 434 per g NDF. Table 2 shows two distinct classes: one with low A_{550} values (Sample No, 1-5) and another with higher values (sample No. 6-21). From Beer-Lambert law, it is known that absorbance is directly proportional to concentration. Thus, it can be concluded that those lines of sorghum which gave rise to low A_{550} values are also low in procyanidin content, and the vice-versa. High procyanidin (tannin) content is associated with bird resistance (8-12). Therefore, samples No. 1-5 with A_{550} values between 2 and 9/g NDF are identified as Non-Bird-Resistant varieties and the rest (No. 6-21) with A_{550} between 177 and 434/g NDF are identified as Bird-Resistant varieties.

4.2. Endogenous Levels of Fe, Zn, Cu, Ca and Mg.

The two classes of sorghum grain have very similar mineral compositions, based on their respective mean values. However, species differences are large as indicated by the large value of the standard [illegible]iation (Table 3).

Table 2. Relative Procyanidin Content (expressed in A_{550}/g NDF) as Measured in Sorghum Grain NDFs.

NO.	Identification	A_{550}/g NDF
1	Gambella	2±1
2	Tegemeo	2±1
3	Melkamasha 79	5±0
4	Buraihi	6±1
5	N-13	9±1
6	BM 10	117±10
7	SVR 8	187±14
8	Susa	198±12
9	BM 27	203±2
10	3K X 72/1	217±4
11	SVR 157	223±12
12	E-1291	225±0
13	Ikinyaruka	228±3
14	5 DX-160	236±6
15	Serena	261±20
16	5 DX 135/13/1/3/1	295±4
17	Dobbs	304±1
18	Is 8686	314±18
19	E 525HT	352±10
20	Seredo	425±18
21	MW 5020	434±16
Mean, NBR		5±3
Mean, BR		267±78

Table 5. Endogenous Levels of Minerals in Whole Seed
(μg mineral/g grain)

No.	Identification	Fe	Zn	Cu	Ca	Mg
1	Gambella	47.1±1.5	13.7±0.4	3.2±0.1	1099.2±50.0	762.8±7.4
2	Tegemeo	37.0±3.1	19.0±0.9	10.0±0.2	1710.1±44.4	755.4±9.3
3	Melkaomasha 79	43.5±2.5	15.9±0.3	3.1±0.5	478.2±1.4	795.9±1.1
4	Baraihi	36.8±0.6	19.4±0.1	10.0±0.2	1066.2±3.4	760.2±6.1
5	N-13	42.2±3.1	13.3±0.2	3.4±0.7	1332.3±17.3	854.7±1.1
6	BM 10	44.9±1.2	15.5±0.8	10.1±0.2	1332.1±39.9	871.5±11.4
7	SVR 8	49.8±3.0	19.1±0.9	10.9±1.0	1832.3±4.9	873.2±13.6
8	Susa	37.6±2.5	18.7±0.8	10.4±0.4	1359.1±6.4	845.8±11.0
9	BM 27	47.5±2.5	15.5±0.7	10.1±0.1	1588.3±40.1	817.6±9.5
10	3K X 72/1	36.1±2.2	12.0±0.5	8.6±0.2	1574.2±24.6	789.8±6.2
11	8 VR 157	56.3±2.4	22.7±0.7	11.5±0.2	1982.3±39.9	923.1±8.7
12	E-1291	42.0±0.6	13.3±0.2	10.0±0.2	1506.3±60.1	760.7±8.9
13	Ikinyoruka	26.7±0.2	13.2±0.6	10.1±0.4	1471.1±76.2	799.3±4.5
14	5 DX-160	59.2±1.8	19.1±0.3	9.6±0.4	2194.3±16.7	849.6±9.3
15	Serena	37.0±2.1	13.6±0.7	8.8±0.2	1596.2±1.9	708.3±4.2
16	5DX134/13/1/3/1	15.6±2.8	8.4±0.0	9.1±0.3	1180.0±63.5	542.5±0.4
17	Dobbs	55.2±2.6	14.6±0.7	9.8±0.9	1970.0±25.3	729.6±2.0
18	Is 8686	31.2±1.6	16.2±0.2	9.8±0.6	1246.1±15.8	720.8±5.1
19	E525 HT	50.9±3.0	18.7±0.1	10.3±0.1	1644.4±50.1	853.7±0.1
20	Seredo	43.2±0.2	1[illegible].4±0.9	10.5±0.6	1604.2±5.0	803.0±0.0
21	MW 5020	33.1±2.2	16.4±0.3	3.1±0.1	1158.2±56.7	659.3±6.0
	BR, Mean	40.2±10.9	15.6±3.4	9.5±1.8	1565.2±28.6	779.8±91.5
	NBR, Mean	40.5±9.8	16.3±2.6	5.5±3.2	1137.2±40.2	789.8±34.8

Table 4. Endogenous Levels of Minerals in NDF of Sorghum Grain
(µg mineral/g NDF)

NO	Identification	Fe	Zn	Cu	Ca	Mg
1	Gambella	22.0±0.8	0.00	0.00	0.21	0.08
2	Melkamasha 79	24.0±1.2	0.10	0.00	0.06	0.05
3	N-13	16.5±1.6	0.00	0.00	0.00	0.06
4	Susa	26.5±1.3	0.00	0.02	0.02	0.10
5	SVRJ57	38.5±1.0	0.00	0.06	0.05	0.10
6	Ikinyaruka	34.5±1.1	0.10	0.00	0.07	0.07
7	E525HT	24.0±0.5	0.02	0.00	0.07	0.05
Mean	BR	30.9±5.9	0.03±0.04	0.02±0.02	0.04±0.01	0.08±0.20
	NBR	20.8±3.2	0.03±0.04	0.00±0.00	0.02±0.02	0.06±0.00

The order of abundance is Ca > Mg > Fe > Zn > Cu. Comparison of the mineral contents to the recommended daily allowances shows that eating about half a kilogram of sorghum -based diet will satisfy the needs for all the mineral except for Zn in which case about 800 g grain is needed (Table 5) by an average man.

Table 5 Daily Dieatry Allowances for Minerals for an Average Man (70 kg Body Weight) as Compared to Their Availability in Sorghum Grain.

Element	Daily allowance (mg)	Mineral content of sorghum grain (mg/g)	grain sorghum to be eaten/day (g)
Fe	13[a]	0.040	325
Zn	13[a]	0.016	812
Cu	4[a]	0.010	400
Mg	300[b]	0,780	385
Ca	800[c]	1.563	513

a. Ref. 51 ; b. Ref. 54; c. Ref. 56.

Both BR and NBR grains retained part of their Fe after treating with NDS. Larger amount of Fe was retained by the NDF from BR compared to the NDF from NBR, suggesting the possible association of Fe with sorghum grain procyanidins.

4.3. Analytical Ranges of Concentrations

The range of concentrations within which the Beer-Lambert law is obeyed depends, among several factors, on the composition of the solvent. In this investigation, GSS was used as the solvent system with TEA/HCl as the buffer. Though both solutions were prepared in water, the presence of other components such as TEA and glucose may interfere with the absorbance of the cations to be determined. At higher concentrations the cations form insoluble hydroxides leading to deviation from the Beers law. These problems necessitated determination of the analytical range of concentrations to be used.

Figure 8 shows the plot of concentrations (absorbance) as read on the AAS versus the volumes of the cation stock solutions. Based on the linear range of thecurves, the maximum concentrations for the cations were found to be Fe, 12.5 ppm; Zn, 4 ppm; and Cu, 15 ppm (after correcting for the factor of dilution with the buffer solution).

4.4. Mineral Binding by Sorghum Grain NDF.

The selection of the P^H range for the investigation was governed by (1) the stability of the cations in the solvent system and (2) the relevance to physiological P^H ranges. Salts of transition metal cations such as Fe, Zn, and Cu underge hydrolysis to form sparingly soluble hydroxides under alkaline conditions and cause difficulties in spectrophotometric determinations of the cations. This limitted the upper P^H range to 6.5. It has been noted that most of the mineral cations are absorbed from the stomach and the small intestine whose P^H varies with the species of the animal and the composition of the diet eaten. A P^H of 2 in

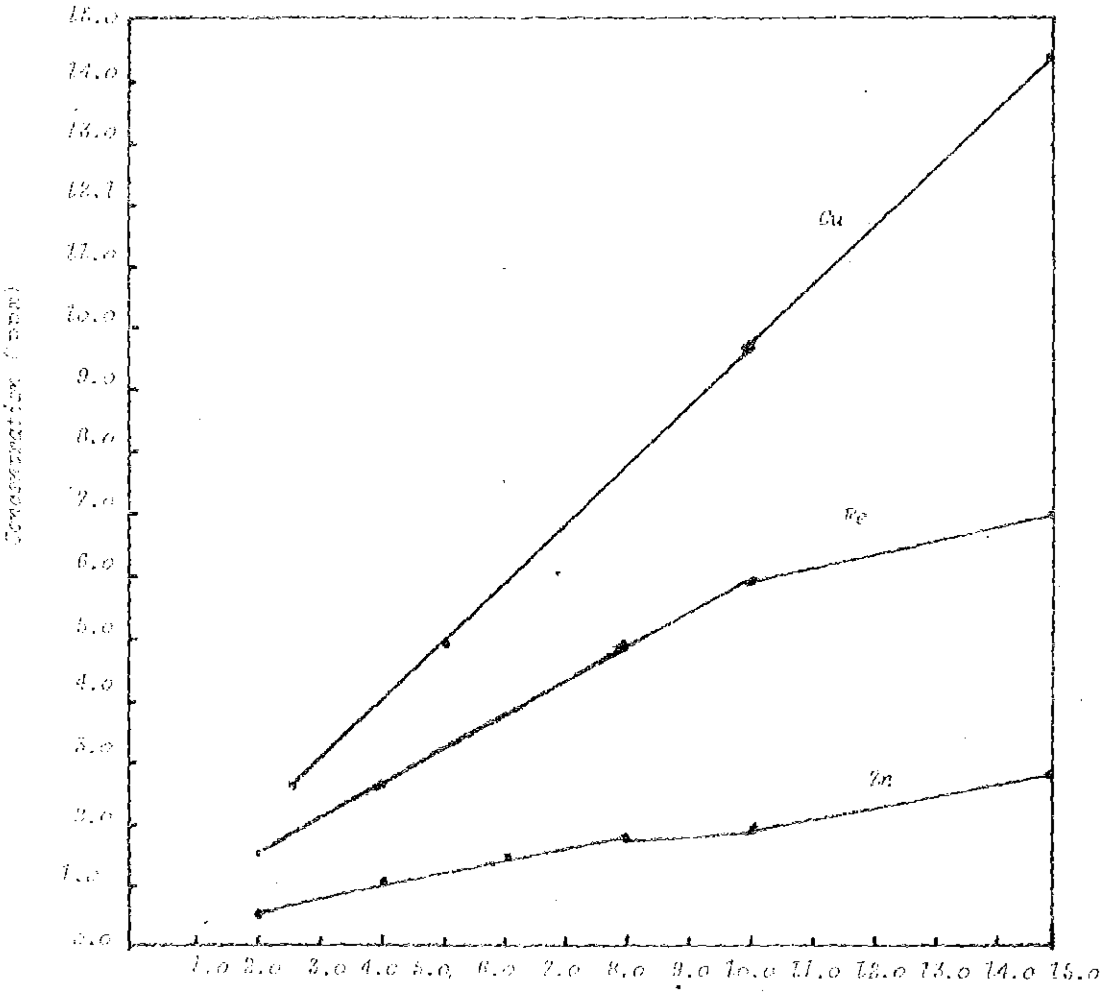


Figure 8. Analytical ranges of concentrations.

(Each point corresponds to the means.)

the pyloric region of the human stomach has been reported (71) and samples aspirated from sites ranging from the proximal to the distal ends of the human deoaenum have shown a p^H range from 3.8 to 7.8 (72). Therefore, choice of a p^H range from 2.5 to 6.5 will give meaningful results that can be correlated to the physiological availability of the minerals.

The level of minerals in the digestive tract affects mineral availability for absorption. The mineral assimilated in the body organs are in dynamic equilibria with that in the digestive tract (54). Change in either direction is controlled by homestatic mechnisms. Dietary fiber interferes with mineral balance by binding the minerals which are lost in the feces (59). The amount bound by the dietary fiber and hence removed through feces depends on the concentration of the cation, the amount of fiber ingested and the fiber particle size (21). The influence of these factors depend on the composition of the dietary fiber (21).

As the two types of grain samples (NBR & BR) are similar in their chemical composition except in their procyanidin content, the NBR samples were used as controls in reporting cation binding by procyanidins, i.e. the difference in binding between the BR & NBR is approximated to the binding by procyanidins.

4.4.1. Effects of p^H on Mineral Binding by Sorghum Grain NDF

The results and possible effects of varying the p^H of the medium are summarized in Table 6.

Table 6 . Effect of p^H on Mineral Binding by NDF

(Fig. 9 & 10) .

p^H range	Observation	Conclusion
2.5-3.5	(1) No significant Binding (2) Leaching of Minerals	Neither procyanidins nor grain NDF could interfere with mineral absorption from the stomach.
3.5-4.5	(1) A slight rise in binding (except Cu, and Zn-BR) (2) No apparent difference between BR and NBR	Procyanidins may interfere with mineral absorption from the duodenum.
4.5-6.0	(1) A sharp rise in binding (except Ca) (2) Large difference between BR and NBR bound minerals	Both sorghum grain NDF and procyanidins can interfere with mineral absorption from the small intestine.
6.0-6.5	Dependance on p^H is lower for the BR sorghum NDF	(1) Procyanidins are not the sole components governing the p^H dependance behavior (2) Procyanidins are saturated with cations. (3) Procyanidins mask other binding sites on NDF.

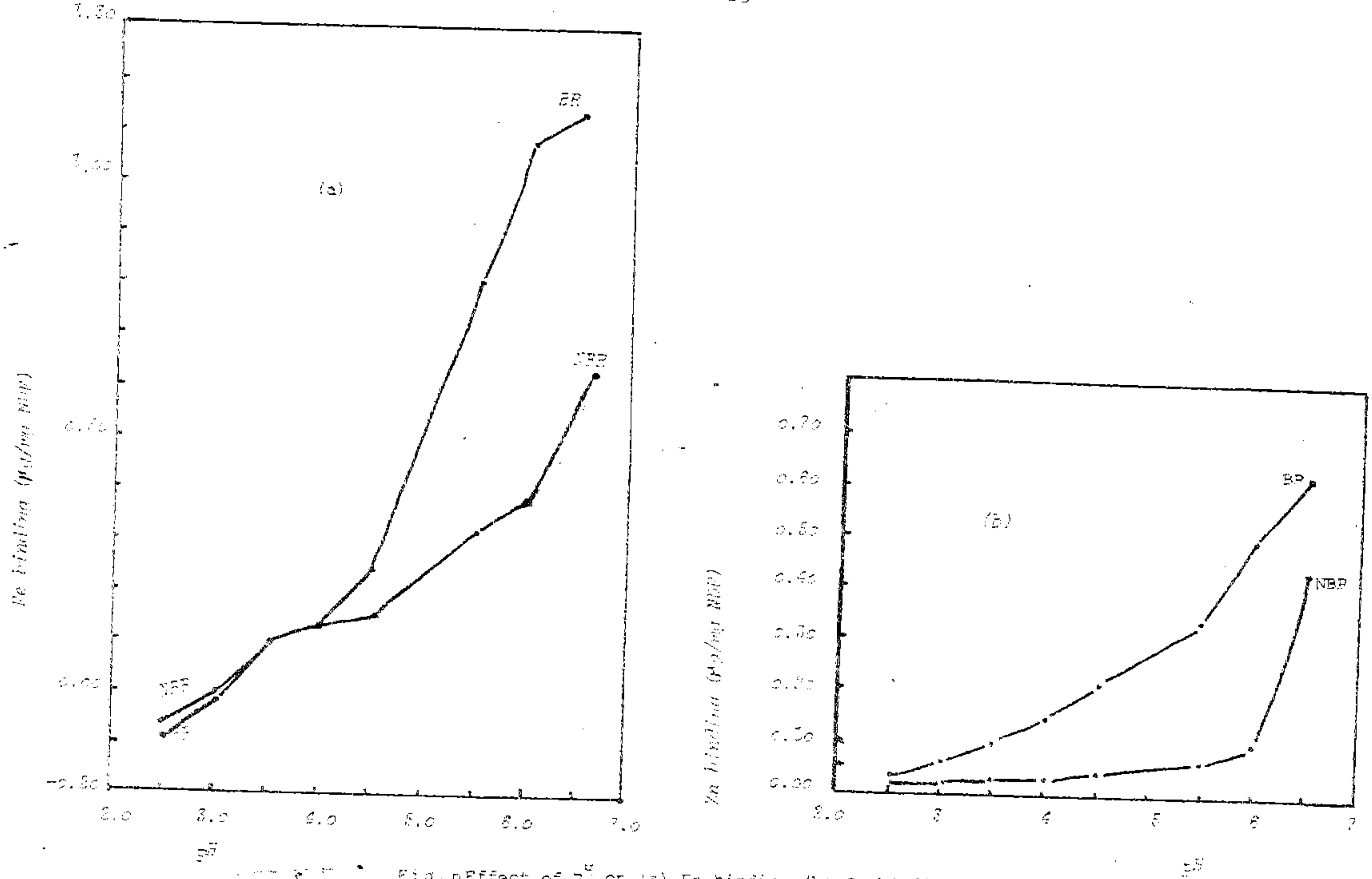


Fig. 9 Effect of pH on (a) Fe binding (b) Zn binding

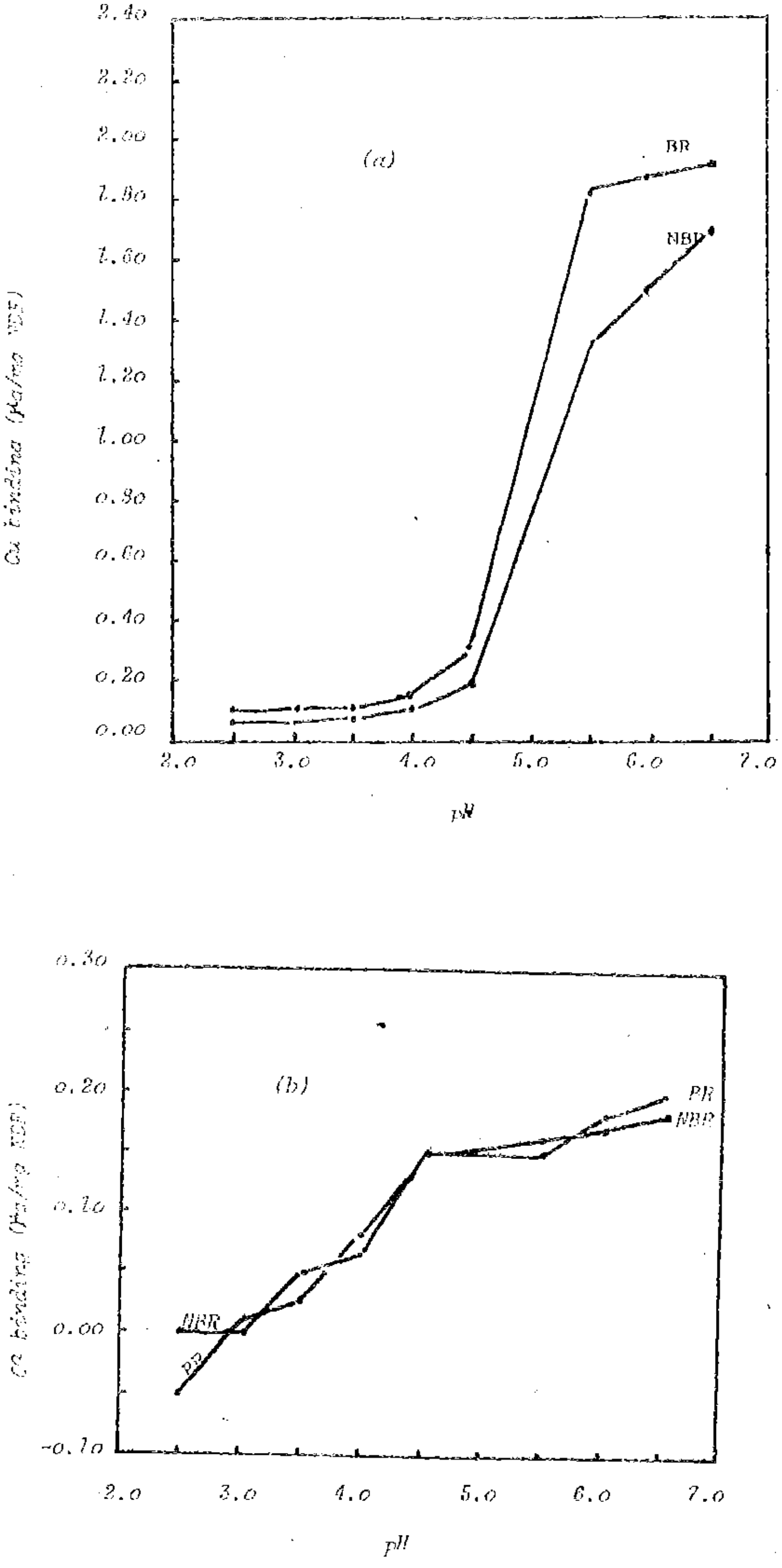
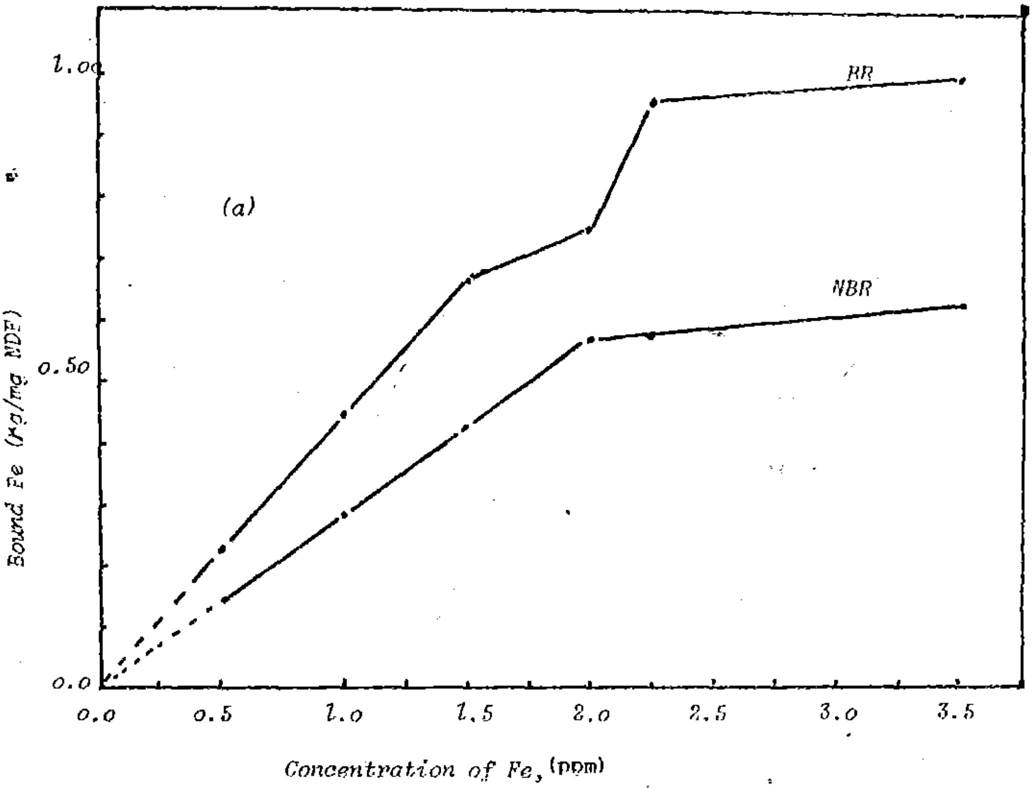


Fig. 10 Effect of P^H on (a) Cu binding (b) Ca binding by Sorghum grain NDF. Each point on the curve corresponds to mean ±0.1 mean deviation.

The p^{Ka} of procyanidins in estimated to be around 9.5. Hence, in moderately basic and netural media, the existence of the phenolate ion is expected. As a result, an enhanced metal ion binding would result. In highly basic solution, the production of phenolate to the extent of 100% might be achieved. Hence, increased complex formation is expected at higher P^H values. Unfortunately, in highly basic solutions, the metal ion exists in the hydroxy form which is unreactive and complex formation with the procyanidins is hampered. In neutral solutions, the metal ion exists in several forms of the hydroxo-aquo species. The presence of a hydroxide adjacent to a water molecule around a central metal ion would labilize the water molecule and an attack of the metal ion by the phenolate group of procyanidin would be enhanced. Once the monodentate liganid has been formed, intra or intermolecular attack would follow resulting in the formation of multidentate procyanidin - cation complexes. In acidic media, the procyanidin molecule exists mostly in the undissociated form. Though the metal ion would be highly reactive in acidic media owing to the formation of the aquo species, the phenolate anion will not be available under acidic conditions and complex formation is not expected.

4.4.2. Effects of Varying Cation Concentrations on Mineral Binding by Sorghum Grain NDF.

The effects of varying the concentrations of the cations on the binding capacities of sorghum grain NDF samples were investigated (Fig 11 and 12). In almost all the cases, the cation binding capacities of NDF samples from both sorghum varities, increase with increasing cation concentrations until the saturation points were reached. The limit of saturation was approached sooner by the NBR fiber samples, which might be ....



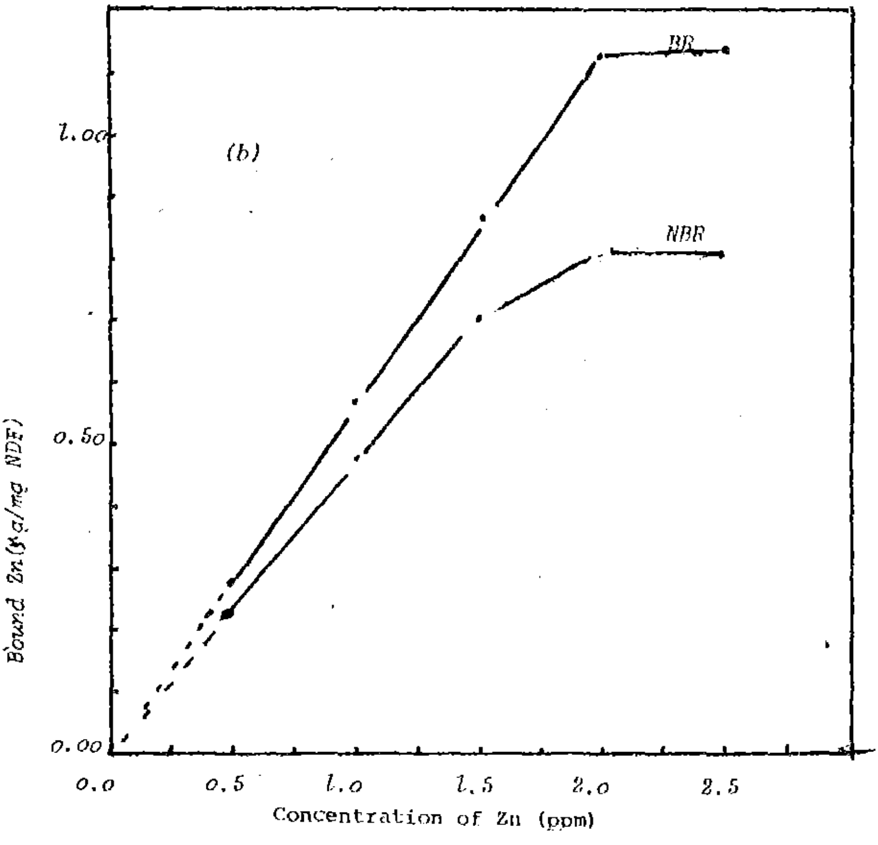


Fig. 11 Effect of (a) Fe concentration (b) Zn concentration on NDF binding capacity. Each point on the curve corresponds to mean ± o.1 mean deviation.

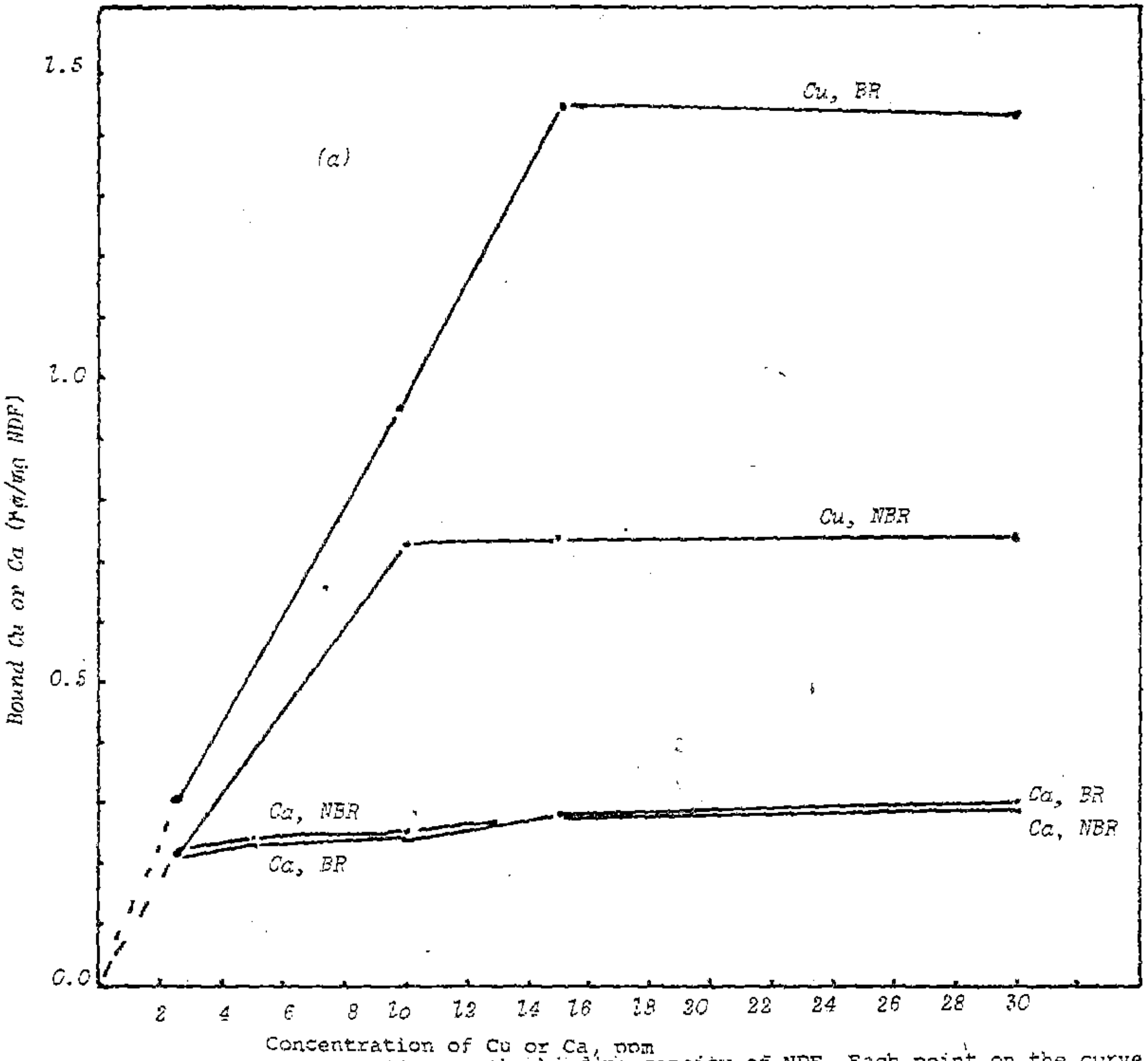


Fig. 12 Effect of Cu or Ca concentration on the binding capcity of NDF. Each point on the curve corresponds to mean ± mean deviation.

explained by their lower binding capacities. At lower cation concentrations, binding varies with the first power of the concentration of the cation. Ca-binding by sorghum dietary fiber appeared to be independent of concentration.

The variation of cation binding by both sorghum grain NDF and procyanidins is consistent with the law of mass action for Fe, Zn, and Cu.

4.4.3. Effects of Varying the Amount and Fiber Particle Size on Mineral Binding.

The effects of amount of fiber in equilibrium with the cations in solution were investigated. The total amount of cation bound increased with increasing amount of fiber, whereas the amount bound per mg fiber decreased (Fig 13 and 14). In contrast to the observations obtained in studying the effects of cation concentration, the difference in binding between the two fibers (NDF from BR and NBR sorghum) remained almost constant through out the range of fiber amount studied.

The effects of fiber particle sizes on cation binding were also studied using different fractions of fiber sieved through a series of screens. It has been observed that with increasing fiber surface area (decreasing fiber particle size) cation binding decreased (Fig. 15). The results obtained in this investigation resemble those obtained by varying the amount of fiber and therefore, owe the same explanation.

The fact that the difference between NDF samples from BR and NBR sorghum grain remained almost constant irrespective of the amount of fiber or particle size shows that the interaction between procyanidins and mineral cations involves

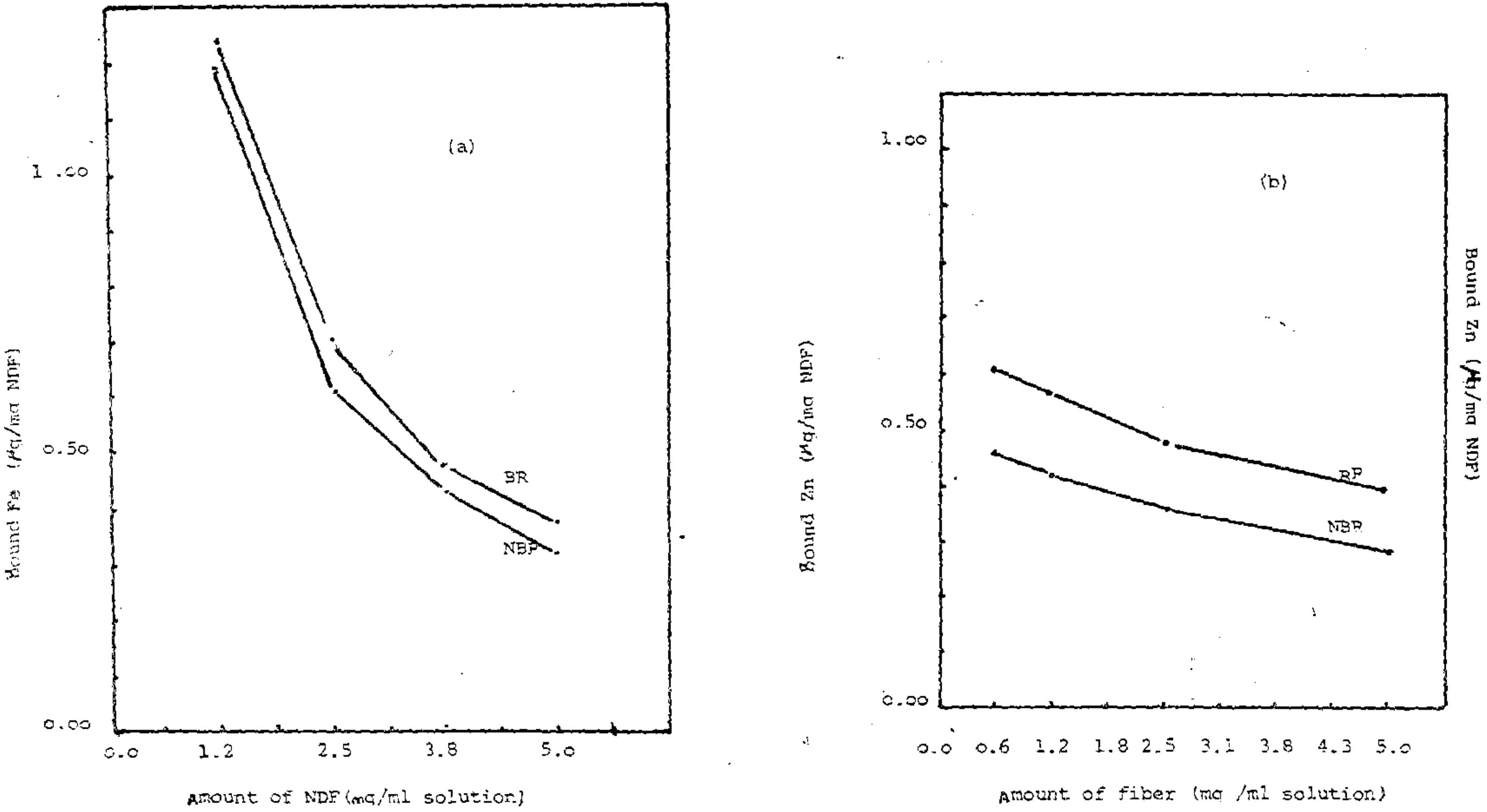


Fig. 13. Effect of amount of fiber on (a) Fe-binding (b) Zn binding by sorghum grain NDF. Each point on the curve corresponds to mean ± mean deviation.

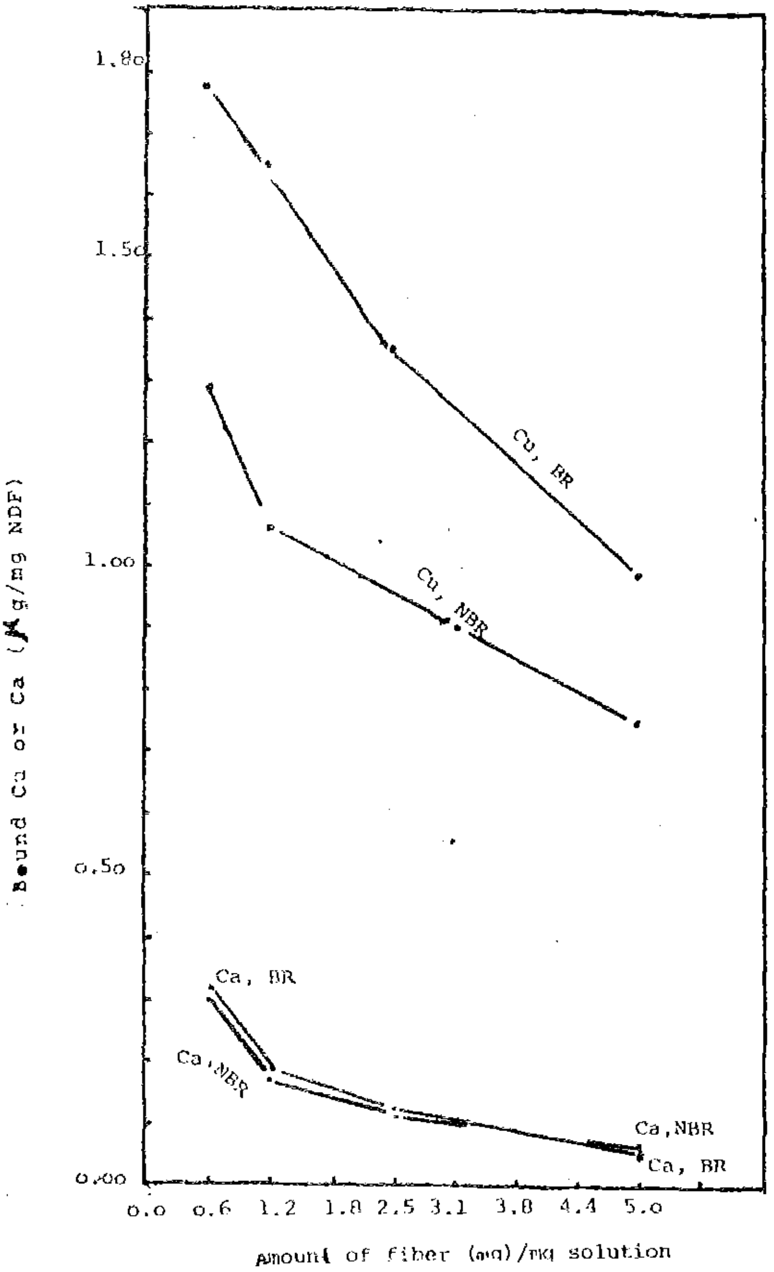


Fig. 14 Effect of amount of fiber on Cu or Ca binding by sorghum grain NDF. Each point on the curve corresponds to mean ± o.1 mean standard deviation.

other types of interactions (probably complex formation reactions) than simple adsorption at the surface of the fiber.

4.4.4. Effect of Competing Minerals

Fe-binding by NDF was decreased by the presence of both Zn, and Cu, with Cu having stronger effect (Fig. 16). The effects of both Zn and Cu increase with increasing concentrations. When the concentration of Zn was increased from 0 to 3.3 ppm, Fe-binding decreased by 29% in the BR and by 34% in the NBR, NDF samples, whereas, increasing the concentrations of Cu from 0 to 6.7 ppm caused a decrease in Fe-binding by 49% in the BR and 48% in the NBR samples. Further increase in concentration of either Zn or Cu had little affect. Similarly, bound Fe decreased by 25% in the presence of 4 ppm of Zn and by 53% in the presence of 8 ppm of Cu.

Zn- binding by NDF was also decreased by the presence of either Fe or Cu (Fig.17)

The effect of both Cu and Fe on Zn - binding increases with concentrations. Variation in the concentration of Fe from o to 4 ppm caused a decrease in Zn - binding by 63% in BR and 70% in the NBR fiber samples. The same change in concentration of Cu caused 41% in both the BR and NBR fiber samples. The amount of procyanidin bound Zn was also decreased by the presence of Cu and Fe upto 2.5 ppm Fe or Cu. Thereafter, the effect of both the ions was negligible.

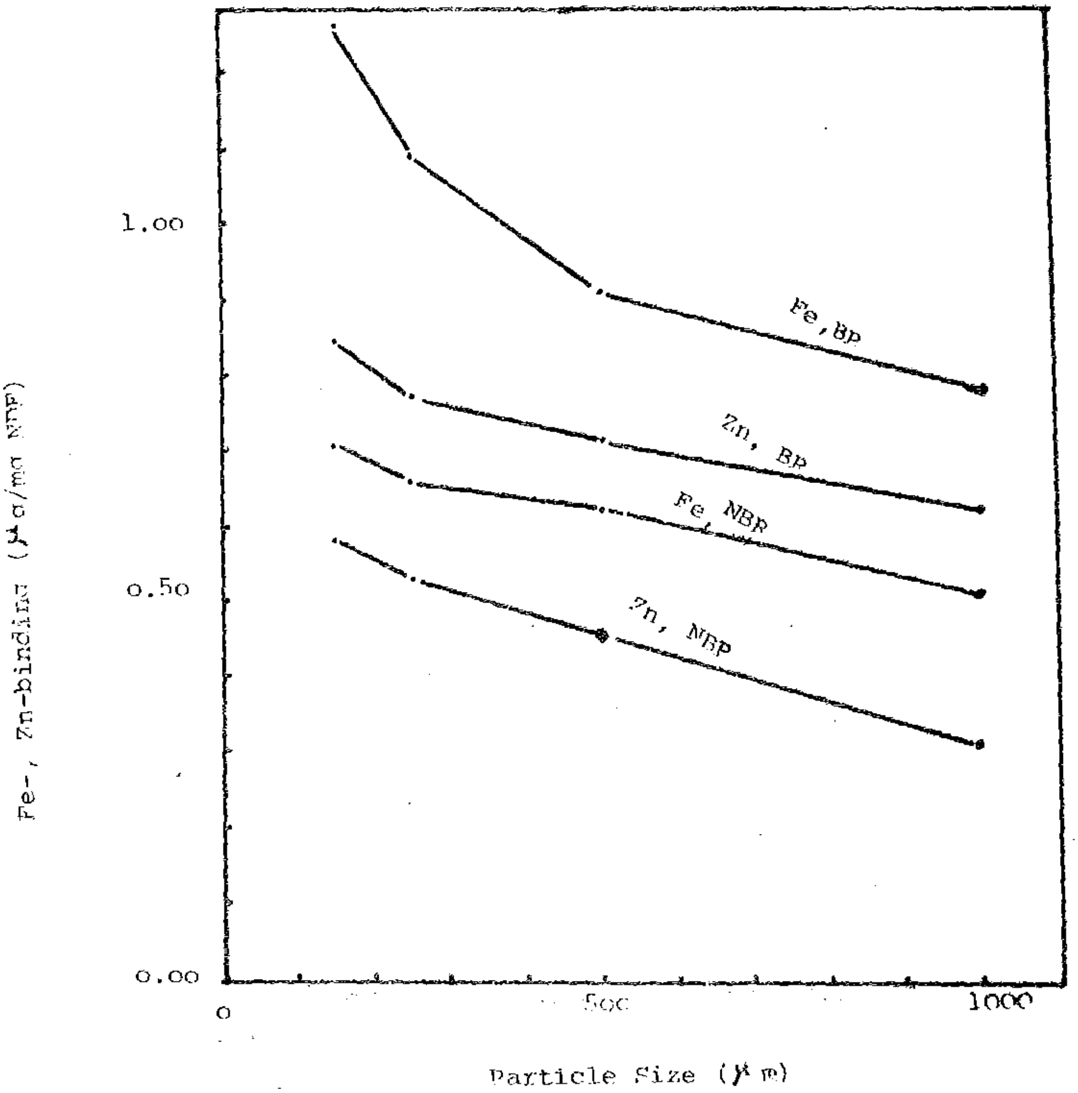


Fig. 15. Effect of fiber particle size on Fe or Zn binding by sorghum grain NDF. Each point on the curve corresponds to mean ± mean deviation.

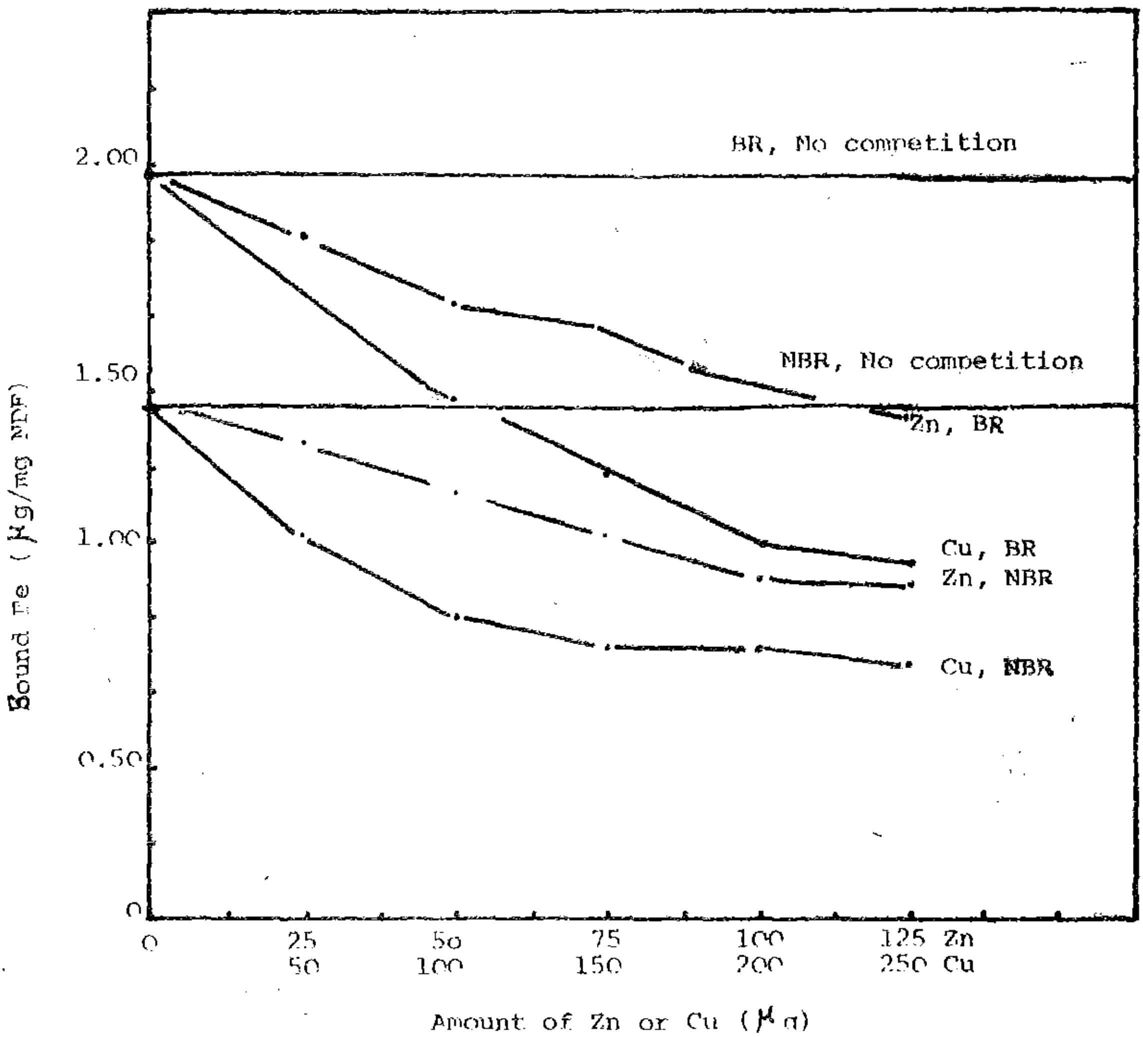


Fig. 16 Effect of Zn or Cu on Fe binding by sorghum grain NDF. Each point on the curve corresponds to mean ± o.1 mean deviation.

Cu-binding by NDF was decreased in the presence of Fe (Fig. 17).The effect of varying Fe concentration had a grater effect on NDF from BR sorghum compared to NDF from NBR sorghum. An increase in Fe concentration from 0 to 3.5 ppm resulted in a decreased binding of 18% by BR and 9% by NBR samples and 48% by procyanidins. These results indicate that Cu has a stronger affinity for sorghum grain fibers compared to Fe. However, Fe appears to be equally or more competative with Cu in its affinity for procyanidins. (The concentration of Cu was twice that of iron).

Cu-binding by NDF samples from NBR sources was enhanced by the presence of Zn. It increased by 8% when the concentration of Zn increased from 0 to 4 ppm. Increasing the concentration of Zn from 0 to 0.4 ppm decreased Cu binding by NDF from BR sources by 8% and Cu binding by procyanidin by 52%. Further increase in Zn concentration enhanced Cu-binding. When the concentration of Zn increased from 0.4 ppm to 2.0 ppm, Cu binding increased by 7% in BR and 16% in procyanidins. This is assumed to be due to the distortion of the fiber structure by Zn which may expose more procyanidins and other molecules of the fiber for Cu-binding. The distortion may have created passages that allow very small atoms such as Cu to interact with NDF but not large enough for Fe-atoms.

The relative affinities of Fe, Zn, Cu, Ca and Mg was determined using a solution containing all of the cations. The order of binding was: Ca > Fe > Mg > Cu > Zn in BR samples and Ca > Mg > Fe > Zn > Cu in NBR samples. Ca and Mg were not bound by procyanidins and comparison of the relative affinities of the trace elements for procyanidins follow the order Fe Cu Zn. The fact that Cu was the least-bound by NDF from NBR sources may be due to the antagonistic effect of Ca or Mg or both.The

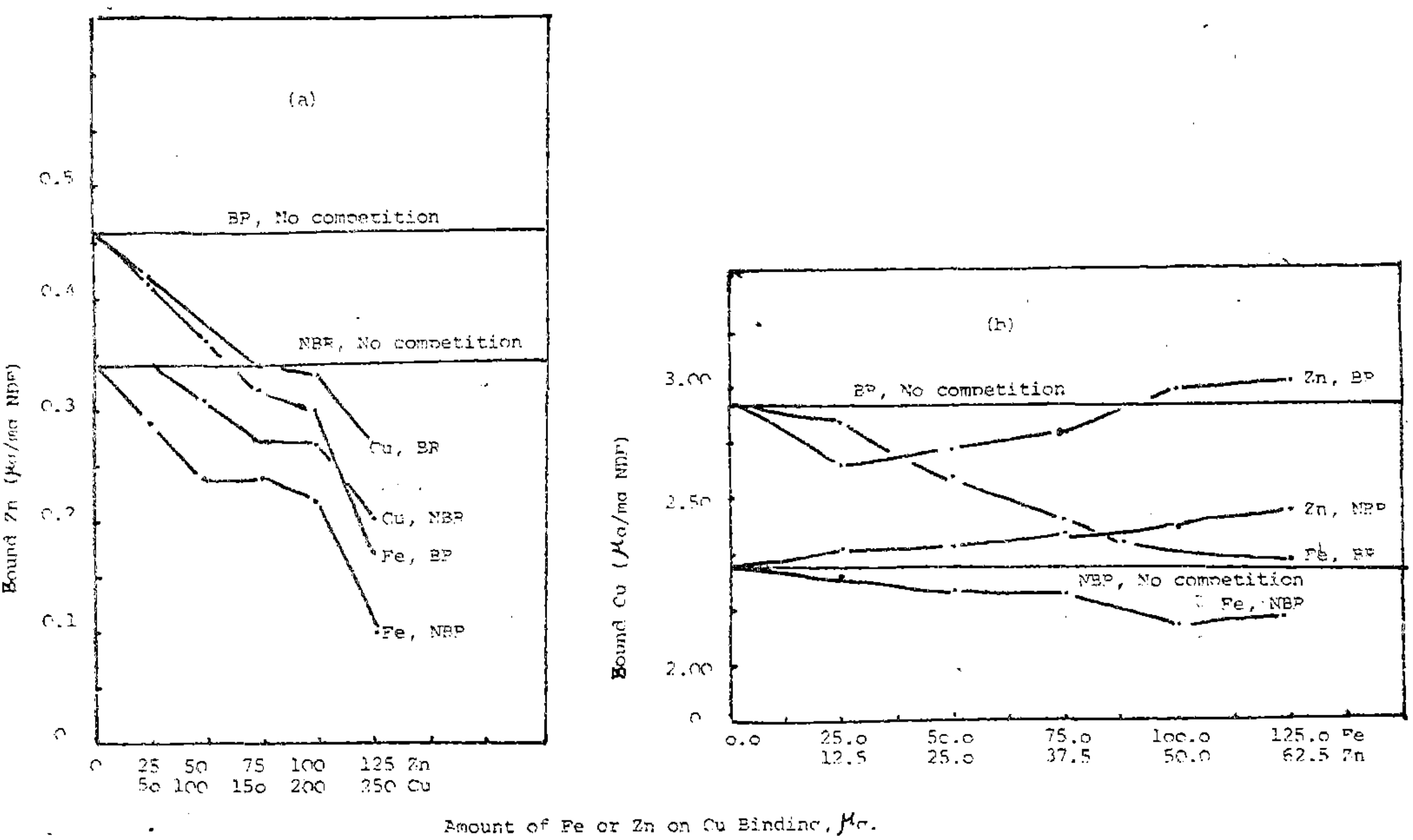


Fig. 17 Effect of (a) Fe or Cu on Zn binding (b) Fe or Zn on Cu binding, by sorghum grain NDF. Each point curve corresponds to mean ± 0.1 mean deviation.

high value for Ca and Mg binding may be due to their high concentrations in the solutions used. More Fe was bound as compared to Cu, despite the presence of Cu over 3 times in excess of Fe. This may be due to the interference of Ca and/or Mg which outweighs the enhancement due to Zn. The amount of cation bound by the BR and NBR grain respectively are: Fe- o.13, o.o1; Zn - o.o3, o.o2; Cu - o.o3, o.o1; Ca - o.38, o37; Mg- o.12, o12 μg /mg NDF. The mean deviation was ±o.1 in all cases.

4.4.5. Mineral Binding by Sorghum Grain NDF under Optimum Experimental Conditions

Based on the results of the studies regarding the effects of experimental parameters on mineral binding, optimum conditions found are given in Table 7.

Table 7 . Optimum Conditions for Mineral Binding by Sorghum Grain NDF

Element	p^H	Cation concentration (ppm)	Amount of NDF (mg)	Fiber particle size (mm)
Fe	6.5o	12.5o	4o	o.5
Zn	6.oo	5.oo	2o	1.o
Cu	5.5o	25.oo	25	1.o
Ca	4.5o	2o.oo	25	1.o

The selectivity of the optimum conditions was based on (1) the value of the binding, (2) the difference between the BR and NBR samples, and (3) the saturation point.

Table 8 . Cation Binding Capacity for Sorghum Grain NDF

(μg/mg NDF)

Identification	Fe	Zn	Cu	Ca
Gambella	0.62±0.01	0.42±0.02	1.58±0.04	0.14±0.02
Tegemeo	0.66±0.01	0.38±0.04	1.76±0.04	0.16±0.01
Melkamasha 79	0.53±0.03	0.36±0.02	1.63±0.00	0.15±0.01
Burraihi	0.70±0.02	0.31±0.01	1.61±0.02	0.05±0.01
N - 13	0.76±0.02	0.43±0.04	1.43±0.01	0.16±0.01
BM 10	1.05±0.07	0.66±0.03	1.92±0.03	0.08±0.01
SVR 8	1.06±0.03	0.62±0.02	2.04±0.04	0.06±0.01
Susa	1.03±0.01	0.65±0.02	2.36±0.08	0.12±0.01
BM 27	1.22±0.02	0.61±0.03	1.89±0.05	0.04±0.01
3K x 72/1	1.17±0.02	0.54±0.02	2.82±0.04	0.17±0.00
8 VR 157	1.01±0.06	0.64±0.01	2.06±0.04	0.13±0.01
E - 1291	0.98±0.07	0.63±0.05	1.97±0.03	0.07±0.01
Ikinyaruka	1.03±0.04	0.65±0.02	2.36±0.03	0.12±0.01
5 Ox - 160	1.00±0.06	0.63±0.03	2.26±0.09	0.12±0.01
Serena	1.16±0.01	0.62±0.02	2.40±0.06	0.15±0.01
5 Dx135/13/1/3	1.13±0.02	0.62±0.02	2.83±0.08	0.20±0.01
Dobbs	1.08±0.02	0.59±0.03	2.22±0.02	0.15±0.00
Is 8686	1.18±0.01	0.64±0.01	2.69±0.04	0.18±0.02
E 525 E7	1.15±0.02	0.55±0.01	2.68±0.04	0.15±0.01
MW 5020	1.12±0.01	0.68±0.01	2.10±0.04	0.18±0.02
verage BR	1.09±0.07	0.61±0.05	2.28±0.31	0.13±0.04
NBR	0.65±0.08	0.38±0.04	1.60±0.11	0.13±0.04
t	11.53	8.89	4.60	0.00
t 0.0005	2.86	-	-	-

Mineral binding by NDF from all of the lines of sorghum were studied under the optimum condition (Table 8). In all cases except for Ca, mineral binding was higher for NDF from BR sorghum compared to those NDF from NBR sorghum.

If grown under the same enviornmental conditions, the two classes of sorghum will have similar chemical compositions except for their procyanidin (polyphenol) contents. Based on this, the **mineral** binding **by** procyanidins may be approximated to the difference between that of the BR and the NBR samples. The calculations have shown that mineral binding by procyanidins as expressed in μg cation per mg NDF are:

Fe: o.44±o.69
Zn: o.23±o.o49
Cu: o.68±o.3o8

calcium was not bound by BR sorghum procyanidins.

The relative binding capcity of procyanidins for Fe(II), Zn(II) and Cu(II), and the impossibility of the formation of procyanidin complexes of Ca(II) and Mg(II) cations can be explained in terms of the Hard-Soft-Acid-Base (HASB) principle (79).

4.5. Determination of Binding Sites of Sorghum

From the studies concerning the effects of experimental parameters, it has been concluded that interactions of sorghum grain NDF or procyanidins involve complex formation reactions. Therefore, it is impossible to explain cation binding by sorghum grain NDF in terms of adsorption isotherms.

However, Scatchard plots were drawn to indicate the number of binding sites for each metal ion based on the data obtained from the studies of the dependance of cation binding on the concentration of the cation.

NDF samples from BR sorghum grain showed three binding sites for Cu, two for Fe and Zn, and one for Ca. Whereas the NDF of NBR sorghum grain showed two binding sites for Cu, one for Fe, Zn and Ca. Each cation had one more binding site in BR sorghum compared to NBR except Ca. This additional binding site in BR sorghum may be due to procyanidins. Since Ca did not bind to sorghum procyanidin, there is no change in the binding sites in both BR and NBR samples.

4.6. Mineral Binding by Soluble Procyanidins

Both extracts from BR and NBR grain bound significant amounts of Fe, Zn, Ca, and Mg but not Cu. (Table 9). Though the extraction technique was not specific for procyanidins (other phenolic substances are also extracted along with the procyanidins), the difference in binding between the extracts from BR (which contains soluble procyanidin) and NBR grain might be used to approximate cation binding by soluble procyanidins. Based on the difference, the average values for procyanidin bound cations are: Fe - o.71± o.o78, Zn -o.12±o.o34, and Cu-o.o2±o.ooo µg/mg grain extract.

Relatively higher amounts of Ca and Mg were bound by extracts from NBR as compared to those from BR grain. This is probably due to the inhibitory effects of procyanidins resulting from their association with Ca - and Mg - binding components in the extract.

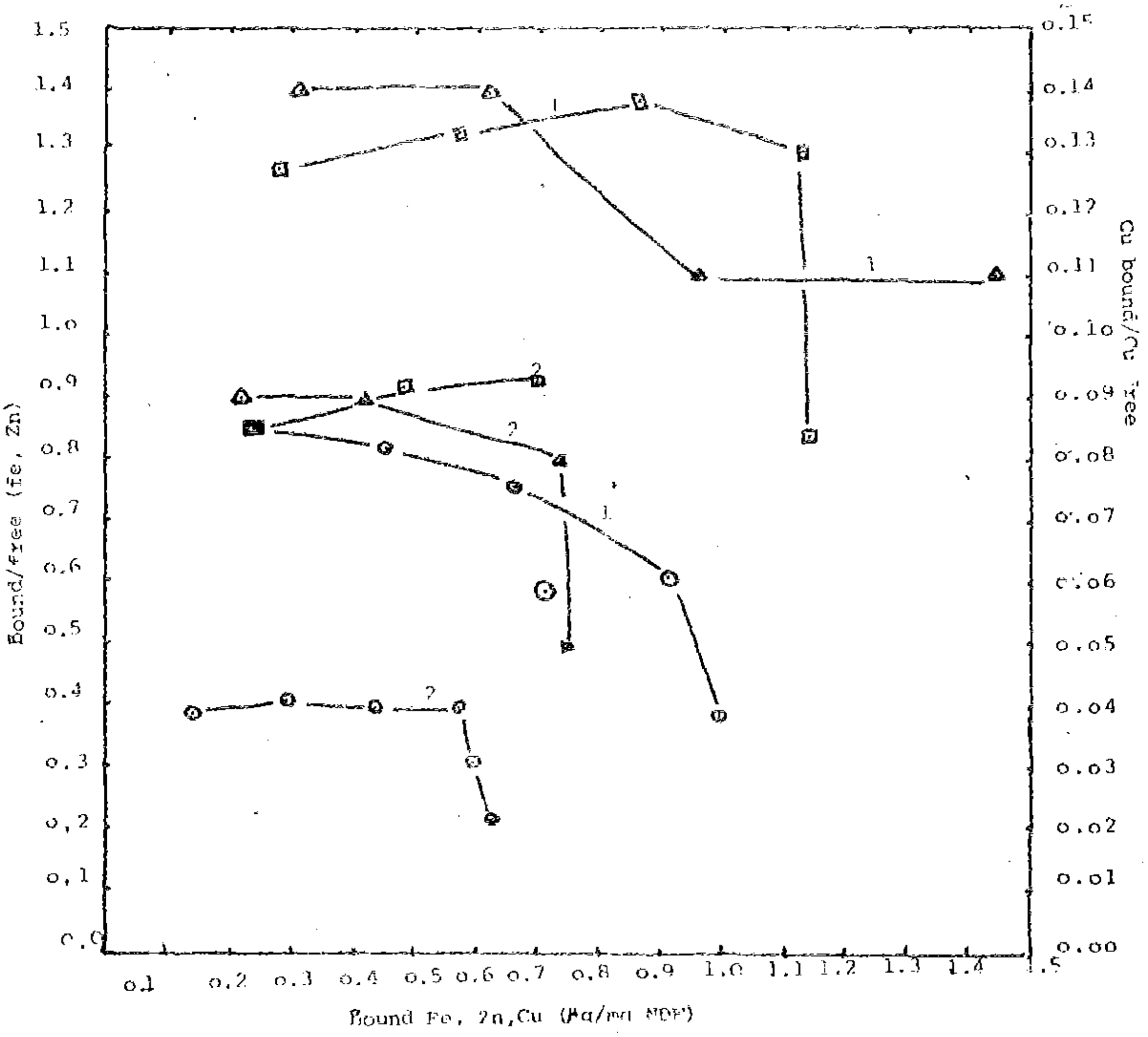


Fig.18 Scatchard plots for Fe (⊙), Zn (⊡), and Cu (△) binding by sorghum grain NDF. Numbers 1 and 2 refere to BR and NBR respectively.

Table 9. Cation Binding by Soluble Procyanidins*

(µg/mg grain extract)

ety	Identification	Fe bound		Zr bound		Cu		Ca		Mg	
		total	procyanidin	total	procyanidin	total	procyanidin	total	procyanidin	total	procyanidin
	Seredo	0.89±0.08	0.64±0.08	0.11±0.01	0.08±0.01	0.05±0.03	0.03±0.03	3.25±0.42	-	1.60±0.10	-
	5DX135X13/1/1	1.07±0.14	0.82±0.14	0.13±0.01	0.11±0.01	0.03±0.02	0.01±0.02	3.92±0.08	-	1.05±0.55	-
	MW 50	0.92±0.04	0.67±0.04	0.18±0.01	0.16±0.01	0.03±0.02	0.01±0.02	3.25±0.58	-	1.15±0.40	-
ean	-----	0.96±0.08	0.71±0.08	0.14±0.03	0.14±0.03	0.12±0.03	0.04±0.1	0.02±0.1	-	1.25±0.75	-
	Tegemeo	0.24±0.10	---	0.01±0.00	---	0.02±0.01	---	3.67±0.17	-	1.55±0.00	-
	Melkamasha 79	0.26±0.02	---	0.03±0.02	---	0.02±0.02	---	3.67±17	-	1.42±0.17	-
Mean	-----	7.25±0.01	---	0.02±0.01	---	0.02±0.00	---	3.67±0.00	-	1.48±0.91	-

* Calculated from the difference betweem total cation binding between BR and NBR sorghum grain.

4.7. Solubility Behavior of the Fe(II) - Procyanidin Complex

A blue colored product was formed when extracts from BR samples were treated with solutions containing Fe(II) cations. The complex associated strongly with the wall of the dialysis tube and made separation difficult. Several solvents including dilute HCl, aqueous acetone, pure acetone, and carbon tetrachloride were tried to get it in solution. Dilute HCl succeeded in dissolving it with a consequent disappearance of the blue color.

5. CONCLUSION

5.1. Techniques

The separation and purification of procyanidins is very difficult because of their chemical nature. Due to this, quantitative determination of their properties remained to be a challenging problem. In these investigations we used samples from two grain classes (BR and NBR). The grain samples have similar chemical compositions except that procyanidins are absent in the NBR samples. Therefore, it is logical to use the NBR samples as controls to study cation binding by procyanidins. We utilized this approach in our investigations. The technique is simple, and enabled us to draw clear conclusions from the results.

The problem of separation became more serious when dealing with soluble procyanidins. In the Yb(III) precipitation methods, besides the organic extracts, inorganic cations and anions added at the various stages made separation more difficult. We alleviated this problem by proposing a new procedure based on dialysis. Dialysis using a membrane of molecular weight cut off (MWCO) of 1000 enabled the removal of contaminating inorganic species and low MW polyphenols from the extratts without loss of the procyanidins. This technique is simple, reproducible and is very useful in binding studies, particularly for those involving homogenous equilibria. However, separation of the procyanidin - cation complexes for further characterization is difficult.

5.2. Effect of Procyanidins on Mineral Availability

From the binding studies, it can be concluded that procyanidins may interfere with mineral metabolism under certain physiological conditions. The effects become significant at neutrality p^H, higher cation concentrations, lower fiber-to-cation ratios, and smaller fiber particle sizes. The presence of one or more competing cations will increase mineral availability. An exception to this is that Cu availability decreases in the presence of high levels of Zn.

5.3. The Nature of the Procyanidin - Cation Complex

The fact that the p^H of the cation solution decreased upon treatment with the fiber samples, and the increase in binding by procyanidin with increasing p^H, indicates the involvement of phenolates in the binding. The strong association of the Fe(II) - procyanidin complex to the wall of the dialysis tube shows that either free OH groups must be present to form H-bonds with the molecules of the wall material or cationic complexes must have been formed. However, the later is less probable as it leads to a coordination number of one for the transition metal cations. Therefore, based on these arguments it is reasonable to propose the structure given in Fig. 9" for the metal-procyanidin complex. However complete characterization of the structure of the complex requires further investigation.

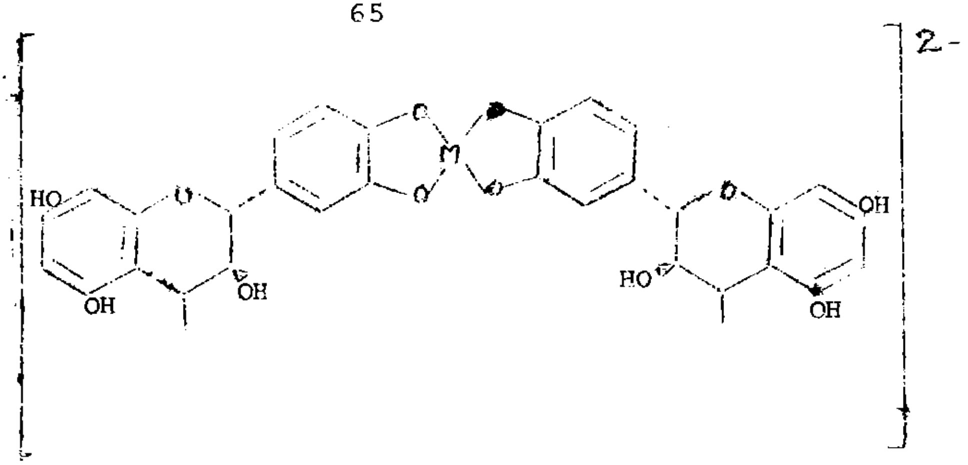


Fig. 9 Proposed structure for the cation [Fe(II, Zn(II), Cu(II)]- procyanidin complex.

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