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PINTO BEAN HULLS, BUT NOT CONDENSED TANNINS, ALTER COPPER BIOAVAILABILITY IN RATS

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PINTO BEAN HULLS, BUT NOT CONDENSED TANNINS, ALTER COPPER BIOAVAILABILITY IN RATS

By

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ABSTRACT

PINTO BEAN HULLS, BUT NOT CONDENSED TANNINS, ALTER COPPER BIOAVAILABILITY IN RATS

By

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Bioavailability of copper in diets containing condensed tannins was determined. Male rats were fed: control, 1.6 % catechin or 25 % pinto bean hulls diet in Expt I, control or 25% hulls in Expt II. The level of catechin was based on the amount of condensed tannins in the hulls diet. No significant differences were found in weight gain and food intake. Liver relative weight was significantly lower in hull-fed rats. Catechin-fed rats had a higher mean tibia weight. Tissue copper concentration were similar in all groups. Hull-fed rats had significantly elevated plasma copper and ceruloplasmin activity but decreased total liver copper. However, condensed tannins fed as catechin had no effect on cooper bioavailability at the levels fed in this study. Thus, these results suggest that in rats the consumption of bean hulls, but not condensed tannins, is associated with apparent mobilization of copper from the liver to the circulation.

For my family

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I wish to express my gratitude to Dr. Wanda L. Chenoweth for her encouragement and understanding throughout my graduate studies. I would also like thank Dr. Maurice R. Bennink and Dr. Dale R. Romsos for their commitment on my research and personal growth, Dr. Pao K. Ku, Department of Animal Science, for the technical assistance during my experiment time, Dr. Greg Fink for his help with statistical analysis.

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INTRODUCTION

Legumes provide a valuable source of food energy to people, especially in developing countries. They are also a good source of protein, dietary fiber, vitamins (B1, niacin, pantothenic acid, and biotin) and certain minerals (iron, copper, and zinc). Increased use of legumes in western diets would bring these diets closer to recommended dietary goals in terms of complex carbohydrates and dietary fiber (Walker, 1982). However, it is also well recognized that mineral bioavailability from plant sources is generally low. The factor or factors responsible for this low bioavailability have not been clearly identified.

In the past years, much attention has been given to destruction of antinutritional factors such as trypsin/ chymotrypsin inhibitors, phytohemagglutinin (lectins), and phytates by appropriate processing. Recently tannins (polyphenols) in many edible plant products have received increasing attention as a result of their possible influence on the nutritional and aesthetic qualities of foods, biochemical and physiological functions and pharmacological implications (Reddy *et al.*, 1985 Garcia-Lopez *et al.*, 1990).

Phenolic compounds occur naturally in many plants including coffee, tea, and legumes. Tannins are known to function as natural defense agents in plants against insects, fungi, and preharvest seed germination (Salunkhe *et al.*, 1990). The presence of phenolics in certain colored legumes may improve their acceptability over white beans, perhaps due to taste.

If present in appreciable quantities, tannins lower the nutritional value and biological availability of dietary proteins. Experiments with a variety of laboratory animals have shown that dietary tannins decrease the growth rate, feed efficiency, and egg production by laying hens (Butler *et al.*, 1986). Tannins have been shown to cause leg abnormalities and ultrastructural changes in the livers of chicks and affect the physical or sexual maturity and even death in hamsters (Jansman, 1994). The major antinutritional effect of dietary tannins is the formation of stable complexes with dietary proteins and perhaps with digestive enzymes, which ultimately results in poor digestion and absorption of dietary nutrients. Tannins have also been reported to form complexes with starch and reduce its digestion.

Tannins also are known to act as an inhibitory factor on iron absorption when tea and coffee are consumed with various diets (Disler *et al.*, 1975; Morck *et al.*, 1983; Brune *et al.*, 1989). Conflicting results have been reported for the effect of tannins in legumes on iron bioavailability (House and Van

Campen, 1994). The effect of legume tannins on copper has received little attention. Therefore the focus of the present study is on the effect of bean hull tannins on copper bioavailability.

LITERATURE REVIEW

CHEMISTRY OF TANNINS

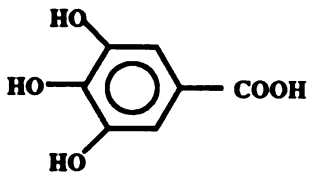
Originally, the term "tannin" was used by Seguin (1796) to describe substances in vegetable extracts which are responsible for converting animal skins into stable product leather (Salunkhe *et al.*, 1990). The substances essential in the tanning process were later identified as polyphenolic compounds with various molecular size and complexity. Tannins are present in a large number of products of vegetable origin used as human foods or animal feeds. The form of the phenolic compounds vary from small monomeric phenolic acid (e.g., gallic acid) to large polymerized polyphenols (e.g., tannins).

Bate-Smith and Swain (1962) defined plant tannins as naturally occurring water-soluble phenolic compounds with a molecular weight between 500 and 3000, containing a sufficiently large number of phenolic hydroxyl or other suitable groups which are capable of precipitating alkaloids as well as gelatin and other proteins from aqueous solution (Gupta and Haslam, 1980). From this definition it is clear that tannins are chemically not well-defined substances but rather a group of substances with some common properties. Polyphenols referred to as tannins have a considerable number of phenolic groups. They are capable of forming effective

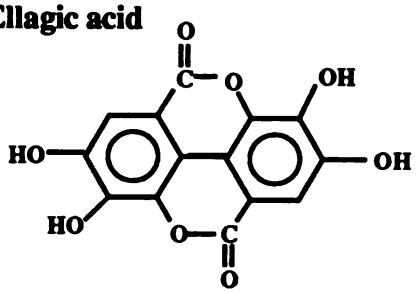
cross-links with other molecules as hydrophilic or hydrophobic complexes. According to White (1957), phenolic compounds with a low molecular weight (<500) do not form stable cross-links with other molecules. However, compounds with a much higher molecular weight (>3000) do not show tanning properties because they appear to be too large to penetrate into the collagen fibrils in hides (Swanson, 1993).

Tannins have been classified by Freudenberg (1920) into hydrolyzable and non-hydrolyzable or condensed tannins which are differentiated by their structure and reactivity toward hydrolytic agents (Gupta and Haslam, 1980). Hydrolyzable tannins are classified as gallotannins (so-call tannic acid) or ellagitannins on the basis of tannic acid structure relative to gallic or ellagic acids. Figure 1 shows the structure of gallotannin and ellagitannin, respectively. Tannic acid is a well-known gallotannin and contains 8 to 10 moles of gallic acid per mole of glucose. Ellagitannin is a hexahydroxydiphenic acid linked with glucose as a diester in addition to gallic acid. Hydrolyzable tannin can be acid, alkaloid, or enzymatically hydrolyzed to yield glucose or some other polyhydroxy alcohol and gallic acid or some phenolic acids related to it (Swanson, 1993). Although many low molecular weight phenols are metabolized in the bodies of higher animals, the metabolic fate of these phenolic compounds is not yet clearly known (Salunkhe *et al.*, 1990). Jansman (1994) suggested that in animals tannic acid is degraded into

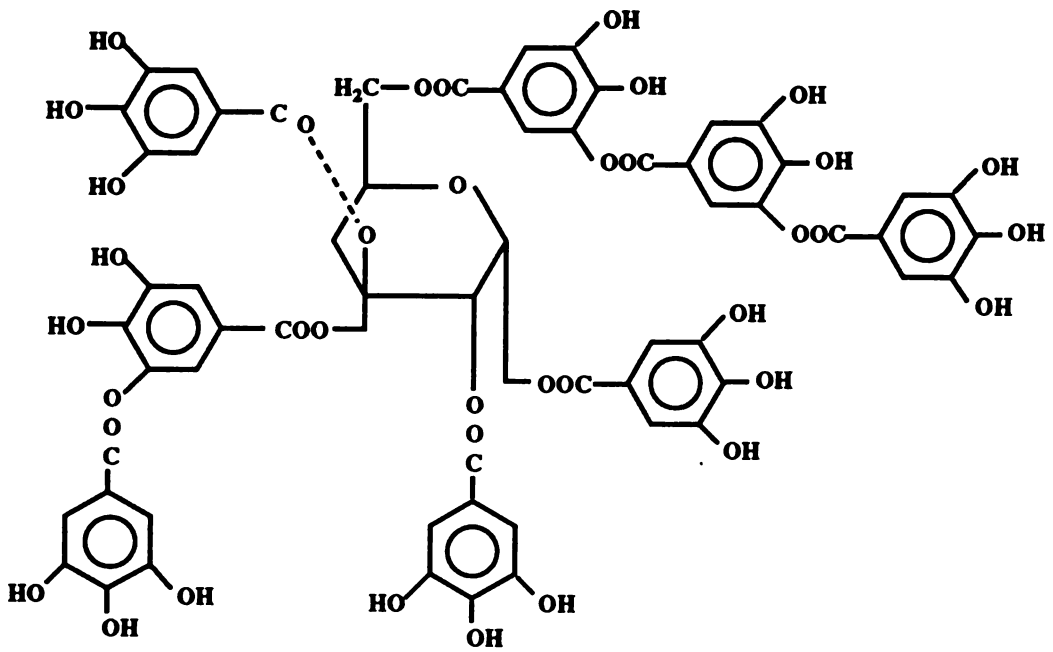
Gallic acid



Ellagic acid



1) Gallotannin (Tannic acid)



2) Ellagitannin

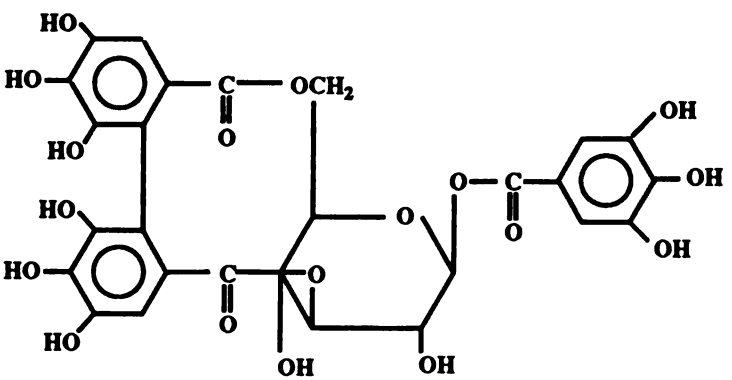


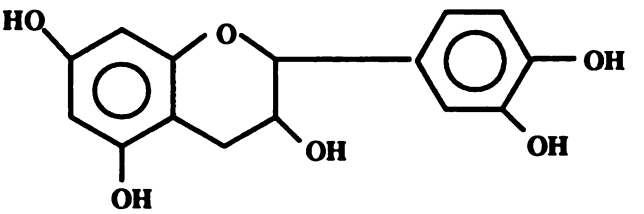
Figure 1 Structure of gallotannin and ellagitannin

gallic acid derivatives, mainly 4-methoxy gallate (4-O-methyl gallic acid). Oral administration of tannic acid to chickens resulted in some gallic acid excretion in the urine but not in the feces. Sources of hydrolyzable tannin are seed pods, bark, woods, fruits and leaves of plants belonging to the family Leguminosae, Fabaceae, Combretaceae, Anaacadiaceae (Salunkhe *et al.*, 1990).

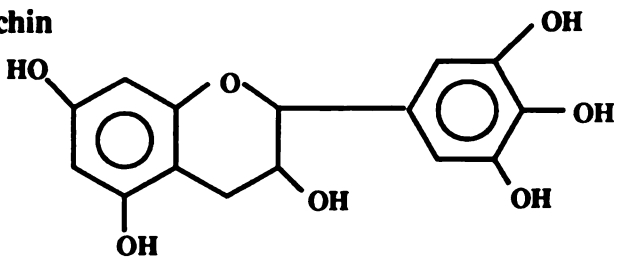
A second category of polymeric flavonoids, the condensed tannins are resistant to hydrolysis in the gut, but on severe acid or alkaline treatment yield less-soluble polymeric phlobaphanes or monomeric flavonoids such as catechin or epicatechin. Condensed tannins contain linkages between the 4'-position of one catechin residue and the 6'- or 8'-position of another flavonoid. They are mainly oligomers of flavan-3-ols (e.g., catechin) and flavan-3,4-diols. Figure 2 shows examples of condensed tannins. The full chemical nature and exact metabolic routes of condensed tannins still have not been elucidated (Gupta and Haslam, 1980; Jansman, 1994). Condensed tannins are also referred to as flavolans or procyanidins. Flavan-3-ols with a molecular weight below 3000 are soluble compounds. Higher polymerized procyanidins become insoluble and are often more closely linked to the structural tissue of the plant (Salunkhe *et al.*, 1990). Flavan-3,4-diols belong to the class of leucoanthocyanidins because they polymerize upon heating in acid solutions not only to phlobaphene-like products (tannin reds), as flavan-3-ols do,

A. Flavan-3-ols

Catechin

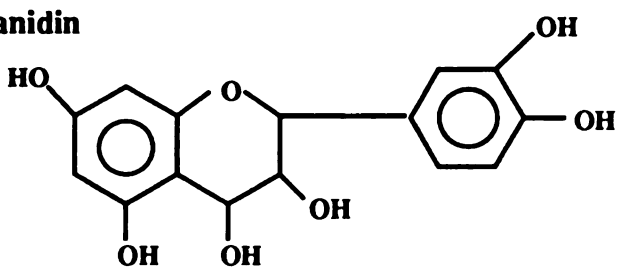


Gallocatechin



B. Flavan-3,4-diols

Leucocyanidin



Leucoanthocyanidin

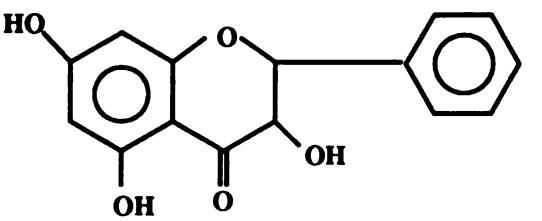


Figure 2 Structures of condensed tannins

but also to anthocyanidin.

Condensed tannins are the predominant class of polyphenols in green vegetables, fruits, cereals, and legumes (Salunkhe *et al.*, 1990). The concentration of tannins in plant tissue used commercially as sources of food may vary from 5 to 50% of the material's dry weight (Singleton, 1981). The pigmented varieties of cereals and legumes contain 2 to 4% condensed tannins, although values of up to 7 to 8% are reported for red high-tannin sorghum varieties (Salunkhe *et al.*, 1990). Other food and beverages, such as cider, cocoa, and red wines, contain considerable amounts of condensed tannins. Strong preparations of these beverages may have as much as a gram of tannin per liter. An intake of dimeric flavans as high as 400 mg/day in the American diet from a variety of sources has been reported (Reddy *et al.*, 1985). The total dietary tannin intakes in such cases would be somewhat higher than dimeric flavans. However, this may depend on the composition of the diet and diet patterns. High bean-based diets may supply higher levels of dietary tannins. For example, analyses of diets consumed in different regions of India indicated that daily intake of tannins varies from 1500 to 2500 mg (Rao and Prabhavati, 1982).

TANNINS IN BEANS

Tannins in legume seeds appear to play a role in the crop's resistance to being eaten by birds. They also play a role in their susceptibility to attack by fungi and pests and in the incidence of preharvest germination. Tannins may form a physical barrier, which prevents water imbibition necessary for germination (Salunkhe *et al.*, 1990).

With regard to the legume seeds, tannins have been found in dry beans (*Phaseolus vulgaris*), peas (*Pisum sativum*), chickpeas (*Cicer arietinum* L.) and lentils (*Lens culinaris*). In most grain legumes tannins are present as condensed tannins (Salunkhe *et al.*, 1990; Jansman, 1994).

Tannin content in beans is unevenly distributed in the seed and varies with seed color (Martin-Tanguy *et al.*, 1977; Deshpande *et al.*, 1982). White beans have been shown to contain a low amount of tannin, whereas colored seeds contained high concentrations irrespective of the method of estimation. Cabrera and Martin (1986) found a clear correlation between color of the flower, seed color and tannin content of faba beans. White-flowering varieties, with no pigments in the flowers, yielded white and grey seeds with low tannin contents. Colored-flowering varieties yielded seeds of different color with the amounts of tannins increasing progressively in seeds having green, red, beige or brown colors.

Condensed tannins are mainly located in the hulls of the colored seeds, with low or negligible amounts in the cotyledons. Desphande and Cheryan (1985) reported that the seed coats contained approximately 0.5 to 7% tannins on a dry weight basis. Rao and Prabhavathi (1982) reported that after decorticating there was very little tannin detectable in cotyledons, indicating that almost all the tannins of the seed are present in the seed coat. Deshpande and Salunkhe (1982) also found that removal of seed coats lowered the assayable tannin content of beans by 68 to 95% and dehulled pinto beans had a 94.6% reduction of assayable tannin content expressed as mg catechin equivalents. Later, Laurena *et al.* (1984) also detected 2 to 3 times greater concentration of assayable tannins in eight varieties of cowpeas when tannins were determined in seed coats alone. Deshpande and Cheryan (1985) showed that when expressed on total seed weight basis, the concentration of assayable tannins in seed coats was 1.1 to 2.5 times compared to the concentration in whole bean flours.

Soaking beans before cooking is a common practice used to soften the texture and hasten the cooking process. Reduction in tannin concentration of several beans by soaking in different solutions was reported by Deshpande and Cheryan (1985). The leaching of bean tannins increased with the time of soaking. Soaking winged beans and cowpeas for 24 hr reduced tannin concentration by 50% and over 50%, respectively, as reported by Sathe and Salunkhe (1981) and

Laurena *et al.* (1986). Since tannins are present in the outermost layers of the seed during soaking, they may diffuse into the seed and bind with proteins to form insoluble complexes. Deshpande and Cheryan (1985) suggested that the apparent loss of tannin after soaking was caused by the formation of such complexes which were not assayable by the method used to determine tannin content.

A reduction in tannin concentration of beans can be seen during cooking, another common process used for softening beans. Cooking whole seeds and discarding the cooking water caused a reduction of 37.5 to 77% in the tannin concentration in beans (Salunkhe *et al.*, 1990). According to Bressani *et al.* (1982), cooking resulted in a 61 to 98% decrease in the tannin concentration of beans and a 17.8 to 50.9% decrease when taking into consideration the tannin concentration in the cooking waters. Price *et al.* (1980) reported that moist heat cooking of grains reduced the assayable tannin from 2.6 to 0.4%; however, this apparent reduction in tannins was not accompanied by a proportional improvement in weight gain or food efficiency in a rat feeding trial. A decrease in the tannin concentration of mung beans, pigeon beans and cowpeas during cooking has also been reported (Gahlawat and Sehgal, 1992). Bressani *et al.* (1982) suggested that the decrease in tannin concentration may be not due to an actual decrease in tannins but due to their binding to other organic substances and protein thus reducing their extractability, since

compounds that have reacted with amino groups of proteins are not extracted by methanol (Reddy *et al.*, 1985; Gahlawat and Sehgal, 1992). Alterations in the chemical structure of tannin as a result of heating have never been shown (Jansman, 1994).

Condensed tannins constitute the major portion of dry bean tannin concentration. Relatively few studies have been reported on the chemical nature of legumes tannins. Martin-Tanguy *et al.* (1977) reported that seed coats of colored-flowering varieties of faba beans (*Vicia faba* L.) contain condensed tannins of the proanthocyanadin type. They found the polymers consist of molecules of flavan-3-ols (catechin and gallocatechin) and flavan-3,4-diols (leucocyanidin and leucodelphinidin) and are joined together by a carbon-carbon linkage between C4 of one unit and C6 or C8 of other units. Chains were linearly linked with a flavan-3-ol at the terminal end.

TANNINS ANALYSIS

According to Deshpande and Cheryan (1987), one of the major difficulties encountered in polyphenol research is the lack of a standard quantitative method for the analysis of phenolics that would be suitable for a wide range of seeds, forage crops and food products under varying experimental conditions. Several different methods have been developed for

the measurement of tannins in plants, based on different principles and measuring different structural groups in these compounds, either all phenols as a group apart from nonphenols or specific individual substances or classes of phenols. However, these assays, due to the complexity and heterogeneity of tannins, do not provide completely satisfactory results. They can, nevertheless, be categorized into three groups: colorimetric methods, protein precipitating methods and other methods. TABLE 1 gives the standard and compounds measured in these methods.

TABLE 1

Analytical methods for tannins

Method	Standard	What is measured?
Vanillin	Catechin	Leucoanthocyanidins Proanthocyanidins
Folin-Denis	Catechin	Total phenols
Prussian Blue	Tannic acid	All reducing compounds
Protein Precipitating	Tannic acid	Proanthocyanidins

Adapted from: Salunkhe *et al.* (1990), p 43.

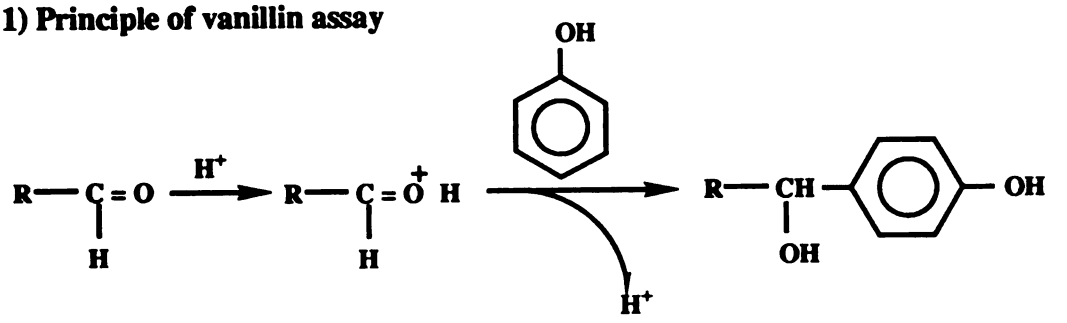
Colorimetric Methods

The methods most frequently used in the earlier experiments on beans are the vanillin-hydrochloric acid assay, expressed as catechin equivalents (Swain and Hillis, 1959; Burns, 1971; Price *et al.*, 1978); the Folin Denis assay, expressed as tannic acid (Gupta and Haslam, 1980; Bressani *et al.*, 1983; Deshpande and Cheryan, 1987) and Prussion blue assay, expressed as tannic acid (Price and Butler, 1977).

The vanillin-HCl assay is widely employed as a method for the quantitative determination of condensed tannins in fruits, sorghum and forage legumes. The assay determines more specifically the polymeric phenols, especially flavan-3-ols, dihydrochalcones and proanthocylanidins. The basic reaction is based on the substitution of aldehyde groups in vanillin for the resorcinol group in flavanols and favanoids, yielding a red colored condensation product which is measured spectrophotometrically at 550 nm. Figure 3 summarizes the principle and shows an example of the condensation reaction. For convenience, catechin, a monomeric flavan-3-ol unit, is used as the standard substance and the assay gives no reaction with substances such as gallic acid and tannic acid without resorcinol groups (Brune *et al.*, 1991).

Polyphenols of cereals and legumes are predominantly condensed tannins (Thompson *et al.*, 1983). Bressani *et al* (1983), therefore, stressed a need to have more specific methodology to determine tannins and their chemical nature in

1) Principle of vanillin assay



2) Example of vanillin assay

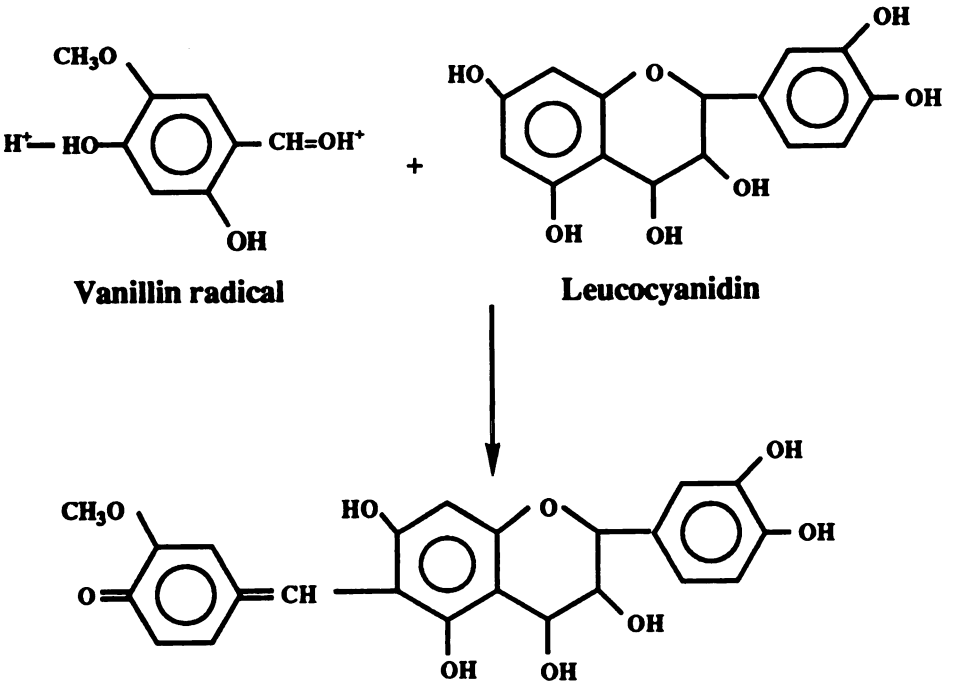


Figure 3 Principle and example of vanillin assay

Adapted from: Salunkhe et al. (1990). p 80.

beans. Recently Deshpande and Cheryan (1985) have critically reevaluated the vanillin reaction for the detection of tannins in legume. They modified the 0.5% vanillin assay as described by Price *et al.* (1978), by using appropriate sample blanks to eliminate variation due to the background color caused by anthocyanin-like pigments. The inherent color variation within a given sample can be minimized by the careful selection of at least three representative samples and grinding them separately prior to the analysis.

The Folin-Denis method is nonspecific and detects all phenolic groups in the extract, including those found in extractable proteins, as well as reacting with reducing substances such as ascorbic acid (Thompson *et al.*, 1983). Since not all phenolics are of nutritional concern, the "total phenol" content of foods as routinely expressed may not be a true index of the nutritional quality of foods. The use of tannic acid as a "reference standard" is also questionable since its biological properties differ from those of tannins of flavonoid origin.

Protein Precipitating Methods

Protein precipitating assays can be used to determine either the tannin content of a sample or the biological activity of tannins (Hagerman and Butler, 1978). Different proteins such as gelatin, casein, bovine serum albumin, hemoglobin and different enzymes have been used in this assay by various investigators (Salunkhe *et al.*, 1990). Each

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protein precipitating assay gives a different response with tannins of different sources. Many factors that may be responsible for the differences have been suggested by Hagerman and Butler (1978) such as the characteristics of the tannins (molecular weight, structural heterogeneity), the protein source (degree of glycosylation, amino acid composition and molecular weight) and reaction conditions (pH, temperature, reaction time, relative concentrations of the reactants). Tannins tend to complex with proteins such as gelatin or specific proline-rich proteins that have a high content of proline resulting in a protein with a loose structure (Asquith and Butler, 1986).

Other Methods

To understand the biochemistry of physiologically active tannins, it is necessary to separate and purify tannins in an unmodified state. Qualitative assays with thin-layer chromatography (TLC) have evolved to quantitative assays with high-performance liquid chromatography (HPLC). More detailed information on the structure and nature tannins can be obtained by mass spectral analysis, droplet countercurrent chromatography, centrifugal partition chromatography (Okuda *et al.*, 1989), nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy (Jansman, 1994).

Some attempts have been made to separate various tannin extracts chromatographically on gel permeation columns. The most successful results were obtained with columns of

hydroxypropylated dextran gel such as Sephadex LH-20 by using alcoholic solvents, aqueous methanol or ethanol. According to Okuda *et al.* (1989), tannins can be separated on the basis of differences in adsorptivity of polyphenolic compounds on the gel rather than on the basis of gel filtration. In a first step using 95% ethanol as eluent, non-tannin phenolic compounds were separated from the tannin-containing compounds. Subsequent elution of the tannin fractions from two sorghum varieties in aqueous acetone (50/50 v/v) yielded different chromatographic patterns. Marquardt *et al.* (1977) used this procedure to purify and fractionate the tannins from the hulls of faba beans into four fractions. The fractions differed in their degree of polymerization which can be attributed to their solubility in methanol and ether. Fraction A was highly polymerized, while fraction B and C were intermediate, and fraction D was found least polymerized. Condensed tannins of faba bean hulls yielded 2 fractions (A and B) on separation with Sephadex LH-20 column. Fraction A contained at least 15 low molecular weight polyphenolics and B, the major fraction, contained soluble condensed tannins. When applying the same chromatography to extracts of a white flowering variety of faba bean, low molecular weight compounds were still found, but no peaks were found in the chromatograms which could be identified as condensed tannins (Marquardt *et al.*, 1977).

PHYSIOLOGICAL EFFECTS OF TANNINS

Most of the effects of dietary tannins in animals are considered antinutritional (Jansman, 1994; Salunkhe *et al.*, 1990). A few beneficial effects related to stimulation of estrogen (Jansman, 1994) and production of a flat blood glucose response (Thompson, 1988) have been attributed to consumption of specific polyphenolic compounds. Singleton (1981) stated that dietary tannins at appropriate levels may have a general antibiotic effect by suppressing the growth of detrimental flora in the alimentary tract. However, it is still questionable whether preventive or pharmacological effects can be expected from tannins occurring in foods and feedstuff.

The influence of tannins on the overall acceptability and utilization of food/feed, including nutritional parameters such as feed intake, weight gain, and utilization of minerals, has been studied by some investigators. Reports on the nutritional consequences of dietary tannins are based mostly on either in vitro studies or experiments with nonruminants. Some of them have been carried out with isolated tannins from feedstuffs or with 'standards' of commercial tannins, such as tannic acid and catechin. Most studies, however, were carried out with raw or fractionated feedstuffs (e.g., hulls of legume seeds) of the same plant species containing different levels of tannins as analyzed by one of the available methods. In

these studies the effects or differences found were fully or partly related to the differences in tannin level in the experimental diet.

Effects on Feed Intake and Mortality

Conflicting reports have been published on the effect of dietary tannins on feed intake. Because tannins are known to have a bitter or astringent taste, a depression of food intake might be expected (Glick and Joslyn, 1970; Reddy *et al.*, 1985). In contrast, it has been suggested that a slightly astringent taste increases the palatability of feed and stimulates feed intake (Gupta and Haslam, 1980). An increased feed intake was found in chicks fed sal seed (*Shorea robusta*), a seed that contains high levels of hydrolyzable tannins (Zomabade *et al.*, 1979). An opposite effect, however, was found in cockerels by Ahmed *et al.* (1991).

The physical basis for astringency may be that tannins bind and perhaps precipitate salivary mucoprotein. This effect would reduce the lubricating property of saliva, give the mouth the feeling of dryness and affect the ability to swallow the food (Mole, 1989). Direct binding of tannins to taste receptors may be a second more direct mechanism by which tannins affect palatability of food. Glick (1981), however, reported the effect of tannin (especially gallic acid) on food intake is not mediated entirely through taste aversion or through other gastrointestinal factors, since a continuous

daily infusion of a gallic acid solution (2%) resulted in a significant reduction of food intake.

TABLE 2 summarizes some effects of dietary tannins in several feedstuffs on the performance of rats, poultry and pigs. The level and type of tannins as well as differences among animal species may explain the conflicting results with respect to the effect of tannins on feed intake (Jansman, 1994).

A high mortality rate was reported in chickens and rats due to supplementation with 5% tannic acid (Glick and Joslyn, 1970; Vohra *et al.*, 1966), but no mortalities occurred at a 5% level of catechin (Glick and Joslyn, 1970). Similar effects have been observed with the addition of tannins to swine diets (Salunkhe *et al.*, 1990). Glick (1981) and Reddy *et al.* (1985) reported that the ingestion of certain polyphenols by chicks and rats reduced growth. Marquardt *et al.* (1977) reported that chicks fed a diet containing 3.9% of condensed tannins extracted from faba beans had markedly depressed growth rate. Martin-Tanguy *et al.* (1977) found that high tannin content horse beans reduced laying rates of hens. Feeding of tannin-rich faba beans in a longer trial increased mortality (Lindgren, 1975).

Interactions with Protein and Starch

Tannin consumption in animals causes a decrease in protein digestibility (Jansman *et al.*, 1993) and possibly

Table 2

Some effects of condensed tannins in rats, chickens, and pigs[1]

Species	Source	Level of source(%)	Tannin level(%)	Effect	Reference
Rats	cowpea hulls extract	0.057	0.057	None	Chang et al. (1994)
	red kidney bean hulls	2.4	0.13	None	Garcia-Lopez et al. (1990)
	quebrancho extract		1	None	Levrat et al. (1993)
	catechin	1.17	1.17	None	Greger & Lyle (1988)
	faba bean hull extract	20	1.41	None	Jansman et al. (1993)
	faba bean	60	1.99	↓ FI	Jansman et al. (1993)
	catechin	2	2	↓ FI	Glick & Joslyn (1970)
Chick	faba bean	85	(Low/high)	↑ FI	Marquardt & Ward (1979)
	faba bean(hull extract)		2.5	↓ FI	Marquardt & Ward (1979)
Duck	sorghum	80	(Low/high)	↑ WtGn	Elkin et al. (1990)
Pigs	sorghum	90	(Low/high)	↑ FI	Cousins et al. (1981)
	faba bean hull	20	3.3	None	Jansman et al. (1993)

[1] FI=food intake; WtGn=weight gain; (Low/high)= white/colored-flowering sorghum.

also in humans due to formation of insoluble protein-tannin precipitates in the gastrointestinal tract (Butler *et al.*, 1989). Tannins, by definition, are able to form complexes with proteins. Hydrogen bonds and hydrophobic interactions appear to be the principal linkages involved (Artz *et al.*, 1987). Casein, bovine serum albumin, G1 protein from beans, and carob pod proteins resist proteolytic digestion when complexed with tannins (Desphande and Damodaran, 1989). Such complexes may not be dissociated in the range of pH existing in the gastrointestinal tract and thus may be excreted in the feces (Reddy *et al.*, 1985).

Using a competitive binding assay, Hagerman and Butler (1981) clearly showed differences in binding affinities between proteins and tannins. Asquith and Butler (1986) confirmed the earlier reports in studies of quebrancho, wattle and pinto bean tannins. They concluded that condensed tannins from either sorghum or pinto bean had a particularly high affinity for proline-rich proteins.

Griffiths (1981) found that removal of tannin-containing hulls had a significant positive effect on the solubility of faba bean proteins. This effect on protein solubility was not found in low-tannin varieties of faba beans.

Tannins are also known to interact with carbohydrates, particularly starch, although their affinity seems to be less than for proteins. Deshpande and Salunkhe (1982) studied the interaction of tannic acid and catechin with starches of

different legumes. Processed amorphous amylopectin and amylose associated more with phenolic compounds than did native starch. The in vitro digestibility of starches associated with tannic acid or catechin was reduced by 9-17% compared with control group.

Effects of Tannins on Mineral Metabolism

Knowledge about the effect of tannins on mineral utilization is limited. Tannins are known to form complexes with divalent metal ions, rendering them less available for absorption (Salunkhe *et al.*, 1990). Among the minerals, the relationship between polyphenol compounds and iron has been studied the most. Interactions of tannins with other minerals such as copper and zinc has received little attention (Jansman, 1994).

Iron

Tannins from different species have variable effects on iron utilization and tannins did not significantly depress iron absorption in some studies (House and Van Campen, 1994).

The inhibition of iron absorption observed when tea (Disler *et al.*, 1975; Hallberg and Rossnader, 1982; Brune *et al.*, 1989), some wines (Bezowda *et al.*, 1985) or coffee (Hallberg and Rossander, 1982; Morck *et al.*, 1983; Brune *et al.*, 1989; Wang and Kies, 1991) are drunk with a test meal has been ascribed to the content of phenolic compounds in these

beverages. Epidemiological relationships have also been shown between coffee intake and low serum ferritin values in menstruating women (Soustre *et al.*, 1987), between coffee intake and low maternal and infant hemoglobin in pregnant women (Munoz *et al.*, 1988), and between tea drinking and a higher incidence of anemia in children (Merhav *et al.*, 1985). Certain vegetables have been reported to decrease iron absorption in proportion to their content of phenolic compounds (Gillooly *et al.*, 1983; Tuntawiroon *et al.*, 1991). All of the recent investigations have demonstrated poor bioavailability of iron contained in legumes but the factors responsible are poorly understood. Torrance *et al.* (1982) and Rao and Prabhavathi (1982) have suggested that tannins are responsible for the low bioavailability of iron in legumes. Merhav *et al.* (1985) suggested the mechanism is related to a chelating effect of the tannins on metallic iron. Griffiths (1982) found a high iron-binding capacity of extracts from seed coats of colored-flowering varieties of faba beans while white-flowering varieties did not show this property.

House and Van Campen (1994) conducted a series of studies to assess the effects of tannins extracted from hulls of beans. They reported that tannins in single meals did not affect ^{59}Fe absorption by moderately anemic, growing rats. In a long term feeding trial, they observed some impairment in iron absorption in growing rats resulting from consumption of tannin-containing diets at 0.5% level. In contrast, tannins

did not affect ^{59}Fe absorption by mature rats. They concluded that bean tannins consumed as part of typical diets probably have little adverse effect on iron absorption. In a human study using a bean soup test meal, Lynch *et al.* (1984) showed that iron absorption was uniformly low, but not statistically significant from a reference meal. Garcia-Lopez *et al.* (1990) found that in rats iron absorption from legume-containing test meals was reduced only when legumes were fed before and after the test meal but was not reduced when casein was the only protein source in the habitual diet. They suggested that tannins per se did not have an inhibitory effect on iron absorption but habitual feeding of diets with a high tannin content may lead to changes in the intestine which in turn may alter the absorption of iron.

A series of studies were conducted to explain the different effects of phenolic compounds on mineral absorption observed with coffee/tea and legumes. Using a weanling rat model, Greger and Lyle (1988) reported that ingestion of 1.17% catechin did not affect iron metabolism. However, Gillooly *et al.* (1983) found that addition of tannic acid to a broccoli meal dramatically reduced iron absorption from 29.7 to 1.5 percent in human subjects. Similarly, Siegenberg *et al.* (1991) reported the addition of tannic acid to white-bread test meals resulted in a dose-related inhibition of iron absorption. The maximal inhibitory effect was achieved between 12, 26, and 55 mg tannic acid/ 80 g bread. The

dose-response curve then leveled off, even with very large doses (263 and 833 mg) of tannic acid reducing absorption to about one-fifth. This finding implies that the interference of tannic acid on iron absorption is not related to its protein-precipitating effect, since white bread has a low content of protein.

In human subjects, Brune *et al.* (1989) studied the relationship between the phenolic structure of tannins and inhibitory effect on iron absorption. They found the inhibition of iron absorption by tannic acid was strongly dose-related. The smallest amount (5 mg) inhibited absorption by 20%, 25 mg by 67% and 100 mg by 88%. Gallic acid inhibited iron absorption to the same extent as tannic acid, per mol galloyl groups. However, no inhibition on iron absorption was observed when catechin was added to the test meal. These results showed that the galloyl groups may be a major determinant of the inhibitory effect of phenolic compounds on iron absorption; however, catechin, a phenolic catechol group, seems to be of minor importance in iron absorption. Furthermore, Brune *et al.* (1989) suggested that the inhibitory effect of galloyl groups is due a direct complex formation between the phenolic hydroxyl and the iron ions. They reported that molecules with aromatic rings bearing two hydroxyl (catechol group) or three hydroxyl (galloyl group) positioned at adjacent carbon atoms have iron-binding properties in vitro. The monomer gallic acid and its polymer

tannic acid have ten galloyl groups, and these substances readily yield blackish precipitates when added to an iron solution. Catechin which has one resorcinol (meta-dihydroxy) group in the A-ring and one catechol (ortho-dihydroxy) group in the B-ring does not form complexes with iron.

Zinc

The effects of tannins on zinc utilization have received little attention. Greger and Lyle (1988) reported that rats fed diets with added 1.17% catechin showed increased zinc concentrations in the tibia but not kidney or liver. Greger and Emery (1987) observed that rats fed coffee and decaffeinated coffee that contained 5% polyphenols had elevated tibia zinc concentration. The effect of various forms of tea with different tannin profiles on tissue content of zinc was conflicting. Rats fed tea (desiccated and liquid) tended to have higher tibia zinc; the difference was significant for those given liquid tea (Greger and Lyle, 1988). In contrast, in the same study chronic ingestion of tea was associated with lowered zinc concentrations in kidneys and decreased apparent absorption of zinc.

Copper

Knowledge of copper requirements, absorption, and transport and of the biological roles of copper has accumulated over the past 60 years, but remains frustratingly

incomplete today (Danks, 1988). Much remains to be learned about the transport of copper to and within body cells. Thus, reviewing some basic concepts of copper metabolism will be presented before discussing the effects of tannins on copper bioavailability.

The adult (70 kg) human body contains 80 mg copper, but reported values range from 50 to 120 mg (O'Dell, 1990). The tissue copper concentration is age related, with the newborn and very young values normally being higher than adults. The highest tissue concentration in most animals occurs in liver with variably lesser amounts in the heart, kidney, spleen and brain (Hunt and Groff, 1990). The essentiality of copper is due to its participation as an enzyme activator or inhibitor in critical reactions. The U.S. adult recommended daily safe and adequate range of copper is 1.5 to 3.0 mg, but the average American consumes 1.2 to 0.9 mg copper/day, respectively, from 1982 to 1986 (NRC, 1989). The homeostatic regulation of copper is, therefore, of prime importance.

A steady level of copper in the body depends on a balance between intestinal absorption and biliary excretion, with small additional losses in sweat and skin (Danks, 1988). The short half-life of the copper isotopes (^{64}Cu: 12 hr and ^{67}Cu: 61 hr), the lack of their availability at specific time periods, and the limited use of gamma emitters in humans dictated by human subjects have hampered studies of copper absorption in terms of its incorporation into and release from

copper enzymes (Lonnerdal *et al.*, 1985; Sandstrom *et al.*, 1993). Figure 5 shows the principles of mineral metabolism (Sandstrom *et al.*, 1993). Absorption of copper is maximal in the duodenum with both active and passive components to the system (O'Dell, 1990). Research has shown that up to 12 μg of ^{64}Cu administered orally to rats is absorbed rapidly with peak absorption at 30 minutes, and 30% of the dose is absorbed by 8 hr (O'Dell, 1990). Within the intestinal mucosal cells copper can interact with metallothionein (MT). MT is an unusual protein that contains 61 amino acids, 20 of which are cysteine. Many experimental findings suggest that MT acts to bind and detoxify excess copper especially in kidney and liver (Danks, 1988). O'Dell (1990) suggested that intestinal MT serve primarily as a negative rather than a positive modulator of copper absorption. A role of MT in transport of copper within the liver cells, however, has been suggested since some studies have shown ^{67}Cu to bind to MT as soon as it is taken into liver cells (Danks, 1988).

Newly absorbed copper is bound primary to albumin as the transport form in the portal blood (Cousins, 1985). The conventional view has been that copper is taken up by the liver and incorporated into ceruloplasmin (Cp), a glycoprotein containing six atoms of copper per molecule. Cp is released into the blood where it normally constitutes 90% of the copper present in plasma (O'Dell, 1990). Cp carries copper to nonhepatic tissues for synthesis of cuproenzymes such as

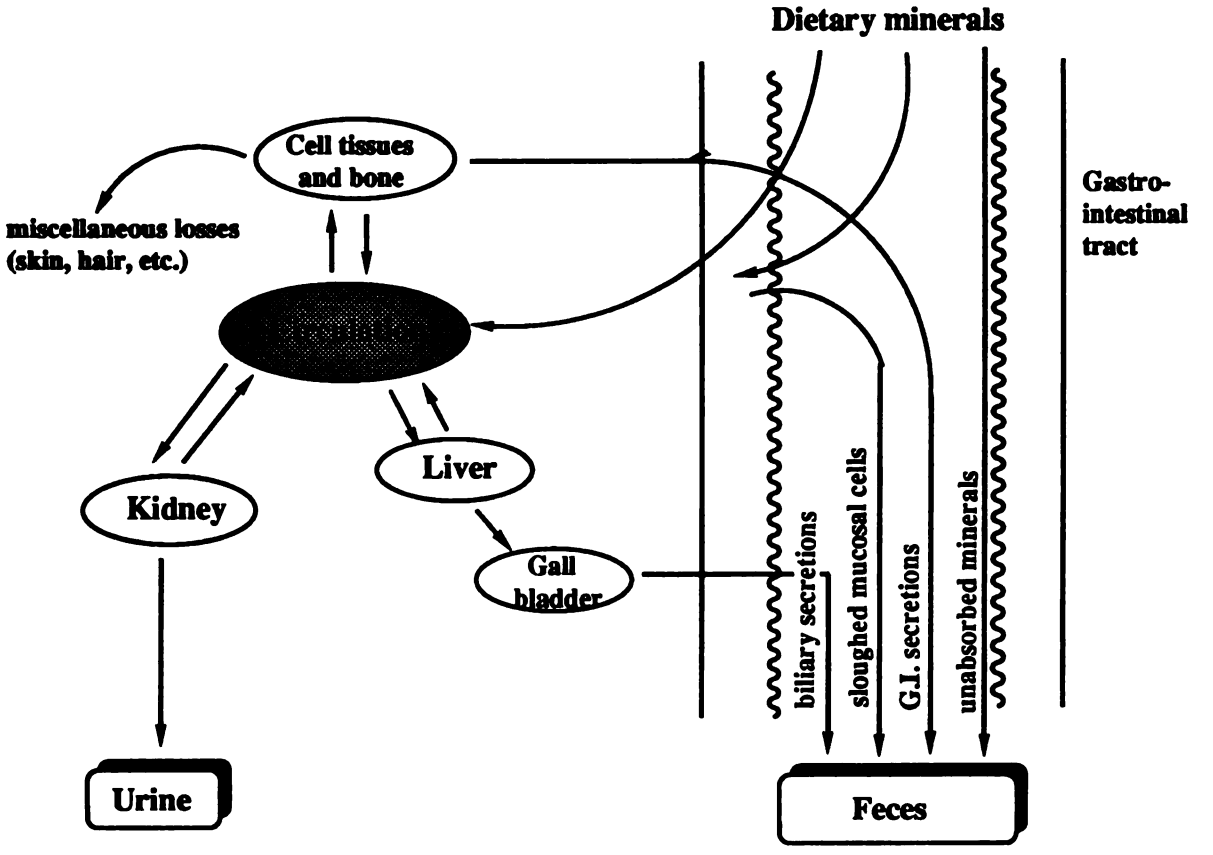

Figure 4 Principle of mineral metabolism

Adapted from: Sandstrom et al. (1993). p 79.

cytochrome c oxidase, superoxide dismutase and lysyl oxidase (Cousins, 1985). However, the mechanism(s) by which Cp copper is made available to cells is unclear (O'Dell, 1990). Cp has a ferroxidase activity that has been considered important in the release of iron from stores. Cp is believed to be necessary for the attachment of iron to transferrin for transport in the plasma by catalyzing the oxidation of ferrous iron to ferric iron (Linder, 1985). It also has amine oxidase activity against a range of biologically active amines.

Copper is primarily excreted via the gastrointestinal tract with < 3% of the intake appearing in the urine. Biliary excretion represents the primary route by which gut-absorbed copper is excreted (Aoyagi and Baker, 1993), although a significant percentage is probably lost through intestinal secretion and sloughing off of cells (Linder, 1985). Biliary excretion increased substantially with excess copper intake (Danks, 1988). The mechanism by which copper eventually enters the bile and is excreted is very poorly understood. There is not even consensus about the molecular form of copper present in the bile (Aoyagi and Baker, 1993).

Factors that influence copper bioavailability have been revealed recently. Amount and type of protein may have an effect on the availability of copper in neonates (Sandstead, 1982). Cow's milk and dairy products contain little copper and that present is poorly absorbable (Lonnerdal *et al.*, 1985; Danks, 1988). Since the digestibility of cow's milk casein

may be low in infants, the bioavailability of copper bound to cow's milk casein may also be low (Lonnerdal *et al.*, 1985). A high dietary intake of ascorbic acid decreases copper bioavailability, impairs copper absorption from ligated intestinal segments of the rat, and lowers blood and liver copper concentrations in guinea pigs and human (O'Dell, 1990; Finley and Cerklewski, 1983). Compared with starch, fructose and sucrose aggravate the signs of deficiency, suggesting a negative relationship between fructose and copper status (Johnson and Flagg, 1986; Fields *et al.*, 1986). Danks (1988) stated that fructose increases the requirement for copper, rather than interfering with its absorption. O'Dell (1990) suggested that both fructose and ascorbic acid are strong reducing compounds and may convert copper to the cuprous state, thereby impairing utilization as well as absorption.

Little has been published on the effects of tannins on copper metabolism (Jansman, 1994). Kies and Umoren (1989) reported in human studies that feeding 21 g of high (red) tannin wheat brans for 14 days had a greater negative effect on apparent copper utilization than low (white) tannin wheat bran. They also observed that after consumption of 8 grams of black tea infusion daily for ten days fecal copper losses in human subjects were significantly increased. Kies and Umoren (1989) thus demonstrated that tannins in wheat bran and tea may have contributed to decreased copper utilization.

In contrast, Greger and Emery (1987) showed that rats fed either coffee or decaffeinated coffee, which has been estimated to contain 5% of tannins (Brune *et al.*, 1989), had significantly higher concentrations of copper in their livers than the control group. Plasma copper and ceruloplasmin and kidney copper levels tended to be greater in rats fed coffee although the differences were not statistically significant. Similarly, Greger and Lyle (1988) showed that in rats ingestion of diets containing more than 1.17% black tea enhanced copper absorption leading to elevated liver copper level. Ingestion of black tea by anemic rats elevate plasma ceruloplasmin activity and tended to elevated plasma copper compared to those of pair-fed control animals. However, when rats were fed the same level of catechin, similar results were not seen, thus suggesting that catechin in black tea was unlikely responsible for this effect. DiSilvestro and Harris (1983) observed that in normal chicks injection of catechin stimulated lysyl oxidase activity in aorta tissue. Lysyl oxidase is a copper-containing enzyme that aids in the crosslinking of elastin and collagen, a role that is integral to connective tissue and blood vessel maintenance. Copper is the major regulator of lysyl oxidase in copper-deficient animals. O'Dell (1990) stated that in copper deficient animals both ceruloplasmin and lysyl oxidase activities respond rapidly and correlatively to copper repletion. DiSilvestro and Harris (1983) suggested that catechin may make

more of the absorbed copper available to the enzyme thus producing the large increase in copper-induced activation of lysyl oxidase in catechin-treated chicks.

Based on the limited reports in the literature, the need for additional research to clarify the effect of tannins on copper utilization is apparent. Experiments by Greger and Emery (1987) and Greger and Lyle (1988), which included only tea, coffee and catechin gave inconsistent results. DiSilverstro and Harris (1983) injected rather than fed catechin and did not measure tissue copper. Furthermore, the mechanism(s) by which catechin from various sources (chemical form, coffee, tea infusion, other plants, or beans) may affect copper metabolism in vivo is unclear. As Salunkhe *et al.* (1990) stated : "..... more research is needed to ascertain the harmful and beneficial effects of dietary tannins, and fix the threshold levels which will again vary with the type or source of tannins and the consumers.". The purpose of the present research was to test the hypothesis that tannins in pinto bean hulls had a inhibitory effect on copper bioavailability.

MATERIALS AND METHODS

MATERIALS

Ten kinds of dry beans including black eye peas, domino black, faba, lentil, lupin, kidney dark, kidney light, pinks, pinto and small white from Morrice Grain and Bean Company (Morrice, MI) were kindly provided by Dr. Uebersax. The following brands of pinto beans were purchased from local sources: Spartan, Sysco and Brown's Best. Catechin-(+), vanillin (vanilaldehyde), *O*-dianisidine dihydrochloride, glycerol and the hemoglobin kit (Catalog No. 525-a) were purchased from Sigma Chemical (St. Louis, MO). Methanol, 30% hydrogen peroxide (H_2O_2), sodium acetate·$3H_2O$, concentrated sulfuric acid (H_2SO_4), copper, zinc, and iron standard solutions (1000 ppm) were purchased from J.T. Baker Chemical (Phillipsburg, NJ). Bovine liver (No 1557a) and wheat flour (No 1567a) were purchased from the National Institute of Standards and Technology (NIST) (Gaithersburg, MD). Metofane (methoxyflurane) was from Pitman-Moore (Mundelein, IL), hydrochloric acid (HCl) and glacial acetic acid were from Mallinckrodt (Paris, Kentucky), ultrapure concentrated nitric acid (HNO_3) was from Fischer Scientific (Pittsburgh, PA).

RESEARCH DESIGN

Preliminary Analyses

Preliminary experiments were done to determine the quantities of condensed tannins in various beans having different seed coat colors using the 0.5% vanillin-HCl assay described by Deshpande and Cheryan (1985). Experiments were done to determine the influence of extraction time on tannin values. Relative distribution of tannins in seed coat and cotyledon was determined in beans from different sources. Mineral content (copper, zinc and iron) of dry beans including whole beans, cotyledon, and hull was determined using atomic absorption spectrophotometry (AAS) (Perkin-Elmer 2380, Norwalk, CT).

Animal Studies

Experiment I: The effect of feeding commercial catechin or condensed tannins in pinto bean hull on copper utilization was determined in weanling Sprague-Dawley male rats (Harland, Indianapolis, IN). After 14 days of adaptation the rats were assigned to three groups (eight rats per group) based on body weight. Rats were fed ad libitum for 21 days one of the three dietary treatments: 1) control diet without bean hulls, 2) control diet with 1.6% catechin, 3) control diet with 25% pinto bean hulls.

At the end of three weeks, all rats were anesthetized with metofane and then killed by open cardiac puncture. Blood

samples collected from the heart were centrifuged at 1500 x g for 20 minutes; plasma was removed and frozen. Livers, tibias, and kidneys were rinsed in saline, cleansed of adhering matter and frozen.

Experiment II: To further clarify the effect of pinto bean hulls on copper utilization, a second experiment was done comparing diets with and without pinto bean hulls. After three days of adaptation Sprague-Dawley male rats were assigned to two groups (eight rats per group) based on body weight. Rats were fed ad libitum for 15 days one of the two dietary treatments : 1) control diet without bean hulls, 2) control diets with 25% pinto bean hulls.

Rats in Experiment II consumed more food and grew more rapidly than rats in Experiment I. In order to have comparable body weights in both experiments, rats in Experiment II were killed after 15 days of dietary treatment. Procedures for collection of samples were the same as those used in Experiment I.

Care and treatment of rats were approved by the All University Committee on Animal Use and Care at Michigan State University.

PREPARATION OF BEANS

Whole bean samples used for tannin analyses were ground to obtain flours of 820 micron particle size (100% of the

samples passed through 20-mesh screen) using a micro-mill (Chemical Rubber company, Cleveland, OH). In order to obtain the hulls and cotyledon, dry pinto beans were soaked in deionized water (4:1 water:bean) for 4-6 or 17-19 hr; the seed coats were then manually removed from the cotyledon and air dried. Dried hulls and cotyledon were then ground separately into flours of 820 micron particle size using a coffee mill (Robert Krups company, Denver, CO). Samples used for mineral analyses were prepared in the same way as described for the tannin analyses. Pinto beans (Brown's Best) for the rat experiment were soaked for 4 hr and hulls were prepared following the same procedures described above.

ANIMALS

Rats were housed individually in suspended stainless steel cages in a temperature- and humidity-regulated room with alternating 12-hour light/dark cycle with lights on at 7:00 am. Temperature was maintained at 20-22° C, humidity at 68-70%. Food consumption and body weights were measured twice weekly. Deionized water was supplied ad libitum throughout the studies using water bottles with plastic caps and stainless steel sipper tubes.

DIETS

The composition of the diets was formulated in accordance with general guidelines prepared by the American Institute of Nutrition (AIN, 1976; Reeves *et al.*, 1993) with modifications in the amounts of cornstarch, fiber, copper, zinc and iron contents (TABLE 3 and 4). The levels of copper, zinc and iron in the diets were adjusted according to the levels present in the pinto bean hull diets used in each study. In each dietary treatment, 25 g/100 g diet of cornstarch was substituted either by the same amount of pinto bean hulls in the hull diets or by the same amount of cellulose in both control and catechin diets. The 25% cellulose was added to the control and catechin diets in order to compensate approximately for the fiber present in hulls diet (Srisuma, 1989). In Experiment I, the catechin and hulls diets were formulated to contain a similar amount (1.6%) of condensed tannins.

ANALYTICAL METHODS

Tannins

The tannin content of beans was determined using the 0.5% vanillin assay of Deshpande and Cheryan (1985) (Figure 5). The procedure was as follows: 200 mg of dried ground beans or 1.0 g of diets was extracted with 10 ml absolute methanol for 20 minutes with mechanical shaking. In a separate experiment

TABLE 3

Composition of diets (g/100g)[1] in Experiment I

Component	Control	Catechin	Hulls
Casein[2]	20	20	20
Cellulose[3]	25	25	—
Pinto bean hulls	—	—	25
Catechin	—	1.6	—
Cornstarch	19.86	18.26	19.86
Mineral mix[4]	3.5	3.5	3.5
Cupric sulfate (mg/100g)	0.407	0.407	—
Zinc carbonate (mg/100g)	0.13	0.13	—
Ferric citrate (mg/100g)	17.23	17.23	—

[1] The following ingredients (g/100g diet) were added to all diets: sucrose, 10.0; soy oil, 7.0; AIN-76 vitamin mix, 1.0; dyetrose (Dyets, Inc., Bethlehem ,PA), 13.2; and choline chloride, 0.14.
[2] Vitamin-free casein (Dyets, Inc., Bethlehem ,PA).
[3] Avicel Food Prototype 174-2, FMC Corporation, Marcus Hook, PA 19061.
[4] Contained (g/kg mineral mix): sucrose, 265.4; $CaCO_3$, 357; KH_2PO_4, 196; K_2SO_4, 46.6; $K_3C_6H_5O_7 \cdot H_2O$, 28; NaCl, 74; MgO, 24; $MnCO_3$, 0.63; KIO_3, 0.01; Na_2SeO_3, 0.01; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.008; $CrK(SO_4)_2 \cdot 12H_2O$, 0.275.

TABLE 4

Composition of diets (g/100g)[1] in Experiment II

Component	Control	Hulls
Casein	20	20
Cellulose	25	—
Pinto bean hulls	—	25
Cornstarch	19.86	19.86
Mineral mix	3.5	3.5
Cupric sulfate (mg/100g)	0.403	—
Zinc carbonate (mg/100g)	1.488	—
Ferric citrate (mg/100g)	13.78	—

[1] Footnotes are as the same as Table 3.

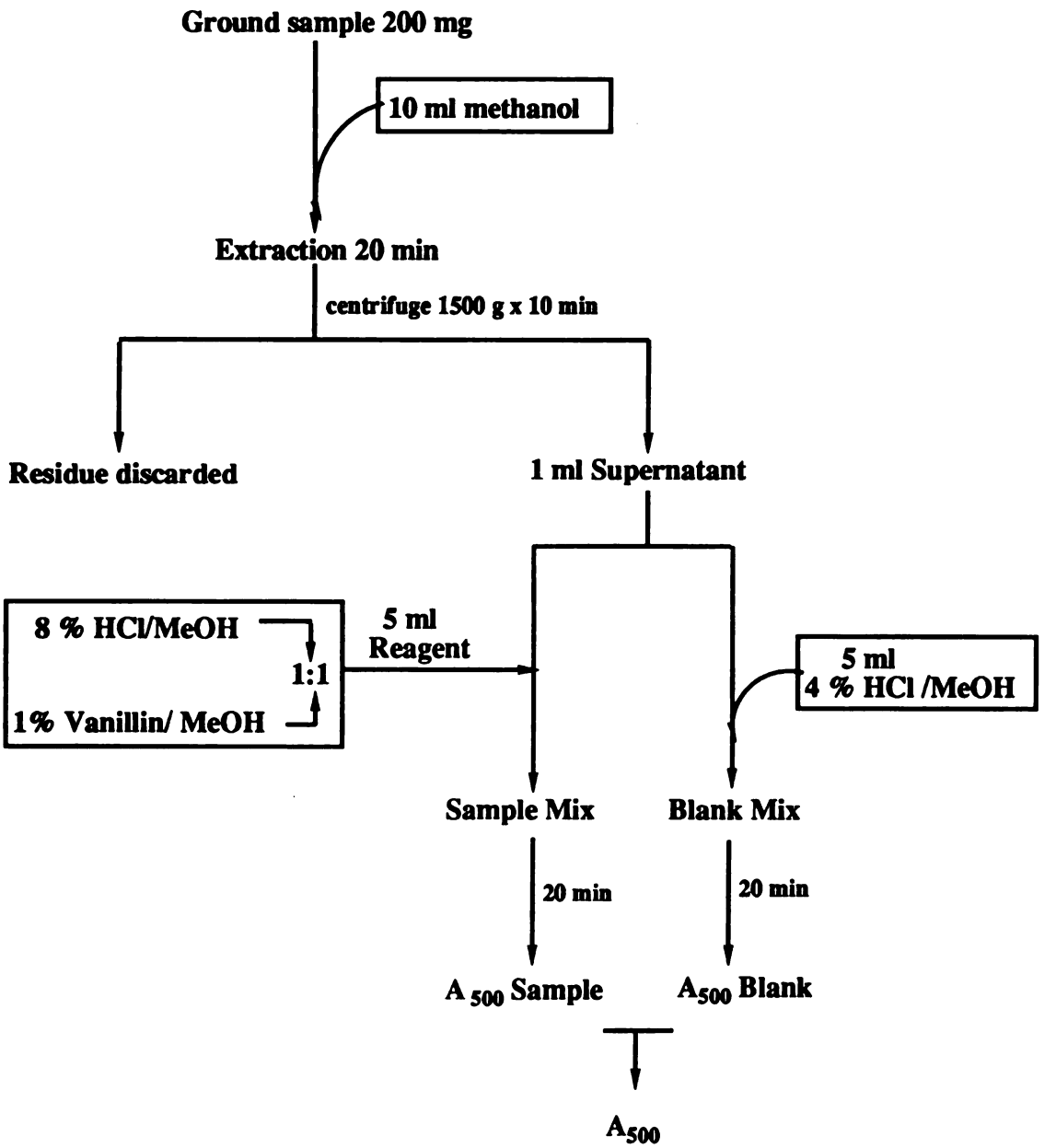

Figure 5 Procedure for 0.5 % vanillin assay

Adapted from: Salunkhe et al. (1990). p 82.

extraction times of 20 min and 24 hr were compared. Samples were then centrifuged at 1500 x g for 10 min and 1 ml of the supernatant was added immediately to 5 ml of the color reagent which was composed of a 1:1 mixture of 8% HCl/methanol and 1% vanillin/methanol. After 20 min, the absorbance was read at 500 nm against a sample blank in which the vanillin was deleted from the color reagent. The tannin concentration was calculated from a standard curve using catechin-(+) standards of 5, 10, 20, 30, 40, 50, 60 and 70 mg catechin/50 ml methanol. The tannin content of the samples was expressed in terms of mg catechin equivalents on a dry weight basis.

All the extractions were carried out at least in duplicate; tannin assays were run in triplicate. All the assays were conducted at room temperature (25±1°C).

Mineral Analyses

Bean and rat diets: Triplicate 1 g samples of ground beans were weighed and placed in 25 ml Erlenmeyer flasks with 10 ml concentrated HNO_3 and 2 to 3 glass beads. The flasks were placed on a hot plate at low heat for 1 to 2 days and then allowed to go to dryness. Five ml of 30% H_2O_2 was added. If a white ash was not obtained, more H_2O_2(5 to 10 ml) was added. When digestion was complete, samples were diluted with 0.1 N HCl (copper, 5 ml; zinc/iron, 25 ml) prior to analyzing by AAS. TABLE 5 summaries the analyzed values for mineral content of the diets used in Experiment I and II.

TABLE 5

Analyzed condensed tannin, copper, zinc and iron content of diets[1]

Diets	Condensed Tannins (mg Catechin equivalent/g diet)	Analyzed Mineral Content (mg/kg diet)		
		Copper	Zinc	Iron
Experiment I				
Control	-[2]	0.95±0.04	12.9±0.9	37.7±1.3
Catechin	15.82±0.11	1.07±0.15	12.9±0.4	38.6±0.3
Hulls	15.59±0.45	1.50±0.17	18.5±0.3	34.5±0.8
Experiment II				
Control	-[2]	1.89±0.07	23.24±0.7	35.66±0.7
Hulls	12.96±0.26	1.99±0.03	23.54±0.4	36.77±2.5

[1] Each value represent mean ± SEM (n=16).

[2] Not detected.

Plasma: In Experiment I, plasma samples were diluted 1:7 and 1:21 with deionized water for analysis of copper and zinc, respectively, by atomic absorption/ emission spectrophotometry (AAES) (Smith-Hieftje 4000, Thermo Jarrell Ash company, Franklin, MA). To determine total plasma iron, samples were deproteinized with 20% trichloroacetic acid (TCA) in a ratio of 1:2 and incubated at 90° C for 15 minutes (Olson and Hamlin, 1969). After protein precipitation, samples were cooled and centrifuged at 1500 x g for 15 min. Iron standards were also prepared and treated with TCA in the same way as samples and then analyzed for iron by AAES.

In Experiment II, plasma was analyzed for copper and zinc only. Plasma samples were diluted 1:1 and 1:7 with deionized water and analyzed for copper and zinc, respectively by AAS (Perkin-dlmer Model 2380, Norwalk, CT). Standard solutions were made by diluting stock standards with 50 and 12.5% glycerol solutions for copper and zinc, respectively. The glycerol solution was used to compensate for the viscosity of the plasma.

Tissues: Livers, kidneys and tibias were freeze-dried (Vacudyne, Chicago, IL) to a constant weight. Liver samples were crushed manually. Duplicate 500 mg liver samples , one kidney (approximate 0.28 g) and one tibia (approximate 0.43 g) were soaked overnight in 10 ml concentrated HNO_3 to allow tissues to dissolve in the acid. Samples were further heated 1 to 2 days until dry; then 5 to 10 ml of H_2O_2 was added to

complete ashing. Ashed samples were diluted with 0.1 N HCl: 5 ml for copper, 25 ml for liver and kidney zinc and iron, 50 ml for tibia zinc and iron. If a white ash was not obtained or particles were present in the flask when diluted with 0.1 N HCl, repeat ashing was necessary.

NIST Standard: Standard reference materials of bovine liver and wheat flour were digested and analyzed using the same procedures as for tissues samples to check the validity of the methodology.

Ceruloplasmin Activity

Ceruloplasmin activity of plasma samples was determined by a colorimetric procedure described by Schosinsky *et al.* (1974). This assay relies on the diamine oxidase activity of ceruloplasmin which reacts with the diamine groups of *O*-dianisidine dihydrochloride.

Hemoglobin

The concentration of hemoglobin in blood samples was determined using the cyanomethemoglobin method described by Crosby et al. (1954). Hemoglobin concentration was expressed as g/dl.

Statistical Analyses

Experiment I: Data were subjected to a one-way analysis of variance at the 95% confidence level to determine the significance of differences between means. Significant differences among the treatments were determined by Fischer's least significant differences (LSD) test when appropriate.

Experiment II: Student's t-test was applied to compare all data. In order to see the overall effect of the hulls diet in both experiments, data were analyzed using a 2 x 2 randomized ANOVA.

RESULTS

PRELIMINARY ANALYSES

The tannin concentration of the ten different kinds of dry beans varied widely, ranging from non-detectable to 1139 mg catechin equivalent (CE)/100g whole bean with an extraction time of 20 min (TABLE 6). With an extraction time of 24 h values ranged from non-detectable to 232 mg CE/100 g whole bean. Tannins were not detectable in the white seeded beans such as small white and lupin beans. Among the colored seeds, pinto beans contained more tannins than did the kidney beans. Light kidney beans had more tannins than did dark kidney beans, whereas dark colored back eye pea had the lowest tannin concentration of these beans. Tannin concentration in all ten dry beans after 24 h extraction time, the recommended extraction time (Burns, 1971), had actually decreased.

Data on relative distribution of tannin in four brands of pinto beans are shown in TABLE 7. Different brands of pinto beans had similar tannin concentrations in the whole beans. Brown's Best had the highest tannin value both in the whole beans and in hulls. The lowest whole bean tannin value was seen in the Morrice brand. Cotyledons from all the brands consistently contained non-detectable amounts of tannins.

TABLE 6

Tannins concentration of ten different dry beans[1]

Dry Beans	mg Catechin Equivalent / 100 g dry wt of beans	
	20 min extraction	24 h extraction
Black eye peas	250±15	ND
Domino black	320±28	158±10
Faba	166±15	ND
Lentil	391±27	ND
Lupin	ND	ND
Kidney dark	460± 7	46± 4
Kidney light	575±18	156±12
Pinks	233±16	ND
Pinto	1139±17	232±22
Small white	ND	ND

[1] Mean ± SEM (n=3); ND = not detectable.

TABLE 7

Relative distribution of tannins and mineral content in four brands of pinto beans[1]

Brands	Morrice	Spartan	Sysco	Brown's Best
Tannin content (mg Catechin Equivalent / 100 g dry wt)				
Whole	1013± 33	1131±253	1378±45	1694± 34
Hulls	3497±136	—	—	11847±297
Cotyledon	ND	ND	ND	ND
Iron (µg/g)				
Whole	50.6±8.8	48.0±4.4	61.5±3.4	—
Hulls	—	145.4±1.8	140.2±7.8	161.6±7.0
Cotyledon	33.6±1.0	38.4±3.1	—	—
Zinc (µg/g)				
Whole	25.4±0.1	21.4±1.3	21.9±0.6	—
Hulls	—	28.2±2.0	29.8±0.7	33.7±2.0
Cotyledon	26.0±1.0	20.1±0.4	—	—
Copper (µg/g)				
Whole	8.8±0.2	7.8±1.0	5.1±0.1	—
Hulls	9.6±0.1	5.9±0.5	11.6±0.7	4.8±0.1
Cotyledon	—	8.4±1.1	—	—

[1] mean ± SEM (n=3); soaking time (hr): Morrice, 19; Spartan, 17; Sysco, 6; Brown's Best, 4.
—=Not Determined; ND= Not Detected.

TABLE 7 also summarizes the relative distribution of mineral concentrations in the four brands of pinto beans. Cotyledons contained somewhat lower concentration of iron than whole beans, while hulls were the most abundant source of iron in these beans, irrespective of the brand. The iron concentration both in hulls and cotyledons was quite similar irrespective of brand or the length of soaking time. For zinc, both cotyledons and hulls contained amounts similar to those in whole beans. A wide range (4.8 to 11.6 ppm) of copper concentrations was found in hulls in the various brands; however similar concentrations of copper were observed in cotyledons.

ANIMAL STUDIES

Food Intake, Body Weight and Food Efficiency

In Experiment I, food intake and body weight gain of rats fed diet diets containing 1.6% catechin or pinto bean hulls with an equivalent tannin concentration were not significantly different from those of controls (TABLE 8). Weight gain tended to be somewhat lower in rats fed the pinto hulls diet and resulted in a significantly decreased efficiency of food utilization compared to rats fed the control diet. Although food intake was similar among the three groups, total copper, zinc and iron intakes were different because the analyzed concentrations of minerals in the diets were different (TABLES

TABLE 8

Body weights, food intake and food efficiency ratios[1]

Diets	Initial Weight (g)	Final Weight (g)	Weight Gain (g/d)	Food Intake (g/d)	Food Efficiency Ratios (%)[2]
Experiment I[3]					
Control	160.6±4.4	286.0±9.6	6.0±0.3	20.5±0.4	29.0±1.0[a]
Catechin	160.5±3.9	280.9±6.8	5.7±0.3	20.8±0.3	27.5±1.1[ab]
Hulls	160.6±4.7	271.3±6.3	5.3±0.1	20.9±0.4	25.2±0.5[b]
Experiment II[4]					
Control	165.25±2.13	288.1±3.8	8.2±0.3	26.5±0.5[a]	30.9±1.1
Hulls	165.25±2.75	292.6±6.1	8.5±0.3	29.2±0.3[b]	29.0±0.8

[1] Each value represent mean ± SEM (n=8).
[2] Food Efficiency Ratio = (weight gain + total food intake) x 100%
[3] Different letters within a column indicate significant differences at $p<0.05$ as determined by ANOVA and LSD.
[4] Different letters within a column indicate significant differences at $p<0.05$ as determined by student's t-test.

5 and 9). Rats fed the pinto hull diet had higher total intakes of copper and zinc but lower intake of iron than rats fed control and catechin diets.

Compared to controls, rats fed 25% pinto bean hulls in Experiment II showed significantly higher food intake but not body weight gain or food efficiency (TABLE 8). Although diets had similar concentrations of minerals, total copper, zinc and iron intakes were higher in the hull-fed group because of their greater intake (TABLE 9).

Organ Weights

Results in both experiments showed rats fed the pinto bean hulls diet had smaller relative liver weights (TABLE 10). In Experiment I, liver weight and kidney dry weight were significantly lower in hull-fed rats (TABLE 11). Tibia dry weight was higher for rats fed the catechin diet. In Experiment II, the weights of liver, kidney and tibia were similar in control and hull fed rats.

Copper

In both experiments rats fed bean hulls showed significantly elevated plasma copper (TABLE 12). In Experiment II, hull-fed rats had a significantly higher plasma ceruloplasmin activity and a tendency toward higher values was also seen in Experiment I (TABLE 12). No dietary effect on plasma copper or plasma ceruloplasmin activity, however, was found in the catechin-fed group compared to the control group.

TABLE 9

Copper, zinc and iron intake[1]

Diets	Copper	Zinc (µg/ daily)	Iron	Total Mineral Intake (mg)		
				Copper	Zinc	Iron
Experiment I[2]						
Control	19.5±0.3[a]	264.8± 4.7[a]	774.0±13.6[a]	0.41±0.01[a]	5.56±0.10[a]	16.3±0.29[a]
Catechin	22.3±0.3[b]	268.2± 3.4[a]	783.9± 9.9[a]	0.47±0.01[b]	5.64±0.07[a]	16.9±0.21[a]
Hulls	31.4±0.6[c]	374.2±13.0[b]	721.2±12.7[b]	0.66±0.01[c]	8.12±0.14[b]	15.1±0.27[b]
Experiment II[3]						
Control	50.1±2.3[a]	614.8±10.0[a]	946.0±15.3[a]	0.75±0.01[a]	9.24±0.16[a]	14.2±0.25[a]
Hulls	58.1±1.3[b]	681.6± 5.6[b]	1074.0±14.3[b]	0.87±0.01[b]	10.13±0.08[b]	16.1±0.13[b]

[1] Each value represent mean ± SEM (n=8).
[2] Different letters within a column indicate significant differences at $p<0.05$ as determined by ANOVA and LSD.
[3] Different letters within a column indicate significant differences at $p<0.05$ as determined by student's t-test.

TABLE 10

Organ wet weights and relative organ weights[1]

Diets	Liver	Tibia (g wet wt)	Kidney	Relative Weights (g wet wt/100g body wt)		
				Liver	Tibia	Kidney
Experiment I[2]						
Control	11.78±0.64[a]	1.62±0.06	1.99±0.09	4.10±0.12[a]	0.57±0.02	0.70±0.02
Catechin	11.21±0.26[a]	1.69±0.04	1.97±0.01	3.99±0.06[a]	0.61±0.02	0.70±0.01
Hulls	9.62±0.41[b]	1.61±0.05	1.85±0.05	3.53±0.09[b]	0.59±0.02	0.68±0.01
Experiment II[3]						
Control	13.76±0.39	1.56±0.03	2.58±0.04	4.78±0.13[a]	0.54±0.01	0.90±0.02
Hulls	13.02±0.58	1.58±0.03	2.46±0.03	4.44±0.13[b]	0.54±0.01	0.84±0.02

[1] Each value represent mean ± SEM (n=8).
[2] Different letters within a column indicate significant differences at $p<0.05$ as determined by ANOVA and LSD.
[3] Different letters within a column indicate significant differences at $p<0.05$ as determined by student's t-test.

TABLE 11

Organ dry weights[1]

Diets	Liver	Tibia (g dry wt)	Kidney
Experiment I[2]			
Control	3.57±0.20[a]	0.86±0.03[a]	0.50±0.02[a]
Catechin	3.37±0.11[a]	0.93±0.01[b]	0.50±0.01[a]
Hulls	2.84±0.14[b]	0.87±0.02[ab]	0.46±0.01[b]
Experiment II[3]			
Control	4.16±0.14	0.87±0.01	0.57±0.01
Hulls	3.89±0.17	0.89±0.01	0.57±0.02

[1] Each value represent mean ± SEM (n=8).
[2] Different letters within a column indicate significant differences at $p<0.05$ as determined by ANOVA and LSD.
[3] No significant differences were found.

TABLE 12

Ceruloplasmin activity, plasma copper, zinc, iron, hematocrit and hemoglobin[1]

Diets	Ceruloplasmin Activity (U/L[2])	Plasma Cu	Plasma Zn (μg/dl)	Plasma Fe	Hematocrit (%)	Hemoglobin (g/dl)
Experiment I[3]						
Control	69.8±3.1	97.0±4.5[a]	223.1±5.3	369.2±34.8	42.3±0.6	14.0±0.3
Catechin	70.1±2.7	99.9±4.7[ab]	235.9±6.4	363.7±82.4	40.8±1.4	13.4±0.6
Hulls	74.4±2.7	110.6±3.5[b]	226.5±6.9	201.4±18.0	41.3±1.4	13.3±0.4
Experiment II[4]						
Control	44.5±0.9[a]	72.3±4.0[a]	168.6±5.8			
Hulls	55.9±1.3[b]	82.7±2.8[b]	165.2±3.7			

[1] Each value represent mean ± SEM (n=8).
[2] μmole of o-dianisine dihydrochloride oxidized per minute per liter of plasma.
[3] Different letters within a column indicate significant differences at $p<0.05$ as determined by ANOVA and LSD.
[4] Different letters within a column indicate significant differences at $p<0.05$ as determined by student's t-test.

The total copper in tissues was altered by the hulls diet treatment in both studies (TABLE 13). Hull-fed rats had a significantly lower total amount of copper in the liver compared to those of the control rats. Similar results were found in kidney and tibia in Experiment I. Liver concentration of copper was similar in the three groups in Experiment I, but lower values were found in rats fed the hulls diet compared to control rats in Experiment II. Results showed no effect of the catechin diet on either concentration or total content of copper in tissues.

Zinc

No differences in plasma zinc concentrations were found in either experiment (TABLE 12). In both studies, rats fed the hulls diet had a significantly higher liver zinc concentration compared to control rats (TABLE 14). A similar increase in liver zinc was found in rats fed the catechin diet in Experiment I. Conflicting results were found in the two experiments for the effect of the hulls diet on tibia zinc concentration. Tibia zinc concentration was lower in the hull-fed rats in Experiment I compared to controls but was higher in Experiment II. Total tibia zinc was significantly higher in the catechin-fed rats compared to controls and hull-fed rats in Experiment I.

Iron

The dietary treatments had no effect on plasma iron, hematocrits or hemoglobin values (TABLE 12).

TABLE 13

Concentration and total content of copper in tissues[1]

Diets	Liver	Tibia (μg Cu/ g dry wt)	Kidney	Tissue total copper (μg Cu)		
				Liver	Tibia	Kidney
Experiment I[2]						
Control	10.2±0.3	2.8±0.2	18.5±0.6	36.3±2.0[a]	2.4±0.5[ab]	9.3±0.3[a]
Catechin	10.8±0.2	2.8±0.1	18.6±1.0	36.4±0.9[a]	2.6±0.3[a]	8.8±0.3[ab]
Hulls	10.7±0.5	2.4±0.2	17.7±0.8	28.9±1.3[b]	2.1±0.4[b]	8.2±0.3[b]
Experiment II[3]						
Control	14.6±0.5[a]	5.2±0.4	24.5±1.1	60.7±2.7[a]	4.5±0.4	14.0±0.6
Hulls	13.2±0.2[b]	5.2±0.3	25.2±1.3	51.1±2.3[b]	4.6±0.3	14.3±0.5

[1] Each value represent mean ± SEM (n=8).
[2] Different letters within a column indicate significant differences at $p<0.05$ as determined by ANOVA and LSD.
[3] Different letters within a column indicate significant differences at $p<0.05$ as determined by student's t-test.

TABLE 14

Concentration and total content of zinc in tissues[1]

Diets	Liver	Tibia (μg Zn/g dry wt)	Kidney	Tissue total Zinc (μg Zn)		
				Liver	Tibia	Kidney
Experiment I[2]						
Control	63.6±1.8[a]	198.2±11.4[a]	80.5± 1.3	225.9±11.8	168.8± 8.6[a]	40.2±1.5
Catechin	74.6±1.9[b]	232.0± 7.4[a]	84.7± 3.5	245.5± 2.5	215.3±10.0[b]	42.1±1.3
Hulls	79.1±2.5[b]	158.1±13.7[b]	98.0±17.7	223.4± 9.2	138.7±13.4[a]	44.9±7.8
Experiment II[3]						
Control	88.3±1.5[a]	158.5± 9.5[a]	94.7± 7.6	367.0±10.9	136.8±10.9[a]	54.2±1.3
Hulls	98.1±2.9[b]	186.4± 8.8[b]	90.4± 7.2	379.9±13.9	164.8± 7.5[b]	51.5±1.7

[1] Each value represent mean ± SEM (n=8).
[2] Different letters within a column indicate significant differences at $p<0.05$ as determined by ANOVA and LSD.
[3] Different letters within a column indicate significant differences at $p<0.05$ as determined by student's t-test.

DISCUSSION

PRELIMINARY ANALYSES

It is difficult to compare the tannin concentration of dry beans as reported by different investigators, primarily because of the differences in methods and beans sources used (Price *et al.*, 1978; Desphande and Cheryan, 1985). The tannin concentration for pinto beans in the present investigation was 1139 mg catechin equivalent (CE)/100 mg whole bean. Using a similar method, Desphande and Cheryan (1985) reported that pinto beans had a tannin concentration of 306-562 mg catechin equivalent/100 g whole bean. The factors that may influence the tannin content include storage time, soil and growing season (Salunkhe *et al.*, 1990).

Results showing low or no condensed tannins in white colored beans, compared to colored beans confirms the results of Cabrera and Martin (1986) and Deshpande *et al.* (1982). A wide variation in the tannin concentrations in pigmented beans was observed ranging from 250 to 1139 mg catechin equivalent/ 100g dry wt in present study. Studies on the inheritance of tannin content have shown a high broad-sense heritability (Salunkhe *et al.*, 1990). Although black-seeded beans contained higher amounts of tannin, few plants with nonblack but colored seed had high tannin concentrations. Tannins were

detected only in the colored beans; however, no direct relationship was observed between the intensity of color and tannin content in the present study which was in agreement with the observation of Desphande *et al*. (1982). Light kidney beans had more tannins than did dark kidney beans, whereas dark colored black eyed peas had the lowest tannin content of these beans.

The extraction time is an important factor influencing tannin analysis. In the literature, extraction times of 20 min to 24 hr are used. Many phenols tend to oxidize during sample preparation and extraction (Salunkhe *et al*., 1990). Hence, the longer the extraction time, the lower the tannin content was. Price *et al*. (1978) reported that when the extraction time was extended to 24 hr, there was a decrease in absorbance of 10 to 15% in assayable tannins. Extraction for 24 hr gave overall 40 to 70% lower estimates of tannin for all the sorghum varieties studied by Price *et al*. (1978) as compared to a 1 hr extraction. Desphande and Cheryan (1985) reported that with prolonged extraction periods (> 48 hr) only 10-20% of the total tannins were detected. This decrease related to extraction times has been attributed to the destruction of tannins by HCl, since, even after the extracts were separated from the grain, the absorbance continued to decrease with time (Salunkhe *et al*., 1990). Deshpande and Cheryan (1987) observed that the tannin extraction pattern of dry beans (*Phaseolus vulgaris* L.) is markedly affected by the

solvent used. With absolute methanol, maximum absorbance at 500 nm using the 0.5% vanillin assay of Price *et al.* (1978) was obtained within 10 to 20 min of extraction, and the values slowly decreased with longer extraction times, presumably due to tannin oxidation.

Results showing that tannins are mainly located in the hulls, with negligible amounts in the cotyledons confirms reports in the literature (Rao and Prabhavathi, 1982). Desphande and Salunkhe (1982) found that removal of seed coats lowered the assayable tannin concentration of beans by 68 to 95% and dehulled pinto beans had a 94.6% reduction of assayable tannin concentration. By determining the tannin concentration in seed coats, Laurena *et al.* (1984) also detected 2 to 3 times greater concentration of assayable tannins in eight varieties of cowpeas. Desphande and Cheryan (1985) showed that when expressed on a total seed weight basis, seed coats had a 1.1 to 2.5 times greater concentration of assayable tannins compared to that of dry bean flours.

Different commercial brands of pinto beans had a similar tannin concentration in whole beans. A wide variation was observed in the tannin concentration of hulls, 3.5 to 11.8 g CE/100 g. The length of soaking time for removal of the hulls may have been the major reason for the difference between the Morrice and Brown's Best beans. Soaking winged beans and cowpeas for 24 hr reduced tannin concentration by 50% and over 50% as reported by Sathe and Salunkhe (1981) and Laurena *et*

al. (1986), respectively. Desphande and Cheryan (1983) observed that pinto beans with the highest tannin concentration showed the greatest reduction in tannin concentration after soaking, and the leaching of bean tannins on soaking in distilled water increased with the time of soaking. They reported tannin losses averaging 17%, 41%, and 49% when beans were soaked in water for 6, 12, and 18 hr, respectively. Since tannins are present in the outermost layers of the seed, during soaking they may diffuse into the seed and bind with proteins (Salunkhe *et al.*, 1990). Desphande and Cheryan (1985) further suggested that the apparent loss of tannin was caused by the formation of insoluble tannin-protein complexes which were not assayable by the method employed to determine the tannin content.

Deshpande and Cheryan (1985) reported 6.85 g CE/100g hulls for pinto beans without soaking. The data in the present study showed 3.5 g CE/100 g hulls with 17 hr soaking and 11.8 g CE/100 g hulls with 4 hr soaking. The conflicting results in the two studies could be related to differences in source of beans, preparation or time of storage. Deshpande and Cheryan (1985) stated the storage time was an important factor affecting the assayable tannin concentration of beans especially after milling. They reported a steady decline in tannin concentration of pinto beans with time after milling. In addition, the beans used in their study had been harvested in 1980 and stored for 5 years before analyzing.

Little attention has been paid to the distribution of mineral content in various bean fractions. In present study, hulls were the most abundant source of iron in these beans, irrespective of the brand. Cotyledons had somewhat lower amounts of iron than whole beans. The iron concentration of pinto bean hulls ranged from 140 to 161 ppm. Srisuma (1989) investigated four cultivars of navy beans and reported a wide range (60 to 172 ppm) for the iron concentration in the hulls. For zinc and copper, both cotyledon and hulls contained levels similar to those in whole beans. In the present study, the zinc value in hulls was from 28.2 to 33.7 ppm; Srisuma (1989) showed a similar range from 16.7 to 35.1 ppm. Values for the copper concentration of whole pinto beans, 4.8 to 11.6 ppm, were similar to those reported in the literature (Srisuma, 1989).

ANIMAL STUDIES

Results for the effect of diets containing 25% pinto bean hulls on food intake and efficiency were different in the two experiments. In Experiment I rats fed 25% pinto bean hulls had a reduction in food efficiency and no change in food intake, but in Experiment II hull-fed rats had a higher food intake and no change in food efficiency. When results from the two experiments were combined statistically using 2X2 ANOVA, no significant influence on food intake and body weight

was shown. Similarly, Garcia-Lopez *et al.* (1990) reported no effect on total food intake and food utilization in anemic rats fed 2.4% red kidney bean hulls for 3 weeks. Edwards *et al.* (1973) found no evidence of growth depression after feeding sheep 62% faba bean hulls for 28 days. Levrat *et al.* (1993) stated that in rats food intake and weight gain were not affected by addition of 1% condensed tannins extracted from quebrancho to the diet. Chang *et al.* (1994) reported that feeding rats 0.057% condensed tannin isolated from tea or cowpeas for 28 days did not significantly change the food intake or body weight. Jansman *et al.* (1994) observed that food intake and weight gain of rats consuming a diet of 1.41% tannins extracted from faba hulls did not differ significantly from those of control rats. However Jansman *et al.* (1994), in the same study found significantly decreased food intake and lower weight gain in rats fed 60% of faba bean hulls which were estimated to contain 1.99% condensed tannin. Jansman *et al.* (1993) showed that in piglets incorporation of 20% faba bean hulls estimated to contain 3.3% condensed tannins had a tendency to decrease food consumption and resulted in a significant reduction in weight gain. Moreover, Marquardt *et al.* (1977) previously reported that chicks fed a diet containing 3.9% of condensed tannins purified from faba bean had markedly reduced food intake compared to those of control. In Experiment I, rats fed the 1.6% catechin diet ate normally but tended to have a somewhat lower food efficiency. This

finding is consistent with the observation of Greger and Lyle (1988) using paired-fed rats. Conversely, Joslyn and Glick (1970) reported that diets containing 2% and 4% catechin caused a depression effect of 17% and 45%, respectively, on growth in rats compared to controls. These conflicting results for the effect of tannins on food intake and food efficiency might be explained by both the level and the source of the tannins as well as the animal species involved. Some authors found evidence for differences in the sensitivity among animal species for tannins in sorghum (Elkin *et al.*, 1990; Mehansho *et al.*, 1987) and faba bean (Ford and Hewitt, 1979). These data also suggested a threshold effect of condensed tannins on food intake in rats at a level of about 2% of the diet.

Tannic acid when fed to different animal species has been shown to affect different internal organs. Chang and Fuller (1964) reported fatty livers in chicks fed diets containing tannic acid. Karim *et al.* (1978) observed necrosis of the liver and kidney of chicks fed diets containing 1-3% tannic acid. Jansman (1994) concluded that the effects on the liver and kidney indicate that either tannic acid itself or degradation products of tannic acid (e.g. gallic acid) are absorbed from the small intestine and cause toxic effects. However, it is not completely clear from the literature whether the condensed tannins affect internal organs, especially liver and kidney. This is related to limited

knowledge about whether or not condensed tannins are absorbed from the digestive tract. In the present study, rats fed the hulls diet had significantly diminished liver relative weight and tended to have decreased wet and dry liver weights compared to control-fed rats. Similarly, Marquardt *et al.* (1977) reported that incorporation of 3.9% condensed tannins extracted from faba bean decreased liver weight in chicks. In contrast, Chang *et al.* (1994) found that diets containing 0.057% tannins isolated from cowpea had no effect on either liver weight or relative liver weight in rats. Moreover, Jansman *et al.* (1993) observed no effect on liver weights in piglets fed 3.3% of condensed tannins isolated from faba beans hulls. These conflicting results might be explained by specie differences in sensitivity to the condensed tannins. However, results in the present study showing relative liver weights in rats fed 1.6% catechin similar to those of controls suggest that factors other than condensed tannin in hulls may have contributed to the decrease in relative liver weight.

Components of pinto bean hulls appeared to alter copper bioavailability. Ingestion of 25% pinto bean hulls elevated plasma copper and ceruloplasmin activity but diminished total liver copper significantly and consistently in the two studies. The reduction of total liver copper may have been the consequence of a reduction of liver weight in Experiment I or decrease of liver copper concentration in Experiment II. These findings of decreased liver copper in hull-fed rats

occurred in spite of higher total dietary intakes of copper. If the total liver content of copper, however, is expressed as a percentage of the total dietary copper intake, copper deposit in hull-fed rats was much lower (5.1%) instead of higher compared to those of catechin-fed (7.8%) or control-fed (8.5%) rats. Since the levels of dietary copper (≤ 2ppm) were considered to be marginally deficient, the efficiency of copper absorption and the percentage of copper deposition into the liver would be expected to be high in all groups. Various factors may have contributed to the decrease in total liver copper observed in the hull-fed rats compared to controls. Stabel *et al.* (1993) reported that the liver is particularly sensitive to changes in copper status. Suttle *et al.* (1975) demonstrated that administration of copper after a period of copper depletion, priority was given to repletion of copper in plasma followed by copper pools such as the liver. Therefore, release of liver copper stores with consequent decrease in total liver copper content may explain the increased levels of serum copper and ceruloplasmin seen in hull-fed rats. DiSilvestro and Harris (1983) found that injection of catechin elevated lysyl oxidase activity in the aortas of chicks. Since lysyl oxidase is a copper containing-enzyme and responds rapidly in copper deficient animals, they suggested that catechin might enhance copper absorption. Greger and Emery (1987) observed that rats fed coffee and decaffeinated coffee that contained 5% polyphenols had elevated liver copper. In

contrast to the present results, Greger and Lyle (1988) reported that ingestion of black tea, estimated to contain 1.17% condensed polyphenols, enhanced copper absorption leading to elevated levels of copper in plasma and in liver by anemic rats. However, feeding rats the same level of catechin showed no change in plasma copper or in tissue copper concentration (Greger and Lyle, 1988). They concluded that catechin in black tea was unlikely responsible for the alteration in copper bioavailability. In the present study, thereby, it is likely that pinto bean hulls but not condensed tannins (or at least catechin) in hulls were responsible for the change in plasma copper and total liver copper content because similar effects were not seen in catechin-fed rats. Aoyagi *et al.* (1993) estimated copper bioavailability from feed ingredients derived from plants (peanut hulls and soy mill run) and animals (livers from chicken, beef, sheep and turkey). They concluded that the copper in feed ingredients from plants was less bioavailable than that from animals. They suggested these results were likely due to phytate binding of copper in the plant material. However, it would appear that some other unidentified factor other than phytate in pinto bean hulls was responsible for the alteration in copper bioavailability in the present study because the majority (up to 95%) of phytate is present in the cotyledon of pinto beans (Salunkhe *et al.*, 1990).

Dietary treatments affected liver zinc concentration. Significantly higher liver zinc concentrations were found in rats fed the hulls and catechin diets. However, the hulls diets overall had no effect on tibia zinc, because opposite effects were shown in the two studies. The reason for the conflicting results is not clear. Tibias in rats fed the catechin diet tended to have a higher zinc concentration and significantly elevated total zinc content. Similarly, Greger and Emery (1987) observed that rats fed coffee and decaffeinated coffee that contained 5% polyphenols had elevated tibia zinc. Moreover, Greger and Lyle (1988) reported a significant elevation of tibia zinc concentration in rats fed catechin or tea; however, the mechanism(s) responsible for these changes are unclear. Pinto bean hulls and catechin had no effect on plasma zinc in the two studies.

No effect was observed in iron bioavailability since the hemoglobin and hematocrit did not change in different dietary treatments. These results confirm the observations of Brune *et al.* (1989), Garcia-Lopez *et al.* (1990) and House and Van Campen (1994) that condensed tannins per se were not responsible for the low iron bioavailability of legumes.

CONCLUSIONS

Ingestion of diets containing 25% pinto bean hulls led to a decrease in relative liver weight in both experiments. The significance of this change is unclear. The overall results indicated that feeding rats pinto bean hulls tended to alter copper bioavailability as shown by the decreased total liver copper content and increased plasma copper. However, these effects were not seen in the rats fed the catechin diet. Since both the hulls and catechin diets contained a similar concentration of assayable condensed tannins, we suggest that pinto bean hulls but not condensed tannins are responsible for the alterations which were observed. There could be some other factor(s) present in bean hulls that caused the copper release from liver stores into the plasma.

The catechin and pinto bean hulls diets had no effect on serum zinc but elevated the liver zinc concentration. In the hull-fed rats, this increase might be related to the higher total zinc intake but this can not explain the results in the catechin-fed rats. Condensed tannins, therefore, could be the potential factor responsible for this change. However, the mechanism involved is unclear. Interestingly, we found that the dietary treatment enlarged the size of the tibia in rats fed the catechin diet, but not in rats fed the hulls diet. If this increase in tibia size was related to condensed tannins,

i.e., catechin, it is suggested that there is some other unidentified factors in hulls which overcomes the effect of condensed tannins.

RECOMMENDATIONS

Because increased consumption of beans is being recommended in order to increase dietary intake of complex carbohydrates and fiber, it is important to have a better understanding of other components in beans such as tannins which traditionally have been considered as antinutritional factors. Ideally such research would be done using tannins purified from different varieties of beans in order to fully understand their nutritional effects. The commercially available catechin is extracted from tree bark, and hence it is questionable to use it as a standard reference in bean tannins studies. Obviously bean hulls contain numerous other chemical compounds as well as a variety of different types of tannins. The most common method used to isolate and purify condensed tannins was described by Hagerman and Butler (1980).

Additional research also should focus on investigating if the condensed tannins in the pinto bean hulls were the factor responsible for the alterations in copper and zinc bioavailabilty in rats that were observed in the present experiments. Experimental approaches might include feeding rats diets containing: (1) PVP (polyvinylpyrrolidone), an agent known to bind condensed tannins (2) purified tannins isolated from the pinto bean hulls.

Because of recent evidence suggesting a possible beneficial effect of tannins, other future research in this area should be aimed at investigating the possible role of bean tannins in cancer prevention or their pharmacological properties as an antifungal agent.

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