POLYPHENOLIC SECONDARY METABOLITES IN HEIRLOOM VERSUS COMMERCIAL BEAN VARIETIES: IMPLICATIONS FOR HUMAN HEALTH

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

CRAIG HASSEL, DANIEL DAVID GALLAHER

JANUARY 2009

Acknowledgements

I would like to express my deepest gratitude to my advisers, Dr. Craig Hassel and Dr. Daniel Gallaher for all of their support and encouragement, and to Dr. A. Saari Csallany who graciously met with me and agreed to be on my committee. I would also like to express my gratitude to all of lab 65, especially Cindy Gallaher, Moonyeon Yoon, Natalia Schroeder and Drew Brockman for all of their help and encouragement.

I would like to especially that the people at Dream of Wild Health, namely Sally and John Auger, Donna Chapelle, Diane Wilson, Emily and all of the kids from the summer of 2008. I would like to thank CURA for their support.

I would like to thank my family, especially Christina for listening to me and having patience.

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Introduction to the Thesis Project

A dietary pattern that is high in fruits, vegetables, whole grains and legumes has been identified through epidemiological studies as being associated with a reduce risk of chronic disease [1, 2]. Chronic disease, defined as "non-communicable illnesses that are prolonged in duration, do not resolve spontaneously, and are rarely cured completely" are the leading cause of death and disability in the United States [3]. Legumes, and in particular dried beans, are a culturally significant food to many populations in the US [4]; they are an inexpensive source of nutrients relative to fruits and vegetables. Epidemiological evidence supports the association between reduced mortality in the elderly and high levels of bean consumption [5]. When compiling data from 5 different cohorts in 4 different countries, Darmadi-Blackberry *et al.* observed a 7-8% reduction in mortality hazard ratio for every 20g increase in bean intake among elderly populations [5]. Dietary bean intake has been associated with reduced risks for breast cancer [6], prostate cancer [7, 8], diabetes [9], and heart disease [10]. It is unknown whether a reduction in chronic disease risk associated with legumes is directly related to the composition of legumes, or if legumes contribute to health by replacement of other less beneficial foods.

Plant foods produce metabolites secondary to those required for primary functions of the plant i.e., photosynthesis [11]. Secondary metabolites may confer human health benefits [12]. Polyphenolics and a subclass, flavonoids, are secondary metabolites which may aid in the prevention and or treatment of chronic disease. Polyphenolics can affect cellular differentiation, proliferation and apoptosis, alter immune function, and affect proteins and enzymes involved in metabolism [13]. Their biological activity, which until recently has been attributed mainly to their antioxidant activity, ranges from anti-

inflammatory, antiviral, antibacterial, antiulcer, antiallergic, antihepatotoxic and antiosteoporotic [14]. Common beans (*Phaseolus vulgaris*) are a rich dietary source of polyphenolics.

Evidence of common bean (*Phaseolus vulgaris*) cultivation in the U.S.A. dates back 2,300 years; their origin is likely Mexico and Central America [15]. Native Americans introduced New World settlers to landraces or small red, pinto, pink and Great Northern beans [15]. Along with squash and corn, beans are a culturally significant food for Native Americans; together they form the "Three Sister" crops. Heirloom varieties of seeds have been shared between generations of Native Americans and may possess health benefits that differ from commercial varieties. It is possible that the bean varieties cultivated by Native Americans before European settlers arrived differ from the bean varieties commercially available today. Early bean researchers involved in government research programs in the early 20th century introduced new germplasm and began developing more disease resistant species [15]. Commercial beans are selected annually based on yield, color, maturity time, disease reaction growth habit, and palatability [16], and therefore may differ compositionally from heirloom variety seeds.

The purpose of this thesis is to 1) review current literature on polyphenolics in common beans, and examine the relationship between polyphenolics and chronic disease. 2) perform a systematic literature study examining dietary intervention trials and cohort studies, investigating the effect of bean intake on human health. 3) analyze antioxidant activity, polyphenolic content and flavonoid content of heirloom varieties of beans and market varieties of beans that have been grown under identical environmental conditions from seed. Three different heirloom bean varieties obtained from a Native American seed

saving project were analyzed and two of them were matched with market variety beans of similar color and size for comparison.

Chapter 1 A Review of Phenolics

Polyphenols

Polyphenols are a class of plant secondary metabolites. Secondary metabolites are defined as those not essential to the primary metabolic processes such as cellular respiration or photosynthesis [11]. The production of secondary metabolites, an energy intensive process, probably originated from genetic mutations in compounds essential to primary metabolism [11]. Plant tissues essential to survival and multiplication, such as epidermal and bark, flowers, fruits and seeds, are generally high in polyphenols [17].

The basic structure of polyphenols comes from the diversion of L-phenylalanine and L-tyrosine from protein synthesis to the synthesis of the phenolic polymer [18]. Polyphenols are characterized by two or more phenolic rings (**Figure 1.1**) joined together [13]. Vascular or "woody" plants contain different polyphenolic profiles than non-vascular plants, such as mosses and algae, likely due to the production of lignin by vascular plants [18]. Lignin is a polymer of phenylpropanoid units synthesized from polyphenol [19] that has a dibenzylbutane skeleton (**Figure 1.2**) [20]. The present study deals with polyphenolics in beans, which are vascular plants. Three classes of phenolics are highly represented in vascular plants; they are phenolic acids, proanthocyanins and the flavonol glycosides. The latter two are classified as flavonoids [18].

Phenolic acids are low molecular weight polyphenols, which are universally distributed in plants [13] and are comprised of a singular carboxyl and one or more hydroxyl groups bound directly to an aromatic hydrocarbon (**Figure 1.3)** [19].

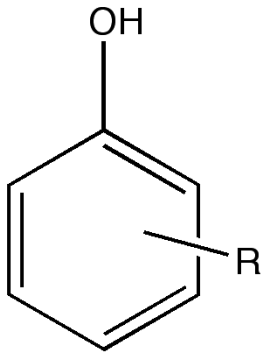


Figure 1.1 Phenol derivatives

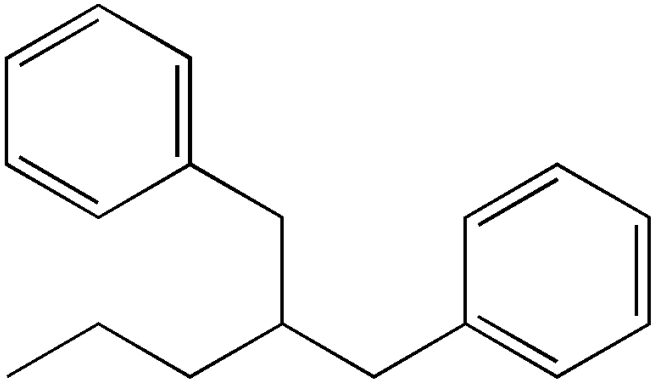


Figure 1.2 Dibenzyl butane (lignin)

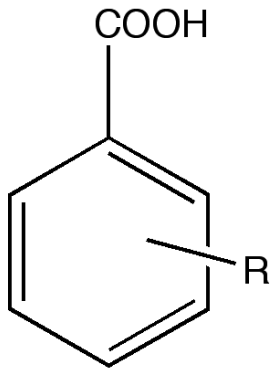


Figure 1.3 Phenolic acid derivatives

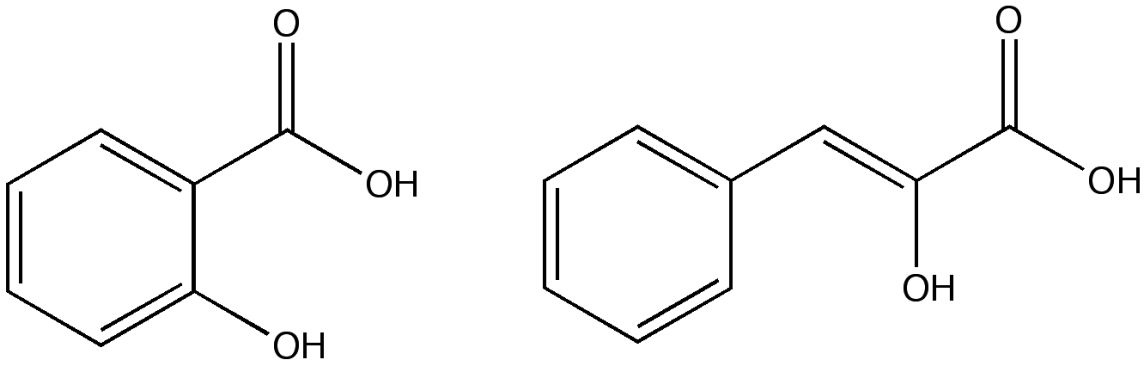


Figure 1.4 Hydroxybenzoic acid (left), hydroxycinnamic acid (right)

The two major classes of phenolic acids include hydroxycinnamic acids (C_6-C_3) and hydroxybenzoic acids (C_6-C_1) (**Figure 1.4**) [13]. Synthesis of phenolic compounds begins with the shikimate and acetate pathways (**Figure 1.5**) [19]. The shikimate pathway is initiated with the condensation of phosphoenolpyruvate (PEP) from glycolysis and erythrose-4-phosphate from the pentose phosphate pathway. After a series of reactions, chorismate is formed and the pathway splits such that some of the chorismate goes to the production of tryptophan and some to phenylalanine and tyrosine. Hydroxycinnamic acids are derived from phenylalanine in a three-step process known as "general phenylpropanoid metabolism" (**Figure 1.6**) [21]. The process begins with the de-amination of phenylalanine, which generates a *trans* double bond in the cinnamic backbone [21]. Subsequent hydroxylation at position 4 yields *p*-coumarate [21]. After this second step, additional hydroxylation and methylation reactions can occur on the aromatic ring, yielding different derivatives of hydroxycinnamic acid [21] including caffeic, ferulic and sinapic acids [22]. The final step is the enzymatic formation of the CoA ester via a ligation reaction [21].

Hydroxycinnamic acids are widely distributed in plants, the most abundant are ferulic acid and caffeic acid (**Figure 1.7)** [23]. Due to their C_6 phenyl ring structure and C_3 side chain, these compounds are collectively called phenylpropanoids and they serve as precursors to structural lignins (as well as other compounds) [22]. Hydroxybenzoic acids are similar to the phenylpropanoids in structure, although they are missing the two-carbon moiety (Figure 1.4) [22]. The plant mechanism for synthesis of benzoic acid is currently unknown [26], however, two possible methods of synthesis of benzoic acid have been suggested [21]. One is the degradation of the acetate side chain of the

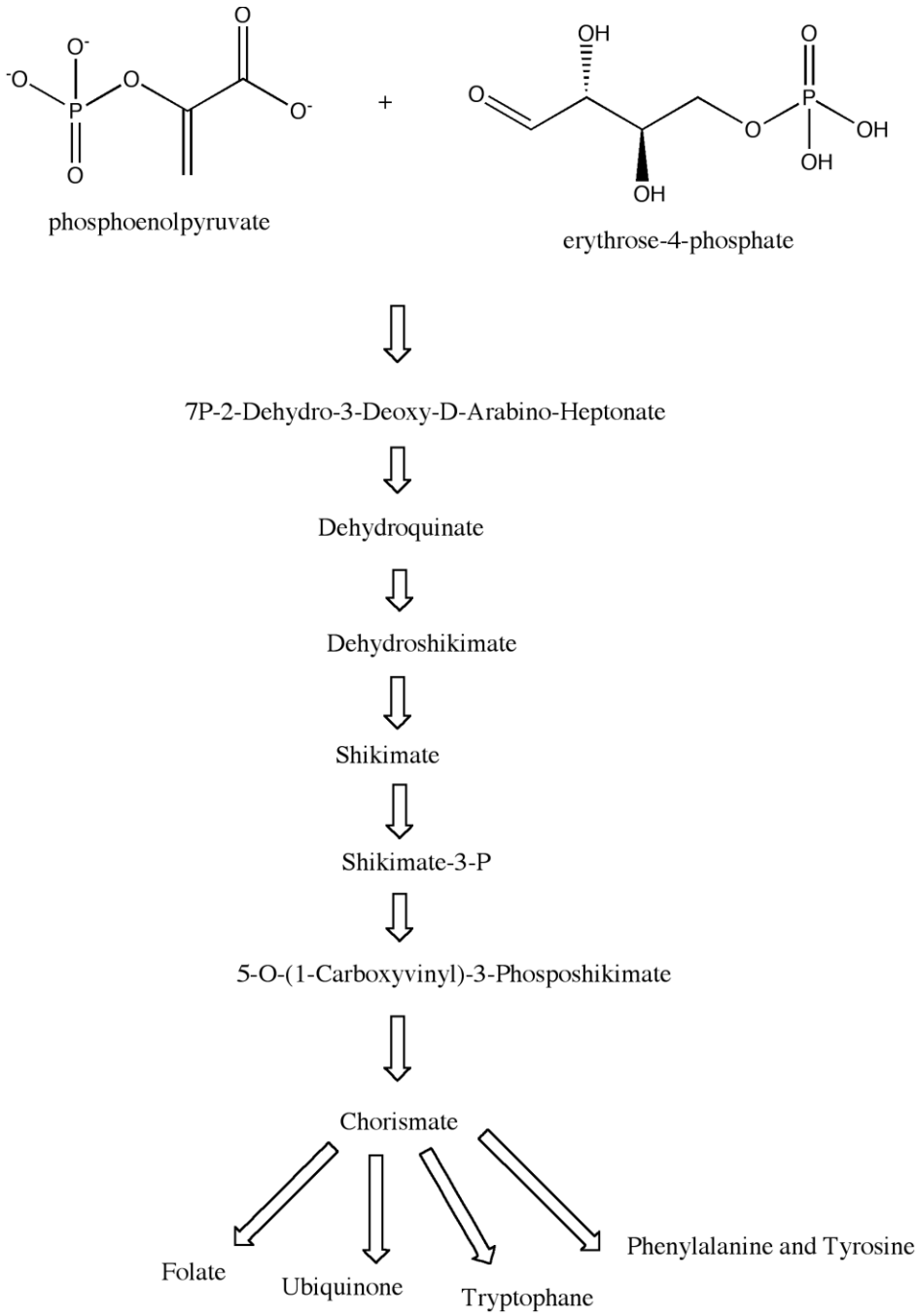


Figure 1.5 Shikimate Pathway [24]

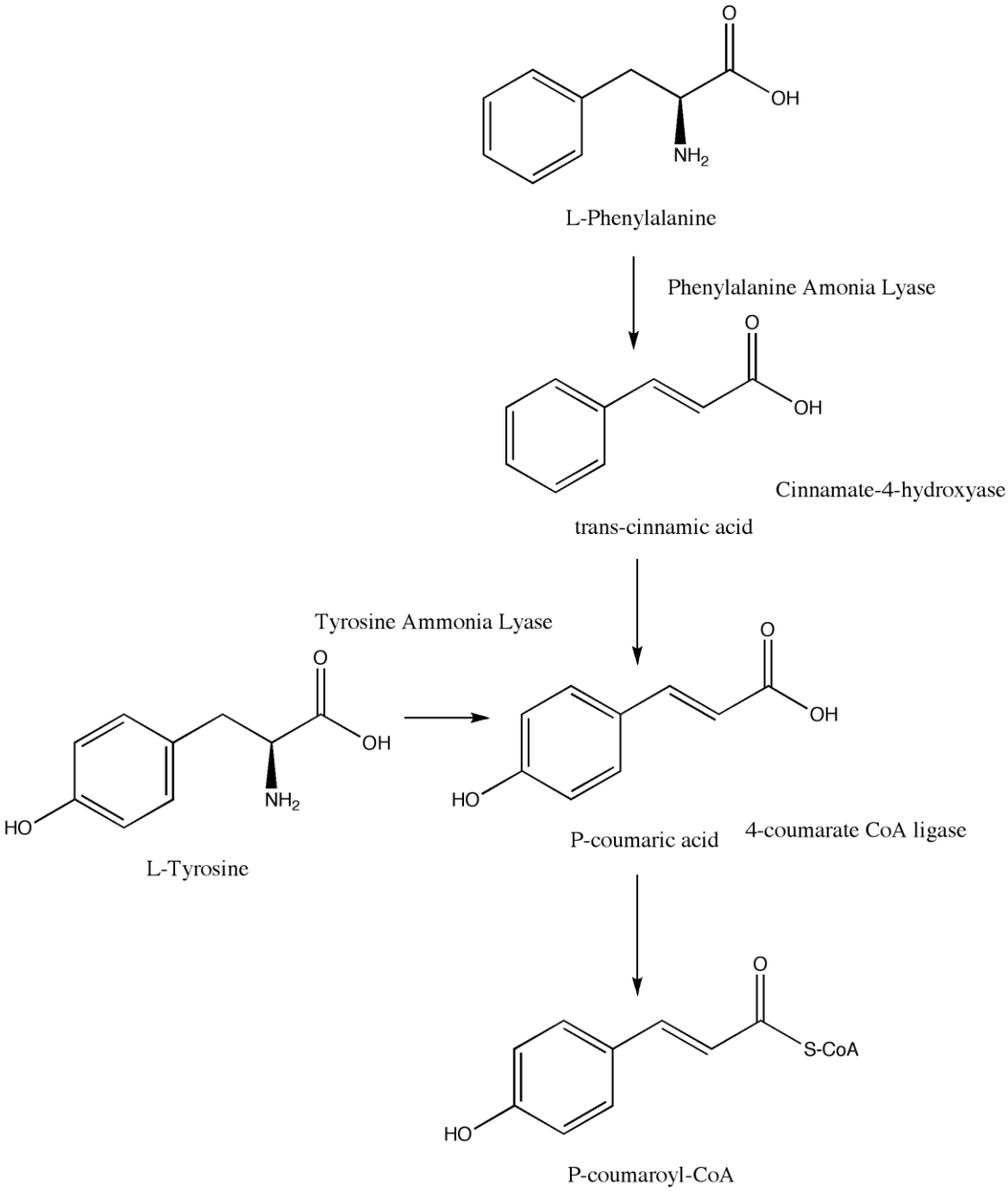
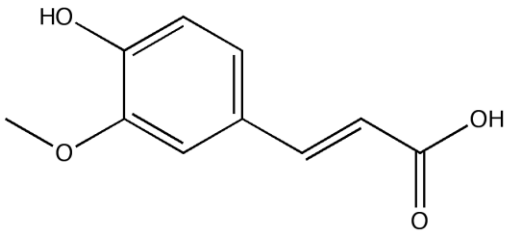
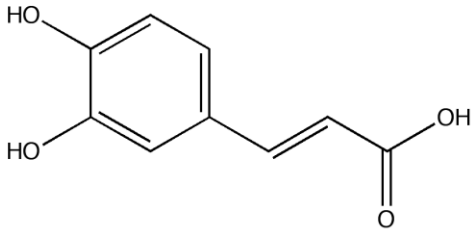


Figure 1.6 General phenylpropanoid pathway [25]



ferulic acid

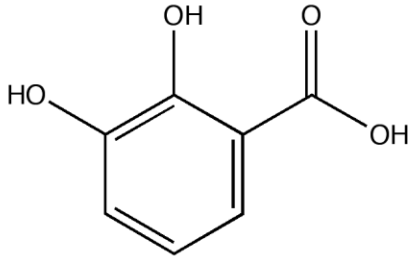


caffeic acid

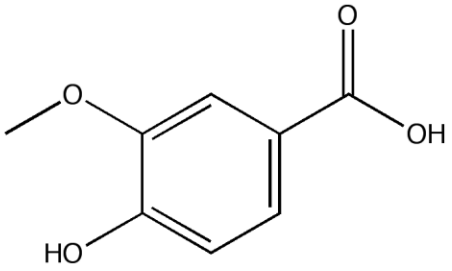
Figure 1.7 Hydroxycinnamic acids commonly found in plants

hydroxycinnamic acid derivatives [21, 22], and the other is an independent pathway branching off of one of the shikimate pathway's derivatives [21]. Hydroxylation and possibly methylation of hydroxybenzoic acid yields dihydroxybenzoic acid, vanillic acid, syringic acid, and gallic acid (**Figure 1.8**) [22].

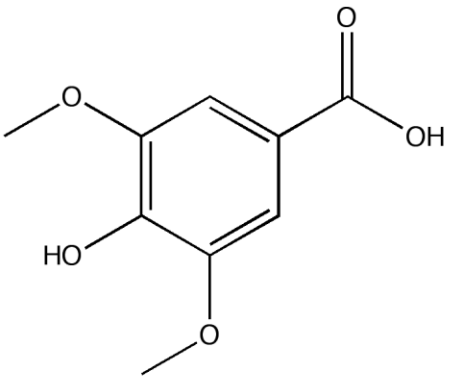
In plants, phenolic acids seldom occur as free acids, but most commonly occur as esters and amides [23]. Hydroxybenzoic acids are often present in bound form as lignins and hydrolysable tannins [22]. Tannins are oligomers and polymers of polyphenolic subunits, which contain enough phenolic hydroxyls to form strong complexes with protein and other macromolecules [27]. Tannins vary according to plant tissue, species and stage of development [28]. Their function is associated with disease resistance [29].
The flavonoid class is diverse, currently over 9000 different flavonoids have been identified [14]. The basic structure is the three ring C_6-C_3-C_6, with rings designated A, B and C (**Figure 1.9**) [30]. The A ring being derived from three condensed acetate units, while the remaining carbons are derived from the synthesis of cinnamic acid [30]. Thus, the A ring is the fused aromatic ring, the B ring is the phenol constituent and the C ring is the heterocyclic benzopyran ring (Figure 1.9) [31]. The flavonoids are organized into subclasses according to oxidation states on the C ring [32]. Seven subclasses of flavonoids include flavones, flavonols, anthocyanidins, flavanols, flavanones, flavanonols and isoflavones (**Figure 1.10**) [33]. These subclasses are further divided into individual flavonoids based on variation in number and arrangement of hydroxyl groups and their alkylation or glycosylation [33]. The flavones and flavonols, which differ only in a hydroxyl group in position 3 of the C ring (Figure 1.10) [33], have been identified in almost all plants [32].



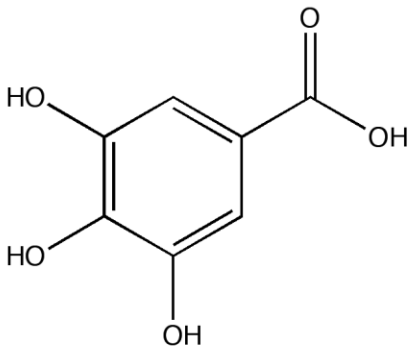
dihydroxybenzoic acid



vanillic acid



syringic acid



gallic acid

Figure 1.8 Hydroxybenzoic acids commonly found in plants

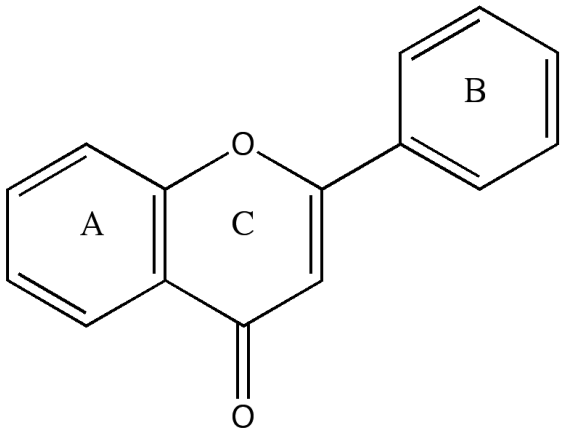


Figure 1.9 Basic flavonoid structure

The flavanols, also called the flavan-3-ols, differ from anthocyanidins in that they lack a double bond in the 2-3 position (Figure 1.10) which, in conjunction with the 3 hydroxyl group on the C ring, creates two centers of asymmetry [33]. Four different isomers of flavon-3-ols are thus found in nature, the most abundant of which are (-)epicatechin and (+)-catechin (**Figure 1.11**)[33]. These flavan-3-ols are the building blocks for proanthocyanidins [31]. Flavanones and flavanonols share an identical structure except for the presence of a hydroxyl group in the 3 position on flavanonols (figure 1.10) [33]. Isoflavones are similar to flavones in structure, except the B ring is attached to carbon 3 instead of 2 (figure 1.10) [32].

Flavonoids usually occur as glycosides in plants [14]; the binding of sugar promotes water solubility, light stability and resistance to enzymatic degradation [30]. The catechins are the only flavonoids that sometimes occur as aglycones [32]. Flavonol glycosides are labeled *O*-glycosylflavonoids or *C*-glycosylflavonoids depending on whether the sugar is linked through a hydroxyl group or through a carbon-carbon bond of the flavonoid aglycone [30]. Linkages are usually β1-2, or β1-6 and although a number of different types of sugar molecules can attach, the sugar at the reducing end is always glucose (**Figure 1.12**) [30].

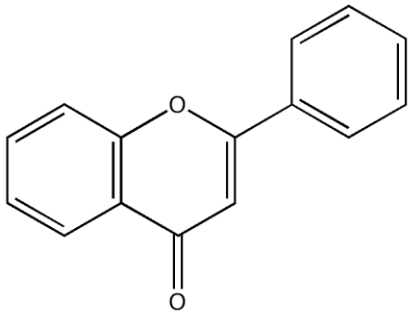
Proanthocyanidins (**Figure 1.13**), also known as condensed tannins, are polymers or oligomers of flavan-3-ol units synthesized via the phenylpropanoid and then flavonoid pathways [31]. The biphenol linkage of condensed tannins is resistant to hydrolysis by cleavage, thus they are termed nonhydrolyzable tannins [27]. Similar to lignins, proanthocyanidins are polyphenolic polymers and that they have a potential role in plant

defense [31]. Different names apply to oligomers and polymers of flavonoids according to the hydroxylation patterns of the monomeric units [31]. For example, the term proanthocyanidins comes from the release of anthocyanins from extension positions observed after being boiled with strong mineral acid. Extension positions are those positions where flavonoids are added to starter units during polymerization. The term procyanidins refers to oligomers and polymers with extension units in a 3'4'-dihydroxyl pattern like (+)-catechin and (-)-epicatechin [31]. Proanthocyanins turn red when treated with acid due to their release of anthocyanin subunits [27]. The method of polymerization of flavon-3-ols to produce proanthocyanidins is currently unknown [31].

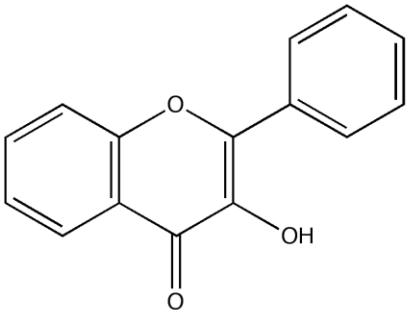
Phenolics In Common Beans

The common beans (*Phaseolus vulgaris*) have over 29, 000 cultivated forms [34], including the dried pinto (mottled), carioca (striped), navy (white), black turtle (purple), kidney (red) and others. *Phaseolus vulgaris* are native to the American tropics, and belong to one of three major gene pools: Middle American, North Andean, or Andean, or a mixed gene pool [29]. When extracts of these three gene pools were analyzed, the highest tannin and polyphenolic contents were observed in the Middle American gene pool, followed by North Andean and Andean [29]. The Middle American gene pool showed significant correlation between tannin content and seed color while the Andean gene pool did not [29].

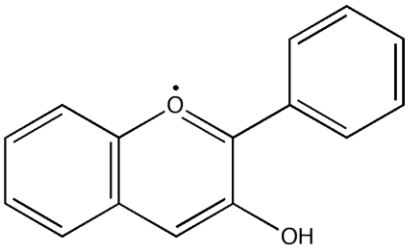
Common bean cultivation in the United States has been in evidence for the past 2,300 years [15]. Selection for specific disease resistance and palatability for commercial growing began in the late 19th century [15]. It is perhaps due to this selective cultivation



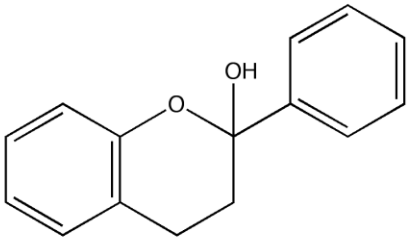
Flavone (apigenin, luteolin)



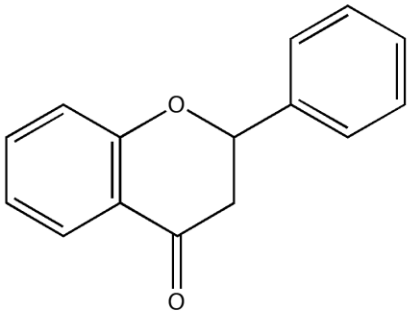
Flavonol (quercetin, kaempferol)



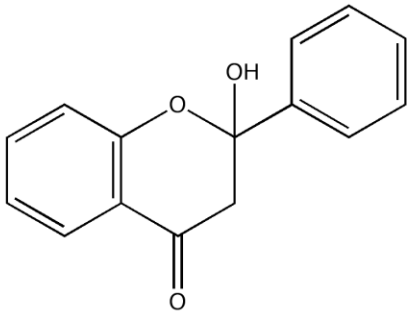
Anthocyanidin



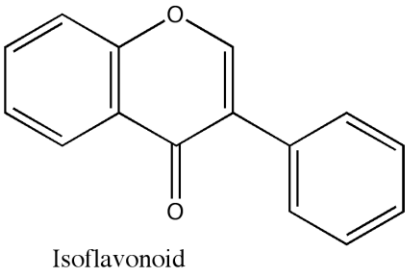
Flavanol (catechin)



Flavanone (hesperetin)

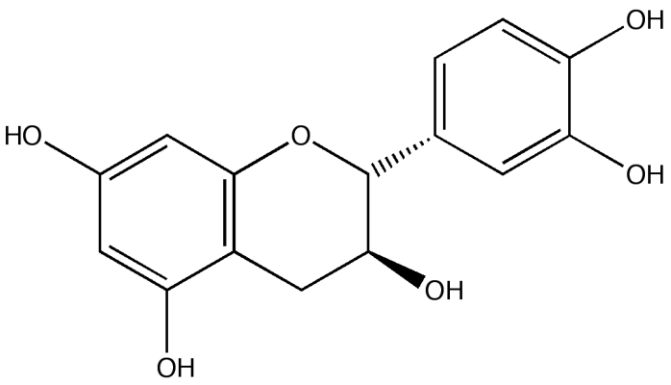


flavanonol

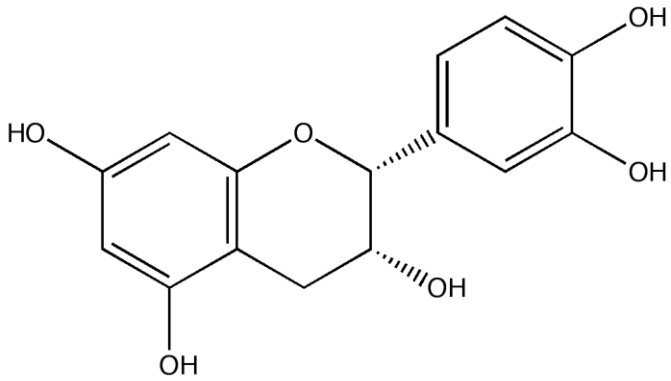


Isoflavonoid

Figure 1.10 Flavonoid subclasses



(+) catechin



(-) epicatechin

Figure 1.11 Isomers of flavon-3-ols

that wild common beans have more extensive genetic variability than cultivated beans [35]. Beans have been selected for based on yield, color, maturity time, disease reaction growth habit, and palatability [16]. Nutraceutical bean characteristics, such as secondary metabolite content, have not been considered in bean breeding programs [35]. Researchers have recently begun investigating the types of secondary metabolites present in wild and cultivated beans [35, 36].

The majority of phenolic acids are in dehulled beans, while the seed coats contain the flavonoids (anthocyanins and condensed tannins)[37]. High concentrations of phenolic acids are found in structural features of plants due to their involvement in the formation of lignin [38]. The predominant phenolic acids found in black beans include gallic acid, caffeic acid, chlorogenic acid, sinapic acid, and trans-cinnamic acid [37]. According to Ranilla *et al*., who characterized 28 varieties of *Phaseolus vulgaris* of varying color, the cotyledon of these beans contained the hydroxycinnamic acids ferulic, synapic and chlorogenic acid, with little difference between samples [39]. Although seed coat color did not significantly influence phenolic acid profiles, the red and black bean cotyledons tended to have higher phenolic acids than the white cultivars[39]. Conversely, no phenolic acids were detected in black or red seed coats, whereas the white varieties contained phenolic acids. Based on HPLC analysis performed by Diez-Banzalla *et al*., hydroxybenzoic acid, vanillic acid, coumaric acid, and ferrulic acid were identified in cream-red, black, and cream beans [40]. Caffeic acid has been identified in the seed coat of black beans in other literature [37].

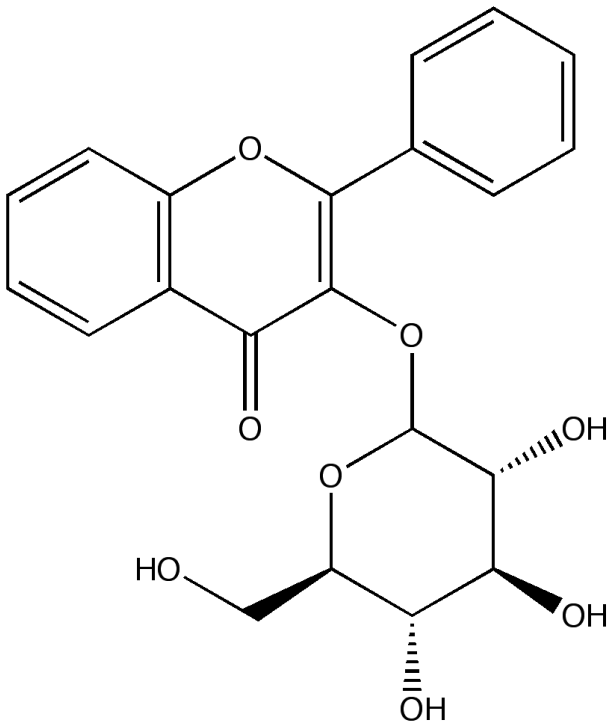


Figure 1.12 *O*-glycosoflavonoid (flavonol glucoside)

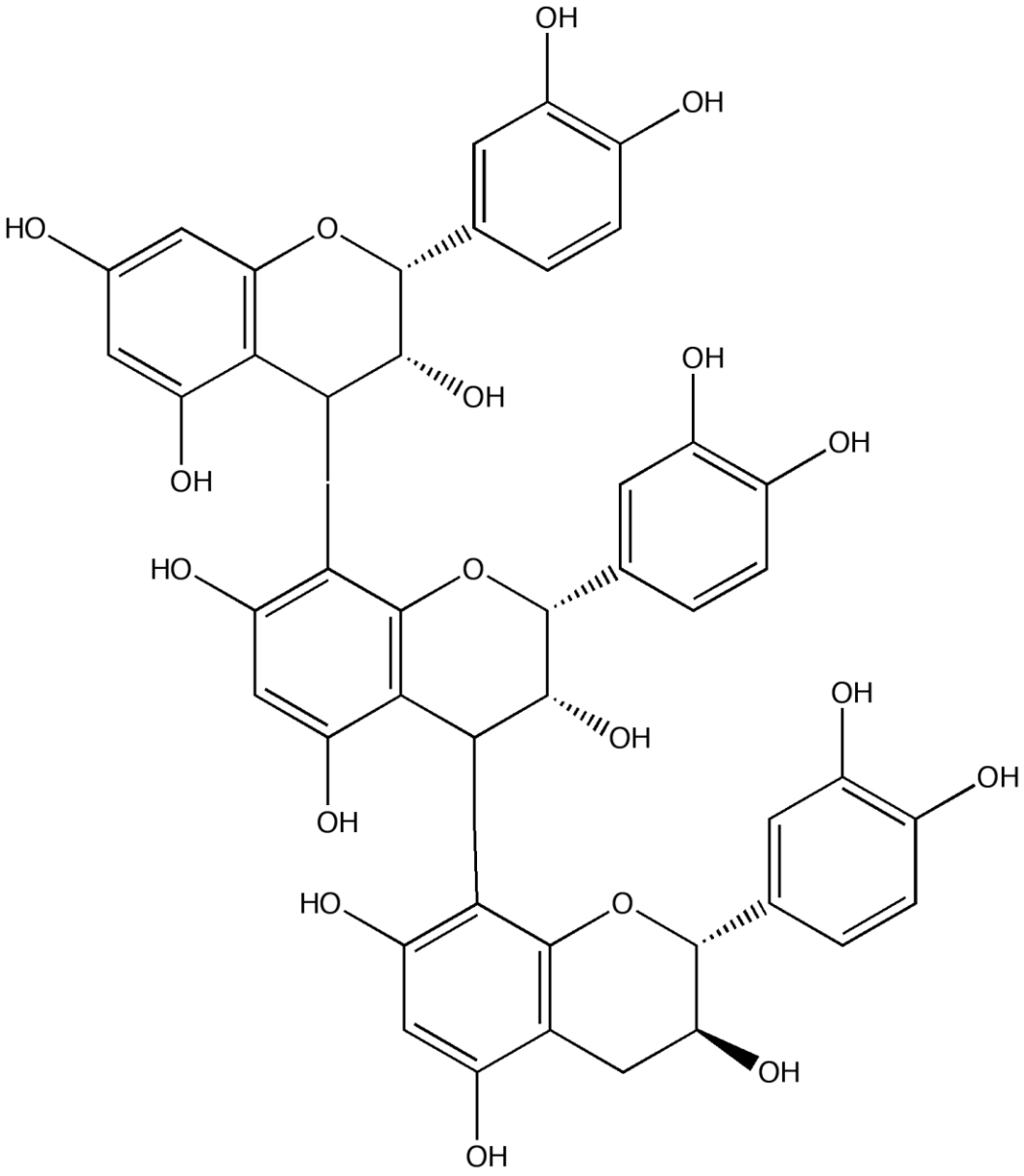


Figure 1.13 Proanthocyanidin

It has been suggested that bean color is determined by concentration of flavonol glycosides, anthocyanins and proanthocyanins [41]. While anthocyanins are mainly found in black or violet colored beans, proanthocyanidins are widely distributed [41]. In a recent analysis of 62 wild and weedy varieties of either Jampa or pinto beans, Espinosa-Alonso *et al.* found no relationship between seed coat color and condensed tannin content [35] or total polyphenolics; however they did find a relationship between anthocyanin content and seed coat color [35]. In measuring varieties of black beans, an inverse relationship was observed between anthocyanin content and condensed tannins [35]. Condensed tannins are the predominant phenolic compounds found in the common bean [42].

In black beans the seed coat has been shown to be the portion of the bean with the highest total polyphenolic content [37]. As reported by Ranilla *et al.*, the seed coats contained anthocyanins, quercetin and kaempferol in black and red varieties, and none in white (although one white variety did contain kaempferol) [39]. Ranilla *et al.* observed no flavonoids in the cotyldon of 22 out of 28 bean varieties; the ones having flavonoids were all brown or yellow in color [39]. When measuring flavonoids from methanol extracts of whole beans using HPLC, Diez-Banzalla *et al.* observed quercetin and kaempferol in cream-red, cream and black beans; the highest quercetin content was in black beans and the highest kaempferol in cream beans, but no association between color and kaempferol content was reported [40]. Perazzini *et al.* observed the presence of kaempferol and kaempferol glycosides in 8 different landraces of Italian *Phaseolus vulgaris* [36]. The flavonol glycosides kaempferol glycoside, quercetin 3-glycoside and myricetin glycoside are common to many beans [41]. Additionally, Laparra *et al.* identified astragalin in three

different varieties of kidney and three varieties of black beans, as well as small amounts of (+)-catechin in the kidney beans and in one black bean variety [43].

Isoflavonoids such as genisten and daidzein are found in soybeans and have been heavily researched for their potential health benefits; however, they are not present in the common bean seed [40] and therefore will not be discussed further.

Factors Effecting Phenolics in Beans

Environmental influences can affect polyphenolic production in plants. An accumulation of phenolic acids is observed in plants during times of environmental stress [19]. Extracts of quinoa were found to have higher polyphenol and flavonoid content when grown in intensely cultivated soil as compared to plants grown in non-disturbed soil [44]. Seeds from bean plants chronically exposed to ozone had an increase in phenolic content as measured by the Folin-Ciocalteu assay, however they also exhibited a significant reduction in some flavonols, including kaempferol and some hydroxycinnamates, and a significant reduction in anthocyanins [45].

Conventional farming practices may use chemical fertilizers, pesticides, irrigation and utilize high-yield crop varieties [46]. Organic farming is regulated such that farmers cannot use genetically engineered crops, and irrigation and soil must be free of synthetic pesticides and herbicides [46]. Sustainable practices promote environmental heath and sustainability; they are difficult to define since they vary from region to region [46]. When matching berries grown in the aforementioned environments, organic and sustainable

varieties had significantly higher total phenolic content than conventional varieties as measured by the Folin-Ciocalteu method [46].

Storage of beans can affect the distribution of phenolic content (free or bound), contributing to the "hard to cook" (HTC) phenomenon [47]. The HTC phenomenon develops in beans when stored in high temperatures or humid conditions [48]. HTC is characterized by decreases in water absorption and increases in cooking times [48]. When comparing phenolic content of varieties of HTC beans, black beans and red beans had a significant reduction in free phenolic acids compared with non HTC bean controls [47]. Increases in free phenolic acid content have been observed in white beans with the HTC phenomenon [48]. There is a correlation between decreases in tannin content and storage time for black beans [47]. Machado *et al.* speculate that the reduction in free phenolic acids and antioxidant activity observed in HTC black and red beans is due to liberation of free phenolic acids from tannins, followed by phenolic acid cross linkages with proteins [47]. Beninger *et al.* observed significant decreases in kaempferol in aged red beans, which was associated with seed coat darkening, the HTC phenomenon, decreases in high molecular weight procyanidin polymers, and increases in low molecular weight polymers [49] which agrees with the speculations of Machado *et al.*

Processing methods for beans for human consumption can reduce the content of polyphenolics. Xu *et al.* observed soaking, boiling and steaming to significantly affect total phenolic content in Eclipse black beans, with steaming under pressure having less reduction compared with boiled [50]. Diaz Batalla *et al.* compared HPLC profiles from various beans and found kaempferol contents were reduced by a 5-71% once cooked [40]. Similarly, quercetin contents were reduced in cooked beans by a 12-65% compared with

raw beans [40]. Mean benzoic acid derivatives and mean cinnamic acid derivatives were also reduced in cooked beans [40].

Bioavailability Of Phenolics In *Phaseolus Vulgaris*

Health benefits of polyphenolics have been demonstrated *in vitro* and in animal studies. These studies may not be relevant to human health, however, due to issues of digestion, absorption, physiological interactions, and the metabolism of these compounds. Polyphenols can interfere or bind with proteins, can be glycosylated, sometimes interfere or interact with one another, sometimes require bacterial digestion in the colon, and have a short half-life *in vivo* [51]. Many *in vitro* studies use higher than physiological doses of polyphenolics, adding to uncertainty of their biological relevance [12]. The pool of literature investigating the bioavailability of polyphenolics in *Phaseolus vulgaris* is small to date, however the bioavailability of polyphenolics in general has been more extensively studied. Anthocyanins are flavonoids commonly found in black and red beans. It has been observed that less than .1% of the digested dose of anthocyanins from blueberries is absorbed in the small intestine [52] and that the remaining anthocyanins are degraded into phenolic acids in the colon, where an additional 3-4% can be absorbed [53]. Quercetin is another flavonoid commonly found in beans. The bioavailability of pure quercetin from an oral administration of ~160 μmol/kg was 16% in rats [54] and 17% in pigs [55]. Most of the quercetin included in these percentage calculations was in the form of conjugated metabolites in the blood [54, 55]. Even after intraveneous injections, quercetin was rapidly conjugated and the parent aglycone was barely detectable in the

blood [54]. However, the flavonol epi-gallocatechin-3-gallate (EGCG) was about 26.5% bioavailable in mice given an intragastric dose of 164 μmol/kg, and 12.4% was found in the blood in an unconjugated form [56]. Several phenolic acids, which have been identified in beans (p-coumaric acid, sinapic acid, p-hydroxybenzoic acid, ferulic acid), were bioavailable to hamsters fed a phenolic rich oat bran powder [57]. The blood of these hamsters after eating the oat bran was more resistant to LDL-oxidation than hamsters not fed oat bran. Furthermore, the blood of these hamsters was even more resistant to LDL-oxidation when the antioxidant ascorbic acid was added to blood samples, indicating that the phenolic acids in the blood worked synergistically with ascorbic acid as an antioxidant defense [57].

Only two studies were identified investigating the bioavailability of polyphenolics in *phaseolus vulgaris*, reflecting a substantial gap in the research. Laparra *et al.* performed an *in vitro* experiment using simulated intestinal digestion to quantify bioavailability of (+)-catechin, p-coumaric acid, hydroxybenzoic acid, ferulic acid, caffeic acid, kaempferol and astragalin in white Great Northern, kidney and black beans [43]. Compared with pre-digestion, the sum of polyphenolics measured post digestion was negligible for the white Great Northern beans, and reduced for the two different varieties of red and black beans measured [43]. Ferulic acid, kaempferol and astragalin were detectable in digested portions of colored beans [43]. Although hydroxybenzoic acid and p-coumaric acid levels were high in some pre-digested samples, neither of these phenolic acids was identified in the digested portion of beans [43]. It has been estimated that 48% of polyphenolics from vegetables are absorbed in the small intestine and 42 % in the large

intestine; the authors report that their results are in agreement with these estimates [43, 58],based on their in vitro digestion procedure.

Kaempferol is affected little by heat, therefore kaempferol content in cooked beans tends to be equal to uncooked beans [36]. Bonetti *et al.* measured urinary excretion of kaempferol from cooked beans and found considerable variability between different individuals as to how much was excreted [59] In general, between 1 and 13% roughly of the kaempferol was absorbed from the beans [59]. They also found differences in excretion time, but not amounts, between genders, indicating that females took longer to excrete kaempferol than males [59] Much of the kaempferol found in beans is present in 0-glycosidic form, which can be hydrolyzed by gut bacteria to be available in its active aglycone form [51].

Investigations quantifying the amount, diversity, and antioxidant activity of polyphenolics are important but bioavailability research is necessary to elucidate the physiological relevance of polyphenolics in the human diet. Currently little research is available on the bioavailablity of polyphenolics in *Phaseolus vulgaris.* The flavonoids appear to be minimally available, and those that are absorbed into the plasma are quickly conjugated by metabolizing enzymes. Health effects of these beans could be due to the metabolites of the flavonoids, or from up regulation of metabolizing enzymes. This data is based on administering individual flavonoids, however. In a study using whole beans [43], almost 50% of the polyphenolics were absorbed in the small intestine, however this study used an *in vitro* simulation of digestion and absorption, and may not be accurate when applied to a human situation. More polyphenoic bioavailability research is needed.

Oxidative Stress

Oxidative stress has been defined as an imbalance of pro-oxidants and antioxidants in favor of the former [60]. It has also been defined as an imbalanced biochemical state leading to the production of reactive oxygen species (ROS) and initiation of a cellular antioxidant cascade [61]. However, oxidative stress can be induced without a disruption of antioxidant levels. For example, in certain conditions, antioxidants can act as pro-oxidants [60]. When excess iron is added to a biological system oxidative stress is induced regardless of anti-oxidant status [60]. Jones *et al.* propose a new definition of oxidative stress as "a disruption of redox signaling and control" [60]. During oxidative stress, there is usually a rise in ROS and reactive nitrogen species (RNS) [62], due to either an inhibition of antioxidant systems or an increase in oxidizing chemistry in the internal environment.

Excessive oxidative stress can trigger a cascade of ROS, which may lead to loss of cell function, apoptosis or necrosis [63]. An example of a cascade of ROS is lipid peroxidation. Lipid peroxidation is a chain reaction, which can occur under enzymatic control for biological benefit to the organism (as in the case of generating lipid derived inflammatory mediators), or non-enzymatically [64]. The non-enzymatic form is often initiated by protonated $^{\bullet}O^{-}_{2}$ [65], and is associated with cell damage and oxidative stress [64]. Initiation of lipid peroxidation begins with the loss of an electron from a polyunsaturated fatty acid (PUFA), generally from a methylene carbon, which lies between the double bonds [65]. The unpaired electron of a free radical abstracts an electron from the methylene carbon leaving a lipid radical [65]. After some molecular

rearrangement this electron loss stabilizes, and the fatty acid can react with molecular oxygen to become a peroxyl radical at a diffusion-limited rate [65]. Propagation can now occur, where the peroxyl radical takes a allylic hydrogen from a neighboring fatty acid creating another carbon centered radical [65]. *In vivo*, the propagation of this cycle typically occurs about 15 times before it is terminated via the reaction of the free radical with another less reactive radical [66]. Alternatively, lipid peroxides can undergo a number of reactions, becoming reactive aldehydes, alkanes, isoprostanes and isoketals [65].

Excessive lipid peroxidation in membranes can destroy cells by disrupting the integrity of the membrane [67]. Lipid peroxides are detrimental to cellular functions [67]. Oxidative stress promotes cell death via many different mechanisms, including activation of mitochondrial death pathways [68]. In the absence of sufficient antioxidants, as in the case of vitamin E deficiency, lipid peroxidation can lead to death of the organism [65].

Many different pathways lead to the production of ROS and oxidative stress. A major contributor to ROS comes from a byproduct of endogenous processes called superoxide radical $^{\bullet}O^{-}_{2}$. Superoxide radical is a reduced molecule of oxygen. It is a free radical, meaning it contains an unpaired electron, and it is capable of standing independent of other molecules. In normal physiological conditions $^{\bullet}O^{-}_{2}$ is converted to hydrogen peroxide (H_2O_2) and oxygen (O_2) by the enzyme superoxide dismutase (SOD). Both H_2O_2 and $^{\bullet}O^{-}_{2}$ by themselves, in solution, are not highly reactive. However, together in combination with traces of non-protein bound iron salts or metal ions, the combination of H_2O_2 and $^{\bullet}O^{-}_{2}$ can lead to the formation of hydroxyl radical ($^{\bullet}OH$) [69]. This is called the Haber-Weiss reaction. First ferric ion is reduced to ferrous iron and superoxide radical is oxidized to molecular oxygen [70]:

$$Fe^{3+} + {}^{\bullet}O^{-}_{2} \rightarrow Fe^{2+} + O_2$$

The second step is the Fenton reaction, where ferrous ion is oxidized back to ferric and hydrogen peroxide is reduced to hydroxyl anion and hydroxyl radical [70]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + {}^{\bullet}OH$$

The hydroxyl radical is highly reactive [69]. It can react with all biomacromolecules and does so at diffusion controlled rates, usually within nanometers of the place of generation [61]. The type of damage produced by a hydroxyl radical is thus dependent on the site of radical formation, ie., $^{\bullet}$OH near DNA will most likely affect DNA (causing base modification or strand breakages) [71]. Hydroxyl radicals often react with biomolecules to form less reactive radicals, which, due to their lower reactivity, can diffuse away from the site of generation [71]. For example, some peroxyl radicals ($RCOO^{\bullet}$) have half-lives in the range of seconds [72], a hydroxyl radical with a short half life could react with a carbon based biomolecule to generate a peroxyl radical, which could then affect a more distant environment [71]. While the generation of $^{\bullet}$OH *in vivo* accounts for some oxidative stress, other reactions likely contribute to the majority of ${}^{\bullet}O^{-}_{2}$ mediated oxidative stress. Formation of $^{\bullet}$OH requires interaction between ${}^{\bullet}O^{-}_{2}$, H_2O_2 and iron, all of which are kept in low concentration by antioxidant scavenging systems [73]. For example, ascorbic acid is present in cells in higher concentrations than ${}^{\bullet}O^{-}_{2}$ and can reduce Fe^{3+}, preventing the Haber-Weiss reaction [73](more on ascorbic acid will be discussed under the section entitled *Antioxidants*).

A second way that ${}^{\bullet}O^{-}_{2}$ might contribute to oxidative stress involves the generation of reactive nitrogen species (RNS) [73]. Nitric oxide ($NO^{\bullet}$) is a stable free radical and a second messenger in neurotransmission. $NO^{\bullet}$ is released by immune cells in

the central nervous system (microglia), along with superoxide radical [61]. Nitric oxide is also produced by endothelium where it participates in the modulation of blood flow, and by immune cells such as macrophages and neutrophils where it is involved in regulation of thrombosis and inflammation [73, 74]. The combination of $NO^{\bullet}$ and superoxide radical gives rise to peroxynitrite ($ONOO^{-}$), a non-radical [72]. RNS are capable of oxidation and nitration of aromatic side chains of tyrosine and tryptophan, a condition known as "nitrosative stress" [61].

The cytotoxic effects of peroxynitrite and the conjugate acid (which accounts for about 20% of peroxynitrite at physiological pH) peroxynitrous acid (ONOOH) include oxidation of free thiols such as cysteine, glutathione, lipids, deoxyribose, guanine bases, methionine and phenols [75]. The tyrosine nitration by peroxynitrite can inactivate antioxidant enxymes like MnSOD, and block tyrosine phosphoylation in signaling pathways, thus inhibiting the signal [75]. In plasma, where thiol concentrations are lower than in cells, peroxynitrite will react with non-thiol targets like LDL and are capable of generating lipid peroxyl radicals [75].

Superoxide radical plays a large role in the generation of (ROS) and is created via a number of pathways. During the mitochondrial electron transport chain (ETC) where electrons are shuttled in the direction of progressively stronger oxidants, a small amount of superoxide radical is formed when electrons are leaked to oxygen [65]. The electron transport chain is comprised of four enzyme complexes. Complex I collects electrons from NADH or H+, and complex II from succinate (the original source of these electrons comes from the catabolism of fats and carbohydrates) and passes them to coenzyme Q (CoQ) creating ubisemiquinone ($CoQH^{\bullet}$) and then ubiquinol ($CoQH_2$) [76]. Ubiquinol

donates these electrons to complex III, which transfers them to cytochrome *c*; they then move to complex IV and finally to ½ O_2 yielding water [76]. The energy generated from this movement of electrons is used to pump protons across the inner mitochondrial membrane creating an electrochemical gradient, the potential energy from which ATP synthase uses to create ATP from ADP and P_i. Approximately 0.4- 4% of molecular oxygen taken up by the mitochondria is reduced to superoxide radical in the ETC when electrons are leaked to molecular oxygen [76]. The production of ROS caused by this process in the mitochondria leads to oxidation of mitochondrial lipids, proteins and DNA [76]. Oxidation of mitochondrial DNA is associated with diseases, which characterize "old age" in mammals [76].

In addition to the ETC, superoxide can be produced by NADPH oxidases, enzymes found on the membranes of most cells [65] particularly phagocytes and macrophages where there is a respiratory burst driven by the NADPH oxidase complex [69]. This complex reduces oxygen to superoxide radical and oxidizes NADPH from the cytosolic pentose phosphate pathway to $NADP^+$ [71].

$$NADPH + 2O_2 \rightarrow NADP^+ + 2^{\bullet}O^-_2 + H^+$$

The respiratory burst serves a bactericidal function in these cells [71]. A dismutation reaction of $^{\bullet}O^-_2$ results in the production of H_2O_2, which can then undergo the Haber-Weiss reaction (as outlined above), or can react with hypochlorous acid (HOCl), produced by myeloperoxidase, or nitric oxide ($NO^{\bullet}$), produced by nitric oxide synthetase, to generate toxic hydroxyl radical [77], as shown below.

$$^{\bullet}O^{-}_{2} + HOCl \rightarrow OH^{\bullet} + O_2 + Cl^{-}$$

$$^{\bullet}O^{-}_{2} + NO^{\bullet} + H^{+} \rightarrow OH^{\bullet} + NO_2$$

The resulting ROS are essential to the antimicrobial activity of these cells, however they are highly toxic [77]. NADPH oxidase is a well-regulated enzyme. It is activated by phagocytizable particles such as bacteria and yeast, certain bioactive lipids, and antibodies [77].

Xanthine oxidase is another source of $^{\bullet}O^{-}_{2}$. Xanthine oxidase is the interconvertible partner to xanthine dehydrogenase, it exists mainly in the liver and catalyzes the conversion of hypoxanthine or xanthine to uric acid [78], a step in purine catabolism [66]. Whereas xanthine dehydrogenase reduces NAD^{+} as a byproduct of catabolism, xanthine oxidase results in the addition of an electron onto an oxygen molecule, generating superoxide radical [78]. When oxygen is low (as is the case during ischemia), ATP levels drop causing calcium increases in tissues that require an ATP dependent calcium pump [66]. Increases in intracellular calcium activate the protease caplain, which converts xanthine dehydrogenase to xanthine oxidase [66]. The subsequent rise in oxygen, which occurs upon reperfusion of the tissue, facilitates the production of high levels of $^{\bullet}O^{-}_{2}$ [66].

Aldehyde oxidase is similar to xanthine oxidase in that it reduces O_2 to $^{\bullet}O^{-}_{2}$ while oxidizing aliphatic and aromatic aldehydes [79]; however, aldehyde oxidase has a lower affinity for purines than xanthine oxidase [80]. Aldehyde oxidase is a cytosolic enzyme found in hepatic cells that catalyzes the catabolism of acetaldehyde, the product of ethanol catabolism by alcohol dehydrogenase [80]. Hepatic aldehyde oxidase has been

shown to be a source of free radicals in ethanol metabolism and to initiate lipid peroxidation as measured by alkane production (explained below) [80].

Cytochrome P450's are a family of Phase I metabolizing enzymes that have many different substrates. In general, metabolizing enzymes are responsible for biotransformation of lipophilic xenobiotics into more water-soluble metabolites to either change the biological activity them, avoid accumulation, change the duration of biological activity or reduce exposure to them [81]. Phase I metabolizing enzymes, such as the P450 system, are involved in reduction, oxidation and hydrolysis reactions which help reveal functional groups in the molecule [81]. Cytochrome P450's with reducing activity can generate $^{\bullet}O^{-}_{2}$ under aerobic conditions because molecular oxygen accepts electrons from a reduced P450 complex. Oxygen can therefore act as an inhibitor of xenobiotic reduction. Superoxide radical can also be formed via redox cycling. Redox cycling is a form of "futile cycling" where a xenobiotic capable of being reduced is reduced by a cytochrome P450, and then oxidized back to the parent compound [82]. In the case of the P450's this occurs in the presence of O_2 with O_2 being the new electron acceptor, making this a source of $^{\bullet}O^{-}_{2}$ [82].

Oxidants are not always created from metabolic processes. X-radiation can create OH radical, ultrasound and microwave radiation can generate ROS, and irradiation with UV light creates radical formation [72]. Accumulation of certain metals, due to their redox potential, can cause an increase in hydrogen peroxide, superoxide radical and hydroxyl radical, leading to oxidative stress [83].

Antioxidants

In a definition by Halliwell and Gutteridge (1989), an antioxidant is defined as "any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate" [72, 84]. Antioxidants can be derived from endogenous sources, such as antioxidant enzymes produced within the cell, or from exogenous dietary sources such as vitamins E, C and A. Some antioxidants are metabolites, such as uric acid [85], ubiquinone [86] or glutathione [87]. Four major cellular antioxidant enzyme families are superoxide dismutase, glutathione peroxidase, thioredoxin reductase and catalase [88].

As discussed earlier, the enzyme superoxide dismutase (SOD) catalyzes the conversion of $\bullet O^-_2$ to hydrogen peroxide and oxygen. In this reaction, one molecule of $\bullet O^-_2$ is reduced and one is oxidized [89]. The equation for this reaction is as follows:

$$2^{\bullet}O^-_2 + 2H^+ \rightarrow O_2 + H_2O_2$$

The SOD family contains a cytosolic, an extracellular, and a mitochondrial form. The cytosolic form (SOD1) (and the extracellular form (SOD3)) have zinc (Zn^{2+}), and copper (Cu^{2+}) as co-factors [90]. The mitochondrial form (SOD2) binds to manganese (Mn^{2+}) in the catalytic site [90]. Although SOD is an antioxidant enzyme, the product of SOD-catalyzed reactions, H_2O_2 can react with superoxide to form the highly reactive hydroxyl radical [91]. The protective effect of SOD from oxidative stress depends on the presence of H_2O_2 detoxifying enzymes [91]. In a disregulated state, an upregulation of SOD2 in absence of sufficient amounts of H_2O_2 detoxifying enzymes has been associated with cancer [91].

Glutathione peroxidases (GPx) are a family of antioxidant enzymes. They catalyze the reduction of hydroperoxides to corresponding hydroxy compounds utilizing electrons from monomeric glutathione (GSH) [92]. Oxidized glutathione, which is glutathione disulfide (GSSG), can then be reduced by glutathione reductase back to GSH using electrons from NADPH [93]. In this way, cytosolic glutathione peroxidase (GPx-1) can reduce hydrogen peroxide levels in the cell [92].

1) $2GSH + H_2O_2 \rightarrow GS\text{-}SG + 2H_2O$ (glutathione peroxidase)

or

1) $2GSH + LOOH \rightarrow GS\text{-}SG + LOH + H_2O$ (glutathione peroxidase)

2) $GS\text{-}SG + NADPH + H^+ \rightarrow 2GSH + NADP^+$ (glutathione reductase) [94]

During oxidative stress there is a decrease in the antioxidant GSH and an increase in GSSG [62]. This ratio is often used as a method of assessing oxidative stress [60]. However, another form of glutathione peroxidase (GPx4) found in the mitochondria and cytosol of cells, which is associated with preventing lipid peroxidation because it is capable of reducing membrane-bound ester lipid peroxides, has no glutathione-binding domain and therefore may utilize other reducing agents [92]. Therefore, the ratio of GSH/GSSG may not be a complete measurement of oxidative stress. While glutathione peroxidase is an endogenous antioxidant source, many forms of it (GPx1, 2, 3, 4) contain selenium in their structure and rely on the presence of dietary selenium to function. The expression of the phospholipid GPx4 is conserved during selenium deficiency, especially in the brain and testes, while the expression of the cytosolic GPx1 is reduced [92].

The thioredoxin system consists of two oxioreductase enzymes, thioredoxin (Trx) and thioredoxin reductase (TrxR) [95]. Thioredoxin is a disulfide reductase responsible for maintaining proteins in their reduced state [95]. Thioredoxin reductase reduces the oxidized disulfide thioredoxin (Trx-S_2) back to dithiol form (Trx-$(SH)_2$) [95].

Trx-1 is expressed ubiquitously in mammilian cells [96]. The presence of Trx keeps the proteins on the inside of a cell a reduced state so that proteins contain free sulfhydryl groups [95]. The outside of a cell is generally a more oxidized environment rich in stabilizing disulfides [95]. During oxidative stress, when peroxyl radicals are generated, one of the first events to occur is oxidation of protein sulfhydryl groups [95]. This can trigger proteolysis of the oxidized proteins [97].

Thioredoxin may help reduce oxidative stress in ways additional to directly reducing proteins. Peroxiredoxin (Prx), is both a cytosolic (PrxI, PrxII) and mitochondrial (PrxIII) protein, which possesses peroxidase activity and relies on Trx as a source of electrons for the reduction of H_2O_2 [98]. In addition to its oxioreductase activity, Trx can translocate into the nucleus and bind to transcription factors modulating their DNA binding activity [96]. In a recent study the overexpression of Trx in *lactobacillus plantarum* resulted in the differential expression of genes involved in DNA repair when exposed to hydrogen peroxide compared with wild type controls [99].

Catalases are a family of antioxidant enzymes that can both reduce H_2O_2 to water and oxidize it to molecular oxygen [100]. Catalase activity occurs in a structurally diverse array of proteins, some heme and some non-heme [101]. Though catalases differ in structure, they catalyze the same reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The heme variety of catalase uses the first hydrogen peroxide to oxidize the heme creating an oxyferryl species where one oxidation equivalent from the iron and one from the porphyrin ring are used, generating a porphyrin cation radical (reaction 1) [101]. The second hydrogen peroxide is used to reduce the oxyferryl compound, regenerating the enzyme to the original state, creating the products water and oxygen (reaction 2) [101].

$$Enz(Por\text{-}Fe^{III}) + H_2O_2 \rightarrow Enz(Por^{+\cdot}\text{-}Fe^{IV}{=}O) + H_2O \qquad (1)$$

$$Enz(Por^{+\cdot}\text{-}Fe^{IV}{=}O) + H_2O_2 \rightarrow Enz(Por\text{-}Fe^{III}) + H_2O + O_2 \qquad (2)$$

Mechanisms for non-heme catalase activity are speculative and are areas of research, however, Mn can be the center of a non-heme catalase [102].

In addition to antioxidant enzymes, dietary antioxidants can neutralize free radicals to and reduce oxidative stress. Vitamin E, also called tocopherol and tocotrienol, is a lipid soluble antioxidant consisting of a polar chromanol ring and a hydrophobic 16-carbon side chain [103]. There are four vitamers of vitamin E (α, β, γ, δ), which differ from each other in the number and position of methyl groups in the chromanol ring [103]. α-Tocopherol is the most abundant form of vitamin E found in human tissues and serum. This is attributed to the α-tocopherol transfer protein, which selectively recognizes α-tocopherol and mediates its secretion into serum [104]. α-Tocopherol is capable of terminating a cascade of lipid peroxidation by donating a hydrogen to a lipid peroxyl radical [105]. An oxidized α-tocopherol molecule is non-reactive because the charge of the unpaired electron delocalizes around its aromatic ring structure [104]. α-Tocopherol radical

is then reduced back to the metabolically active form by vitamin C [106], retinol or ubiquinol [75].

Vitamin C, also called ascorbic acid, is a water-soluble antioxidant, the ingestion of which is essential to human survival [107]. Ascorbic acid is a 6-carbon lactone, the antioxidant activity of this molecule is due to its ability to donate 2 electrons from a double bond between the second and third carbon [107]. These electrons are lost sequentially, however ascorbyl radical usually is not long lived [107]. All known functions of vitamin C are attributed to the property of being an electron donor [107]. Like vitamin E, when ascorbate loses an electron the resulting radical is fairly unreactive, making it a good preventer of oxidative damage [107]. Although vitamin C helps prevent lipid peroxidation in the presence of vitamin E, the effects of vitamin C on lipid peroxidation in microsomal fractions from vitamin E deficient animals was pro-oxidative [108]. This pro-oxidant effect was abolished in the presence of glutathione [108].

Phenolics As Antioxidants

In order for a phenolic to be defined as an antioxidant it must satisfy two conditions- The first is that when present in low concentrations it has the capacity to delay or prevent auto-oxidation or free radical mediated oxidation of a substrate [109], and the second is that the resulting radical formed by the phenol must be stable enough to not propagate the oxidation chain reaction [109]. Polyphenols are good antioxidants because the phenolic ring structures can absorb charges of free radicals, and are capable of decreasing oxygen concentration and binding metal ions [42]. Phenolic antioxidants (PPH) can inhibit lipid peroxidation by rapidly donating a hydrogen atom to a peroxyl radical to form an alkyl hydroperoxide [109].

$$ROO^{\bullet} + PPH \rightarrow ROOH + PP^{\bullet}$$

The resulting phenoxyl radical can then donate another hydrogen forming a quinone, or can react with another radical forming a more stable product [109].

The presence of polyphenols can delay oxidative damage from peroxyl radicals [110], either by regenerating vitamin E, or scavenging radicals [111] as in the reaction outlined above. When LDL particles are incubated *in vitro* with iron and macrophages, vitamin E is able to prevent accumulation of lipid hydroperoxides through antioxidant activity [111]. After all the vitamin E has been used, however, lipid hydroperoxides accumulate [111]. Caffeic acid was shown to act synergistically with vitamin E by recycling alpha-tocopherol radical [38].

Heavy metals, defined as the group of elements with specific weights higher than $5g/cm^3$, contribute to the production of reactive oxygen species by auto-oxidation and by participating in the Fenton reaction [112]. Accumulation of metals can disrupt redox signaling and control by inactivating antioxidant enzymes and depleting low molecular weight antioxidants such as glutathione via phytochelate formation [112]. Additionally, the transition metals have unpaired electrons in their orbitals and can transfer electrons to oxygen resulting in the formation of ROS [112]. Polyphenols have hydroxyl and carboxyl groups able to bind metals, in particular iron and copper [112]. The cinnamic acid derivatives have been shown to protect against Cu^2+ ion induced oxidative damage [38].

The chelation of Cr, Pb, Hg and Mn has been observed by tannins and is probably related to the high nucleophilic nature of the aromatic rings rather than specific hydroxyl or carboxyl groups [112]. Quercetin has the ability to inhibit H_2O_2 induced V79 cell death

and prevent lipid peroxidation initiated by ferric ion in the retina [113]. The antioxidant activity of quercetin is primarily attributed to its ability to chelate iron, as evidenced by the inhibition of strand breakages observed when U937 cells were exposed to *t*B-OOH-derived DNA damaging species [113]. The cleavage of DNA by *t*B-OOH occurs upon exposure of non-metal chelating antioxidants, however it is prevented by metal chelators [113]. Using the *t*B-OOH Sestili *et al*. observed phenylethyl caffeate, benzyl caffeate, 3-methyl-2-butenyl caffeate, quercetin, 3',4'-dihydroxy flavone and phenylethyl 3,4-dihydroxy-hydrocinnamate also prevented DNA cleavage by iron chelation. All of these compounds posess a catechol group in one of the aromatic rings, suggesting two hydroxyl groups in the *o*-position is essential to the iron chelation function of polyphenols [114].

Proanthocyanins, polymers of flavonoids and hydrolysable tannins, which generally present as a glucose molecule esterified with phenolic acids, are believed to have the most potent antioxidant activity due to their ability to form oligomers via phenolic coupling [115]. They are stable molecules yet keep active scavenging sites exposed [115].

Measurement of Antioxidant Activity, Total Polyphenolics, and Flavonoids

Polyphenolics generally comprises a mixture of different polyphenols, therefore measurements of total polyphenolics, flavonoid content and antioxidant activity in whole bean extracts are relevant. Measurement of total polyphenolics in beans varies according to the solvent used for extraction. Compared with ethanol, methanol and various concentrations of acetic acid, the most effective solvent for extraction from tannin rich

beans was 70% acetone, 29.5 %water, and 0.5 % acetic acid [42] Total polyphenolic content reported in beans also varies with the method of measurement.

Due to the different types of antioxidant activity, the quantification of antioxidants in a single assay is not yet possible. Antioxidants work by sequestering heavy metals, by transferring an electron to a quench a free radical, and by hydrogen donation; currently, no one method exists for testing the antioxidant potential of a substance [116]. Additionally, when research groups do employ similar approaches to quantifying antioxidant activity of a substance, methods are often modified slightly by each group, making it extremely difficult to compare results from study to study [116].

In 2004 the First International Congress on Antioxidant Methods was held, during which an attempt was made to standardize methods for three chosen assays, oxygen radical absorbance capacity, the Folin-Ciocalteu method, and trolox-equivalent antioxidant capacity assay (TEAC) [117]. The oxygen radical absorbance capacity method is an example of a hydrogen atom transfer method. It has been said that this is one of the more biologically relevant assays, since it measures the ability of an antioxidant to quench thermally generated peroxyl radicals [118]. According to Huang *et al.* [116], a synthetic fluorescent nonprotein probe called fluorescein is mixed with a standard water soluble vitamin E analogue (Trolox) **(See Figure 1.14)** or sample. A free radical generator, 2,22-azobis(2-amidinopropane) dihydrochloride, (AAPH) is then added to the mixture and the action of peroxyl radicals on the probe decreases fluorescence. The more antioxidants are able to neutralize peroxyl radicals in the solution, the less loss of fluorescence by the probe. [116]

Both the TEAC assay and the Folin-Ciocalteu assay are electron transfer methods. They measure the ability of the antioxidant to transfer an electron to a free radical, as indicated by a color change [118]. The TEAC is described as a simple assay, but it begins with the generation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)(ABTS+) radical, which takes between 8 and 16 hours depending on the temperature [117]. When this radical is neutralized by a sample containing an antioxidant, or by Trolox standard, color loss occurs [117].

The methods reviewed below are employed in the latter part of this thesis and are consistent with some of the most recent literature on antioxidant capacity of beans [37, 42, 50, 119, 120]).

Folin-Ciocalteu Method for Measurement of Total Polyphenolics

The original Folin-Ciocalteu method was created in 1927. It was based on the premise that oxidation of phenols by molybdotungstate reagent causes the molybdenum to go from a yellow to a blue color with absorption between 745-750 nm [117]. Singleton and Rossi modified the methods to make the reaction more specific to polyphenols at optimal absorbance of 765 nm [117]. Problems with this assay result from lack of adherence to the Singleton-Rossi methods, and variable results are often seen depending on assay conditions [117]. The Folin-Ciocalteu method has been reported to overestimate polyphenol content in beans when compared with HPLC analysis, possibly due to lack of sensitivity of spectrophotometric methods, or perhaps to interference from sugars or proteins [47].

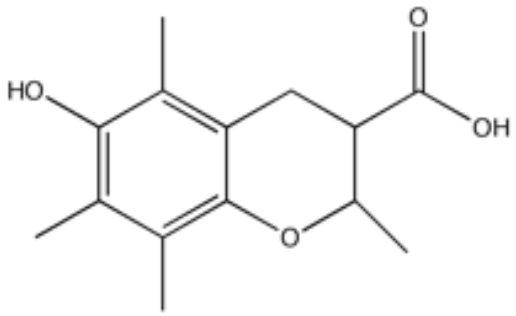


Figure 1.0.14 Trolox

Aluminum Chloride Method for Measurement of Flavonoids

The aluminum chloride method of measuring flavonoid content is a spectrophotometric assay, which measures flavonoid aglycones complexed with aluminum chloride [121]. The chemical procedure involves hydrolyzing the glycosides [121], so that measurements reflect total flavonoid content and not just naturally occurring aglycones [122].

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Capacity Method for Measurement of Antioxidant Activity

The DPPH radical scavenging capacity assay measures the ability of a sample to quench a reactive nitrogen species (RNS). DPPH is a stable, organic nitrogen radical which is commercially available [116]. It has a purple color when in an oxidized state and upon reduction loses its purple color. Although this method is quick and easy to use, the question of biological relevance has been raised [117]. Antioxidants, which react with transient peroxyl radicals in the body, might react slowly or not at all with DPPH [116]. Carotenoids can interfere with the DPPH assay because they overlap in absorbance [117]. Steric access to the nitrogen radical of DPPH can be limiting, thus smaller molecules will give higher readings of antioxidant activity [117].

Antioxidant Activity of *Phaseolus Vulgaris*

Studies have reported a high correlation between phenolic content and antioxidant activity *in vitro* as measured by the Folin Ciocalteu, $AlCl_3$ flavonoid, and DPPH assays [42]. However, Heimler *et al*. observed no correlation between flavonoid content and DPPH radical scavenging ability, and weak correlations between total polyphenols and DPPH scavenging ability using similar methodology to test four different landraces of *Phaseolus vulgaris* [123]. Additionally, Heimler *et al.* used HPLC to quantify specific flavonoids and found no correlation between the presence of kaempferol or quercetin derivatives in the beans and DPPH radical scavenging activity [123]. Ranilla *et al*. also observed no correlation between total flavonoid content or specific flavonoid content with DPPH radical scavenging capacity in Brazilian beans, however they observed significant correlation between total polyphenolics and condensed tannins in seed coats and DPPH antioxidant capacity [39]. Xu *et al*., however, observed correlations between DPPH radical scavenging activity and total polyphenolics and total flavonoid content of seven different varieties of *Phaseolus vulgaris* [120]. Heimler *et al.* sampled Italian bean landraces while Xu *et al.* sampled North American beans. Discrepancies between observed correlations of radical scavenging with polyphenolics may be related to environment in which the beans were grown, subtle differences between laboratory methods, or genotype of the beans. It has been suggested that differences in bean gene pool dictate whether bean color is a predictor of antioxidant activity [29]. Espinosa-Alonso

et al. observed a significant correlation between bean color and anthocyanin content, however total phenols and condensed tannin variation were more associated with genotype than color [35].

Xu *et al*. compared different extraction methods using *Phaseolus vulagris* varieties [42]. Extractions of bean powders using acidic 70% acetone produced the highest measurements of total polyphenolic content and flavonoids in black beans and red kidney beans compared with methanol, ethanol or non-acidic acetone extractions [42]. The DPPH radical scavenging capacity of black beans and red kidney beans was highest in 80% acetone extracts [42], however, the acidic 70% acetone extraction method was used in the present research and is more comparable to the analysis in the latter part of this thesis. Xu *et al.* measured total polyphenolics, total flavonoids and DPPH radical scavenging capacity of seven different varieties of *Phaseolus vulgaris* [120]. Results from their analysis can be found in **Table 1.1**.

Table 1.1
Xu et al. Measurements of Antioxidant Activity, Total Polyphenolics, and Flavonoid content of Several Bean Varieties. [120]

Bean type	DPPH (μmol TE/g)	TPC (mg GAE/g)	TFC (mg CAE/g)
Black turtle eclipse	18.95 ± 0.03	6.99 ± 0.48	3.30 ± 0.11
Black Turtle T-39	14.49 ± 0.14	3.37 ± 0.15	2.51 ± 0.12
Navy bean	1.48 ± 0.04	0.57 ± 0.05	0.92 ± 0.02
Pinto bean	13.79 ± 0.03	3.76 ± 0.06	2.99 ± 0.12
Red kidney	16.81 ± 0.11	4.05 ± 0.05	3.39 ± 0.09
Pink bean	15.49 ± 0.17	3.77 ± 0.19	3.65 ± 0.13
Small red	17.90 ± 0.13	5.76 ± 0.38	4.24 ± 0.10

Abbreviations are: TE: Trolox Equivalents, TPC: Total Phenolic Content, GAE: Gallic Acid Equivalents, TFC: Total Flavonoid Content, CAE: Catechin Equivalents

Inflammation

Inflammation is a physiological response to infection and tissue injury, which functions to repair tissue or kill pathogens [28]. Symptoms of inflammation include heat, swelling, pain, loss of function and the production of and response to chemical mediators [28]. Inflammation can be broken down into two types, acute and chronic. Acute inflammation, which is essential to healing, is self-limiting due to negative feedback regulation [28]. Chronic systemic inflammation, however, is defined as persistent low-grade inflammation indicated by the presence of elevated inflammatory mediators such as C-reactive protein, interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) [124]. The release of pro-inflammatory mediators maintains, regulates and enhances the continuing process of inflammation, leading ultimately to tissue injury [125]. TNF-α and IL-6 are both cytokines whose functions are discussed subsequently. C-reactive protein (CRP) is generally used as a biomarker for inflammation, whose function is not clear [126]. CRP levels can increase 1000-fold in an acute inflammatory response to trauma or infection [124]. In healthy, lean, non-elderly persons blood concentrations of CRP are generally less than 2 mg/L in men and 2.5 mg/L in women [124]. Values of CRP greater than 10 mg/L are considered clinically significant indicators of inflammation [124]. Chronic inflammation is associated with the pathophysiology of chronic disorders by promoting loss of barrier function, infiltration of inflammatory cells into compartments where these cells are not normally found in high numbers, overproduction of reactive oxygen species, cytokines, chemokines, and eicosanoids [28].

Inflammation is characterized by hyperplasia and infiltration of T lymphocytes, which produce cytokines including TNF-α, IL-1 and IL-6 [127]. Cytokines are intercellular signaling peptides that can have autocrine, paracrine or endocrine function [128]. The activity of one cytokine can negate that of another, so net group function is appropriate in their consideration [128]. One such group are the inflammatory cytokines, which include TNF-α, Interleukin-1 (IL-1) (both α and β isoforms), and IL-6 [128]. IL-6 functions as both a pro and anti-inflammatory cytokine, and is often present during inflammatory responses [129]; it is thought to aid in controlling the inflammatory response to prevent it from getting too large [129].

One of the ways in which cytokines are regulators of the inflammatory response is via the transcription factor Nuclear Factor Kappa B (NFκB). When inactive, NFκB is found in the cytoplasm attached to the cytoplasmic inhibitor IxB-a [130]. Activation by cytokines involves the rapid phosphorylation of IxB-a by serine/threonine protein kinases (called IKK) resulting in the degradation of IxB-a and allowing for the translocation of NFκB into the nucleus where it acts as a transcription factor [130]. NFκB activates transcription of CCL2/monocyte chemotactic protein-1 (MCP-1), keratinocyte-derived chemokines (CXCL1/KC), and inflammatory cytokines [131]. In this way, cytokines can activate transcription of additional cytokines in a positive feedback loop. NFκB can also induce transcription of iNOS, growth factors, vascular endothelial growth factor (VEGF), immuno-receptors [132]. Thus NFκB is an important central regulator of the inflammatory response.

Commonly, cytokine activators of the NFκB pathway are TNF-α and IL-1β [133]. TNF-α may be a link between inflammation and ROS. TNF-α stimulates activity of

NADPH oxidase in the endothelium, which contributes to production of ROS [134]. Picchi *et al.* observed increases in the production of $^{\bullet}O_2^-$ and in NADPH oxidase subunits in the coronary arteries of Zucker obese fatty rats, which was attenuated upon administration of either an NADPH oxidase inhibitor or neutralizing antibodies to TNF-α [135]. TNF-α has been shown to increase the production of ROS in type-2 diabetic mouse coronary arterioles. When a neutralizing antibody to TNF-α was given to the animals, there was decreased formation of ROS ($^{\bullet}O_2^-$, $ONOO^-$, and H_2O_2) [136]. ROS activate NFκB, which up-regulate gene expression of pro-inflammatory cytokines that mediates an immune response, causing inflammation [127].

NFκB is a transcription factor for MCP-1 [132]. MCP-1 is a chemoattractant for mononuclear immune cells such as T cells and NK cells, which express the receptor for MCP-1 on their surface [129]. MCP-1 is expressed and secreted by endothelial cells and muscle cells [129]. As well as promoting the attraction of leukocytes to an inflamed site via MCP-1 activation, NFκB induces expression of adhesion molecules such as VCAM-1 and ICAM-1, which promote endothelial adhesion of circulating inflammatory leukocytes [125]. Recent evidence suggests that ICAM can act as a signal transducer, and that it can specifically activate the mitogen-activated protein kinase (MAPK) pathway [137]. MAPKs are a family of protein kinases triggered by growth factors, stress, inflammatory cytokines, which regulate growth, differentiation, apoptosis and inflammatory responses (**Figure 1.15)** [138]. The MAPK superfamily of protein kinases is comprised of 3 different signaling pathways: the extracellular signal regulated protein kinases (ERKs), the c-Jun N-terminal kinases or stress activated protein kinases (JNK/SAPK), and the p38 family of kinases [139]. Each of the 3 families are made up of a cascade of three tiers. Initiation

begins when a stimulus (either growth factors, cytokines or oxidative and environmental stresses ie., heat, osmotic) activates a MAPKKK via a receptor coupled G protein and/or other kinases. The MAPKKK then phosphorylates and activates an MAPKK, which activates a MAPK, which then elicits a cellular response [139] (Figure 1.15). Cellular responses can be apoptosis, cell development, differentiation, inflammation, tumorigenesis, osmoregulation, cell motility or chromatin remodeling, depending on kinase family and cell circumstance **[139]**. MAPKs have been implicated in promoting the production of pro-inflammatory cytokines IL-6, IL-1, IL12, IL23, and TNF, as well as signaling via receptors such as toll-like receptors (TLR) **[140]**. Toll-like receptors (TLR) recognize pathogen associated molecular patterns (PAMPs) and trigger expression of proinflammatory genes **[141]**. Activation of TLRs causes cells to induce expression of the inflammatory TNF-α **[142]**.

CXCL1/KC stimulates the release of IL-1β, which induces the production of prostanoids [143]. The prostanoids behave as autocrine or paracrine lipid mediators, which are synthesized upon mechanical trauma or chemical signaling (such as by IL-1β) from membrane-released arachidonic acid [144]; they are collectively termed eicosanoids [145]. Arachidonic acid is kept esterified until it is mobilized by cytosolic phospholipase A_2 ($cPLA_2$) [144]. Arachidonic acid is released by $cPLA_2$ to one of two isoforms of prostaglandin H synthase (also called COX), an enzyme residing at the endoplasmic reticulum and nuclear membrane. This enzyme converts arachidonic acid into PGH_2 [144] or PGG_2, both of which are unstable peroxide intermediates [145]. COX-1 is an isoform

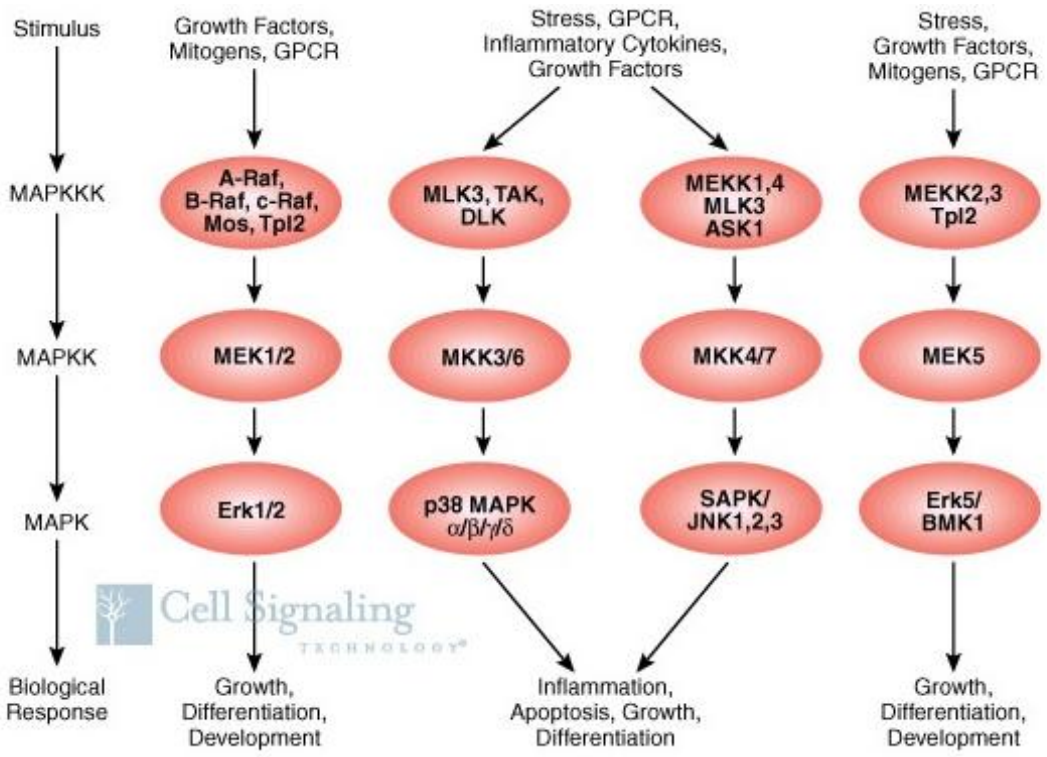


Figure 1.15 Mitogen-activated protein kinase cascade [138]

associated with basal prostaglandin synthesis and COX-2 is up-regulated in an inflammatory response [144]. The enzyme COX-2 is expressed via an IL-1β / NFκB dependent pathway [146], thus NFκB induces expression of IL-1β via CXCL1/KC, and IL-1β activates NFκB to promote expression of COX-2.

The fate of the prostaglandin intermediates depends on the cell type producing them and the isomerase enzymes found therein [147]. Platelets produce thromboxane A_2 via thromboxane synthetase [145]. Thromboxane A_2 is a prothrombotic vasoconstrictor [147]which promotes blood coagulation. Endothelial cells express PGI synthase and synthesize the antithrombotic vasodialator prostacyclin (PGI_2) [145]. Many cells have the capability of producing prostaglandin E_2 (PGE_2) from PGH_2 [145]. PGE_2 is an inflammatory prostanoid synthesized by PGE synthase, which is expressed in leukocytes, vascular smooth muscle cells, endothelial cells and platelets [147]. The intermediates are sometimes produced in one type of cell and taken up by another before they are converted to prostanoids (called transcellular metabolism). For example, PGH_2 can be produced in the endothelium and taken up by a leukocyte prior to enzymatic mediated conversion [147].

Arachidonic acid can be channeled into the lipoxygenase (LOX) pathway in cells expressing the enzyme LOX [145]. The LOX pathway is found primarily in platelets and white blood cells [145]. In this pathway arachidonic acid is first converted to a peroxide intermediate called hydroperoxyeicosatetraenoic acid and then rapidly changed into a hydroxylated arachidonic acid called hydroxyeicosatetraenoic acid. The final products of the pathway are hydroxyeicosatetraenoic acid, leukotrienes, lipoxins, and cytochrome

P450 monooxygenases [148], the type of products being specific to the type of LOX enzyme expressed in that cell [148]. These eicosanoid products can instigate a physiological response, such as cell proliferation or apoptosis [149]. Recent evidence suggests certain leukotrienes have mitogenic effects on macrophages in the absence of growth factors, suggesting direct signaling through the MAPK pathway, or phosphoinositide 3-kinase pathway interactions [148]. There are 5 lipoxygenases in humans. The 5-LOX isoform catalyzes the production of leukotrienes, which mediate inflammatory and allergic responses [149]. The inhibition of 5-LOX metabolic pathways results in decreased leukotriene synthesis with corresponding anti-inflammatory effects [150].

Nitric oxide is formed from L-arginine by nitric oxide synthase (NOS) and several isoforms of NOS have been identified [151], including neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) [152]. Small amounts of NO are regularly synthesized by eNOS and nNOS, with protective roles in the cardiovascular system [152]. NO released by eNOS relaxes adjacent vascular smooth muscle cells promoting blood flow [153]. Endotoxins or cytokines (inflammatory mediators) induce macrophages, endothelial cells and smooth muscle to express the calcium independent inducible NOS (iNOS), which generates NO [151]. In addition to expressing iNOS, these cytokine activated cells also assemble NADPH oxidase subunits resulting in the production of superoxide radical [154]. The combination of $NO^{\bullet}$ and $^{\bullet}O_2^-$ has a generates reactive nitrogen species (RNS), ROS as a sort of cytotoxic defense mechanism [154]. Excessive levels of ROS and RNS in these cytokine activated cells can result in increased susceptibility to apoptosis [154].

The inflammatory response involves a complex and interconnected signaling network. Inflammation and oxidative stress often co-participate in immune response of the cell. Chronic inflammation involves elevation of inflammatory cytokines such as TNF-α [124]. TNF-α is an activator of the inflammatory transcription factor NFκB. NFκB activates the inflammatory response via transcription of several different signaling proteins, the downstream affects of which involve cytokine and cell adhesion molecule production, as well as activation of the MAPK and eicosanoid pathways.

Polyphenolics Commonly Found in Beans and Inflammation

It has been argued that polyphenolics have a low bioavailability, therefore observed health benefits are likely not due to the radical scavenging abilities of polyphenolics directly, but are mediated by their ability to act with protein and lipid signaling cascades [33]. One example of this is that polyphenolics have been shown to inhibit acute [155] or chronic inflammation in animal models [156]. As discussed above, inflammation involves a complex network of signaling cascades. Below is a review of the different ways observed in which polyphenolics have interfered with inflammatory signaling.

Polyphenolics demonstrated abilities to attenuate arachidonic acid induced ear edema when applied topically. Inhibition of eicosanoid synthesis pathways may be the underlying mechanism [157]. Individual polyphenolics can interfere with eicosanoid synthesis. Polyphenols have been shown to inhibit COX-2 and LOX, causing a reduction in prostaglandin and leukotriene production [132]. In human neuronal cells, 5-LOX expression can be stimulated by the HIV protein gp120 leading to lipid peroxidation and

cell death. This was prevented by caffeic acid [158], indicating that polyphenolics may directly inhibit signaling involved in 5-LOX synthesis. Anthocyanins are potent *in vitro* inhibitors of human neutrophil granulocyte 5-LOX probably via uncompetitive inhibition. Conjugated anthocyanins are more potent inhibitors than their aglycones [149]. The anthocyanins D3p-glucose and D3p-galactose had very low half maximal inhibitory concentration values and can be absorbed unmodified as intact glycosides [149]. Quercetin has been shown to inhibit 5-LOX product synthesis, but has also demonstrated inhibition of PLA_2 enzymes, indicating that quercetin mediated inhibition of inflammation might be, in part, due to reduced arachidonic acid supply to COX and LOX enzymes [157]. Taken together, polyphenolics may be beneficial in reducing inflammation via blocking the production of inflammatory eicosanoids.

Polyphenolics have been shown to inhibit inflammatory signaling by inhibition of NFκB, which is activated by inflammatory cytokines [132]. When a procyanidin extract (60μg/mL) was incubated with macrophages in culture, a 40% reduction in NFκB translocation to the nucleus was observed [159]. In cell culture, trimers and oligomer rich procyanidin fractions at concentrations of 5-30 μg/mL inhibited expression of iNOS in macrophages after they were stimulated with pro-inflammatory lipopolysaccaride and interferon gamma, however the monomeric catechin and epicatechin did not [159]. The production of the COX-2 catalyzed PGE_2 was also inhibited by trimers and oligomer fractions of procyanidins [159]. NFκB sites have been identified in the promoter region of both iNOS and COX-2, [159] and it is possible that inhibited expression of iNOS and COX-2 was due to polyphenolic interference with NFκB. Oligerimized polyphenols from grape seeds reduced the overexpression of monocyte chemoattractant protein

(MCP-1) and tumor necrosis factor (TNFα) in adipocytes in coculture with macrophages. Transcription of NFκB was reduced in the macrophages and phosphorylation of ERK1 and 2 (members of the MAPK family) was decreased [160]. Taken together, this evidence suggests that in macrophages oligimerized procyanidins may have an anti-inflammatory effect via reduction in NFκB activity and subsequent reductions of several proinflammatory genes.

Another effect of polyphenols *in vitro* is the inhibition of mitogen-activated protein kinase (MAPK) pathways, through which polyphenolics can reduce the expression of inflammatory proteins and alter cell cycling [132]. Sandoval *et al.* mixed a water-soluble extract of polyphenols from beans with human endothelial cells subjected to peroxynitrite, and cell apoptosis was inhibited at concentrations which caused apoptosis of macrophages [161]. Apoptosis, or programmed cell death, can be regulated via MAPK pathways [139]. However it is unclear from the experiment by Sandoval *et al.* whether polyphenolics were involved in signaling via the MAPK pathways, or whether scavenging of peroxynitrite inhibited endothelial cell apoptosis.

Phorbol 12-myrisate 13-acetate (PMA) is a protein kinase C activator known to induce membrane bound NADPH oxidase, and can be used to generate a model of oxidative stress [137]. The polyphenol quercetin has been shown to prevent depletion of the antioxidant GSH in a PMA-induced environment of oxidative stress in endothelial cell cultures [137]. ICAM-1 is a cell adhesion molecule, which is released by leukocytes and endothelial cells during an immune response and is associated with early stages of inflammation [162]. Quercetin reduced the up regulation of the inflammatory marker ICAM-1 expression and concurrently reduced inflammatory mediator PGE_2 synthesis

[137]. In another study, quercetin inhibited IL-1β mediated degradation of the inhibitory IKKB protein, which results in inhibition of translocation of NFκB to the nucleus and ultimately the prevention of inflammatory cytokines and ICAM-1 [162]. Furthermore, the inhibition of ICAM-1 expression was partially inhibited by activator protein-1 (AP-1), the expression of which involves several MAPK pathways [162]. Kaempferol inhibited TNF-α provoked ICAM-1 expression in human endothelial cells partially via attenuation of the JNK pathway, which activates c-jun mRNA expression. c-Jun is a part of the AP-1 transcription factor, which was shown to be required for TNF-α stimulated ICAM-1 expression [163]. Thus it appears that quercetin and kaempferol may have anti-inflammatory effects on cells in culture by mediating transcription of inflammatory mediators through the NFκB or MAPK pathways.

Chronic inflammation, characterized by increases in inflammatory cytokines [129], infiltration of T- lymphocytes [127], release of matrix metaloproteinases [127], and production of ROS [136] is partially mediated by the transcription factor NFkB [130] and by the MAPK pathway [137]. In cell cultures oligomerized polyphenolics reduce NFkB activation and downstream gene transcription [160], resulting in reduced inflammatory responses. Polyphenolic extracts from beans inhibited peroxynitrite induced apoptosis of human endothelial cells in cell culture, possibly via MAPK pathways [161]. Individual flavonoids from beans inhibited TNF-α stimulated ICAM expression [163] resulting in reductions of leukocyte adhesion and representing reductions in the inflammatory cascade. ICAM reduction was mediated partially via attenuation of the JNK pathway, and partially via NFkB [163]. Inflammatory prostaglandins and leukotrienes are produced via the COX-2 and LOX pathways respectively. Their synthesis is inhibited by polyphenolics

in vitro [132] and with topical application of anthocyanins [157]. *In vitro* models based on measurements of inflammatory mediators suggest that bean polyphenolics might be beneficial in reducing chronic inflammation.

Phenolics In *Phaseolus Vulgaris* and Chronic Disease

Chronic diseases are "non-communicable illnesses that are prolonged in duration, do not resolve spontaneously, and are rarely cured completely." They are the leading cause of death and disability in the United States, and include heart disease, stroke, cancer, diabetes, arthritis and obesity [3]. According to the Centers for Disease Control and Prevention (CDC), many chronic diseases are believed to be preventable through "Adopting healthy behaviors such as eating nutritious food.." [3]. It is estimated that 80% of all heart disease cases and diabetes cases and 40% of all cancer cases are preventable through elimination of "tobacco use, poor diet, and inactive lifestyle"[164]. The CDC identifies nutrition patterns associated with chronic disease risk as being low intake of fruit and vegetables and a high intake of saturated fat [3]. While evidence suggests a pattern high in fruits and vegetables reduces chronic disease risk [7, 8, 165-167] the mechanism by which these dietary patterns contribute to a reduction in risk of chronic disease is not well understood. Do plant materials possess health benefits themselves, or do they simply reduce the amount of animal products in the diet and reduce chronic disease risk? Recent attention has been given to polyphenolics as a potential component

of plant foods that might have some health benefit in the reduction of chronic disease risk.

Cancer is the leading cause of death worldwide, accounting for 13% of all deaths in 2004 [168]. According to epidemiological data, diets high in saturated fats with low intakes of fruit and vegetables increase the risk of breast, colon, esophagus and prostate cancer [169]. These dietary factors are thought to account for 30% of cancers for people in developed countries [169]. The number of people in the US with diagnosed diabetes has increased by 3 million between 2007 and 2009, meaning that nearly 8 % of the US population now has diabetes [170]. The vast majority of diabetes cases are type-2, which is believed to be preventable through moderate weight loss and exercise in high-risk adults [171]. The most common type of heart disease is coronary heart disease (CHD), where coronary arteries buildup plaque causing them to become narrowed and hardened (called atherosclerosis) [172]. CHD is the leading cause of myocardial infarction (heart attack) due to decreases in the blood supply to the heart from occluded arteries [172]. According to the National Heart, Lung and Blood Institute, heart disease is the leading cause of death in the US, and eating "a lot of fruits and vegetables" reduces the chances of getting heart disease [173].

The treatment for one or more chronic diseases accounts for about 75% of U.S. healthcare cost annually [174]. Understanding the mechanism by which fruits and vegetables reduce risk of chronic disease could help reduce health care costs by helping people to make dietary choices which facilitate disease prevention. Possible mechanisms by which this dietary pattern can reduce chronic disease risk are many, including the replacement of calorie dense foods with low calorie foods, increased fiber, vitamin and

mineral intake, and increased intake of dietary polyphenolics. Oxidative stress and inflammation are two hallmarks of many forms of chronic diseases [111], and polyphenolics might help reduce disease through reduction of ROS and inflammation. This section is a review of *in vitro* and animal studies of the effects of polyphenolics on chronic disease.

Overweight (defined as BMI >25) and obesity (defined as BMI >30) have been linked with chronic disease risk [175]. Between 1985 and 2005 rates of obesity have increased dramatically in the US, and the percent of children who are overweight has more than doubled [174]. Diagnosis of childhood chronic disease has quadrupled over the past 40 years [174]. Obesity is associated with chronic systemic inflammation. Recently, it has been suggested that low-grade systemic inflammation contributes to the pathogenesis of chronic diseases including heart disease, cancer and type-2 diabetes [132]. Polyphenolics may help prevent obesity associated chronic disease by contributing to weight loss, or by reducing oxidative stress and inflammation.

It has been reported that polyphenolics have an anti-mitogenic effect such that they inhibit pre-adipocyte proliferation [176]. Inhibition in pre-adipocyte proliferation decreases the number of adipocytes. Polyphenolics have this effect by inhibiting different signaling proteins in the mitogen activated protein kinase family (MAPK) [176]. In 3T3-L1 adipocytes PPARγ is involved in adipogenesis [177]. This transcription factor is down regulated in response to phenolic compounds [176]. Catechin and genistein are both upregulators of PPARα, which controls the expression of genes involved in fatty acid oxidation [178]. Procyanidin rich grape seed extracts (GSE) induced long term lipolysis in 3T3-L1 adipocytes, while monomers did not [177]. GSE demonstrated additional anti-

obesity potential by inhibiting pancreatic lipase activity required for triacylglycerol digestion [177].

The anti-obesity effects of polyphenolics have been shown in animal models. Rats reduce their energy intake when fed a diet supplemented with procyanidin extract from grape seeds [177]. The anti-obesity effect of grape polphenolics is not just attributed to a reduction in food intake. A recent study using hamsters found that gavaging the animals with GSE in a human amount equivalent to consuming 2 glasses of wine per meal, and feeding them a high fat diet decreased body weight without altering food intake compared with high fat fed controls [179]. Liver antioxidant enzymes superoxide dismutase and glutathione peroxidase were significantly upregulated in the high fat diet group, and superoxide dismutase was upregulated, but to a lesser extent in the high fat/GSE [179]. As a measure of oxidative stress Decorde *et al*. measured NADPH-dependent superoxide production in the heart. They observed a significant reduction in superoxide anion production in the left ventricle of the high fat/ GSE group as compared with a high fat or standard diet group [179].

Adiponectin helps to decrease gluconeogenesis in the liver and increase beta-oxidation of fats in muscle [179]. Decorde *et al.* observed increases in adiponectin levels with GSE. Terra *et al*. observed increases in adiponectin levels with polyphenolic consumption in a 19 week study in which Zucker fa/fa rats were fed a high fat diet, low fat diet, or high fat diet plus grape seed polyphenols (PE)[159]. Although isocaloric diets were consumed, the high fat diet group gained more weight that low fat, and high fat/PE gained fat but to a significantly lesser extent then high fat alone [159]. Terra *et al.*

hypothesize that TNFα, which has been shown to inhibit adiponectin expression in vitro, and IL-6 were decreased in response to PE supplementation via the NF-*k*B pathway [159].

Taken together, polyphenolics appear to be preventive for obesity in some animals, and there is evidence suggesting that they act at the transcriptional level via the MAPK pathway, NF-*k*B, PPARα, or PPARγ. As reviewed in sections above, the MAPK and NF-*k*B pathways are involved in the propagation of inflammatory signaling as well as adipocyte proliferation and adiponectin expression. In humans, obesity and inflammation often accompany chronic disease, and polyphenolics may benefit human health by inhibiting or activating genes at the transcriptional level. Clinical trials investigating the effects of polyphenolics on adipogenesis and lipolysis are needed to determine whether a high polyphenolic diet might be recommended in obesity prevention.

There are many different types of polyphenols. Recently it has also been shown that polyphenolics of similar structure can have cell specific effects [132]; a particular molecule might induce apoptosis in one type of cell, but not another. Therefore polyphenolics are a good prospect for cancer research, as they may be cytotoxic to some tumor cell lines while preserving healthy cells [13].

In cell culture, selective growth inhibitory activity of cancer cells by polyphenols and protection from irradiation induced damage has been observed [41]. Black Jamapa bean polyphenol extract has an antiproliferative effect on HeLa human adenocarcinoma cells, but showed less aggressive antiproliferative action on pre-cancerous cells [180]. When the bean extracts were fractionated and the individual polyphenols were added to cell cultures there was a reduced antiproliferative effect [180], suggesting that

polyphenolics may work synergistically; the sum of the parts are not as great as the whole extract [180]. The greatest antiproliferative effects were attributed to proanthocyanidin rich fractions [180]. It is unknown whether the anti-cancer effects of bean extracts are due to the antioxidant activity of the polyphenolics, pro-oxidant activity (polyphenolics can have antioxidant and pro-oxidant effects) [122]), or some other signaling behavior. In a recent study by Aparicio-Fernandez *et al.*, a high free radical scavenging capacity of black Jampa bean extract was observed [181]. Upregulated expression of apoptotic proteins in human cervix adenocarcinoma cells was observed when the extract was added to the cells [181]. The concentration used was only 35 μg/mL extract, although the amount required to quench 50% DPPH radical was 208 μg/mL [181]. The authors stated the possibility that pro-oxidant activity of the extract could be responsible for increases observed in apoptotic proteins [181].

When given a high flavonoid polysaccharide bean extract, rats administered azoxymethane exhibited decreased numbers of abberant crypt foci compared with rats not fed a bean extract [182]. In an ob/ob mouse model, whole navy beans, a navy bean extract containing polyphenolics, and the insoluble fraction of navy bean residue all demonstrated a protective effect against colon carcinogenesis [183]. Similarly, diets supplemented with pinto beans were found to decrease azoxymethane-induced aberrant crypt foci formation in the colons of Fisher rats [184]. Increased levels of hepatic glutathione-S-transferase were also observed in the rats fed dried beans, perhaps indicating an increase in conjugation and detoxification of the carcinogen as a potential mechanism to reduction in crypt formation [184].

Flavonoid extracts are not always protective, and can have mutagen effects when administered at high doses. Azevedo *et al*. demonstrated that physiological effects of 50 mg/kg anthocyanin extract fed to mice failed to protect against DNA mutations and actually promoted DNA lesions in blood peripheral leukocytes [185]. However, in the same study, Azevedo *et al.* observed reductions in DNA lesions when mice were fed cooked and dehydrated black beans in the amounts of 1, 10 and 20% of the diet [185]. Supplementation with individual flavonoids does not always have the same effect as eating a flavonoid rich diet.

From this research, it appears that whole bean extracts of polyphenolics can be protective against certain types of cancer in cell cultures and in rats, however the mechanism remains unknown. Dose and composition of the extract affect whether the polyphenolic is antiproliferative of cancer cells or mutagenic. Cancer type may also affect the action of polyphenolics. In rats, an increase in conjugating enzymes was observed when the animals were fed polyphenolics from beans, indicating that metabolizing enzymes might have been a preventative mechanism against cancer formation [184].

Type-2 diabetes is characterized by decreased sensitivity to insulin resulting in high levels of blood glucose, and is associated with increased ROS formation [63], and inflammation [186], which may contribute to the etiology of the disease [187]. Oxidative stress may be responsible for disturbed β cell functioning in diabetes [188]. β cells have a naturally low expression of certain antioxidant enzymes, which makes them susceptible to oxidative damage [188]. In a balanced physiological environment, this low antioxidant enzyme expression is important to β cell function [188]. Due to low expression of

antioxidant enzymes, hydrogen peroxide levels build up in β cells and serve as signaling molecules for the release of insulin [188]. When ROS get too high they can affect lipids, proteins and DNA. Evidence suggests that β cells release a transcription factor to produce high levels of antioxidant enzymes in response to high levels of ROS, resulting in decreased levels of hydrogen peroxide and impaired insulin release [188]. Another theory is that sub-clinical grade inflammation which often accompanies obesity may be the cause of pancreatic β cell dysfunction leading to type-2 diabetes [189]. Polyphenolics have antioxidative and anti-inflammatory potential, [190] and, therefore may be beneficial in the treatment of type 2 diabetes mellitus.

Glucose can auto-oxidize in the presence of transition metals, forming hydrogen peroxide and causing subsequent lipid peroxidation via a free radical mediated chain reaction [191]. The pro-oxidant effect of glucose on LDL can be attenuated by vitamin E [191]. Wu *et al*. observed inhibition of *in vitro* LDL oxidation in a high glucose environment when samples were incubated with flavonoids luteolin, narigenin, kaempferol, quercetin, rutin, naringin, epicatechin and catechin, and that luteolin, naringenin and kaempferol prevented a-tocopherol consumption under pro-oxidative conditions [191]. Wu *et al.* observed that the protective effect of flavonoids was partially related to direct binding of flavonoids to the lipoproteins, with aglycone flavonoids demonstrating greater inhibition of oxidation [191].

Diabetic rats treated with procyanidin extract showed reductions in hyperglycemia [177]. Some flavonoids have been shown to inhibit glucose absorption in the gut by inhibiting digestive α-glucosidase activity [192]. Anthocyanins, kaempferol and daidzen are some of the inhibitors of α-glucosidase activity [193]. However, glucose-

lowering effects of polyphenolics have been observed in a postabsorptive environment also. Myrcetin flavonol, when injected intravenously into diabetic rats helped reduce hyperglycemia during a glucose challenge [193]. Results from studies using IV injections of flavonoids are not reflective of what happens in conditions of ingestion, however.

Procyanidins extracted from grape seeds have insulinomimetic activity in some cell cultures; they activate insulin-signaling pathways and cause glucose transporter-4 (GLUT-4) receptor translocation [177]. GLUT-4 is a glucose transport receptor necessary for insulin mediated glucose uptake. When fed an oligomeric proanthocyanidin extract, db/db rats had attenuated hyperglycemia, inhibition of ROS and lipid peroxidation, and reductions in NF-kB [194]. Retinol binding protein-4 (RBP-4) is an adipokine associated with reduced GLUT-4 expression of adipocyes [195]. In a diabetic mouse model, the feeding of cyanidin 3-glucoside (an anthocyanin glycoside) attenuated hyperglycemia by improving insulin sensitivity. The improved insulin sensitivity was a result of the upregulation of GLUT-4 and the downregulation of RBP-4 [195]. Kaempferol and quercetin were also shown to improve insulin sensitivity [196]

In summary, polyphenolics have antioxidant activity, which might aid in the prevention of type 2 diabetes by keeping ROS levels down so that normal β cell function is not impaired. Some polyphenolics can interfere with glucose absorption, which may help to prevent toxic effects of an abnormal glucose load. Some flavonoids improve blood glucose levels when injected intraveneously, but this is not a typical physiological method of flavonoid ingestion. Lastly, some of the flavonoid oligomers improved insulin sensitivity via upregulation of GLUT-4 transporters and down regulation of RBP-4, which was observed in both animals and cell culture experiments. While none of these

experiments used polyphenolic mixtures from bean extracts, many of the polyphenols mentioned are found in beans. No animal and cell culture experiments investigating effects of polyphenolic bean extracts on type 2 diabetes pathogenesis were identified at the time of this review.

ROS in excess are implicated in the initiation and progression of cardiovascular dysfunction, which accompanies hypertension, chronic heart failure and ischemic heart disease [197]. ROS mediate signaling pathways that cause vascular inflammation in cardiovascular disease [197]. The majority of cardiovascular disease (CVD) results from atherosclerosis, which may begin with the transport of oxidized LDL (ox-LDL) into the artery wall [197]. When certain white blood cells (monocytes) come upon lipoproteins, they ingest them, which causes monocyte differentiation into macrophages. Once macrophages generate ROS and inflammatory cytokines, ROS further oxidize the ox-LDL, which is then taken up by macrophages to generate foam cells [197]. Foam cells comprise the fatty streak that ultimately progresses into a fibrous plaque, the rupture of which can cause occlusion of vessels [197]. The accumulation of plaques along the artery wall it is called atherosclerosis, and in extreme cases it can lead to artery blockage.

Hypertriglyceridemia, characterized by elevated levels of blood triglycerides, is associated with atherosclerosis and is a risk factor for coronary heart disease (particularly in amounts excess of 1000 mg/dL) [198]. Research on the effects of a procyanidin extract suggests dietary flavonoids can reduce hypertriglyceridemia by enhancing bile acid stimulated gene transcription pathways. A procyanidin extract from grape seeds (GSPE) was shown to enhance bile acid stimulated FXR receptor, and subsequently inhibited expression of SREPB-1 and upregulated ApoA5 expression in the liver [199]. ApoA5 has

been shown to reduce plasma triglycerides in western diet fed mice and decrease atherosclerotic lesion development [200]. SREBP-1 participates in the insulin-mediated expression if hepatic genes involved in lipogenesis and cholesterol synthesis [201]. GSPE reduced post-prandial triglyceridemia in wild type mice by 40%. This reduction is attributed mainly to FXR activity enhancement, as no differences in triglycerides were observed in FXR null mice [199].

Hypertension is a pro-inflammatory state associated with heart disease risk [202]. Hypertension is sometimes related to decreased blood flow due to plaques formed by artherosclerosis, but sometimes appears independently from coronary artery disease. Vascular inflammation is a common link between artherosclerosis and hypertension [202]. Polyphenolics from beans have been shown to attenuate hypertension in rats, and to reduce macrophage infiltration associated with inflammatory signaling [203]. Macrophage infiltration is an immunopathogenic mechanism involved in the pathogenesis of hypertension [204]. Recently a proanthocyanidin polyphenol extract from adzuki beans was shown to suppress heart rate elevation in spontaneously hypertensive rats, and also decrease macrophage number in the heart and kidney compared with untreated animals [203]. The anti-oxidant and anti-inflammatory action of polyphenols could contribute to reductions in macrophage number by inhibition of NF-*k*B activation, thereby reducing the production of signaling chemokines [203].

Polyphenolics appear to reduce risk factors for heart disease, including reducing macrophage infiltration, hypertriglyceridemia, hypertension, and inflammation. Polyphenolics from beans specifically were shown to attenuate hypertension and decrease macrophage infiltration and inflammatory signaling in rats.

Bean polyphenolics can prevent chronic disease risk in animal models. A common link was the observed inhibition of the inflammatory response, which is common to diabetes, heart disease, obesity, and cancers. The next section of this paper is a review of human studies investigating the effect of bean polyphenolics on chronic disease risk and pathology.

Chapter 2 A Systematic Review of Beans and Chronic Disease

Introduction

Chronic disease, such as cancer, heart disease and type-2 diabetes, account for the most common, and costly, health complications in America today [205]. Chronic diseases are believed to be preventable through lifestyle modification, including the incorporation of a healthy diet [205].

Half of the grain legumes consumed in the world are dried beans, however most of this number is accounted for by developing countries [184]. Dried bean consumption is comparatively limited in the western world [184], and few human dietary studies are available focusing on beans and health [167]. Some epidemiological evidence suggests that beans are a component of a diet pattern associated with longevity in the elderly [5]. A 7-8% reduction in the mortality hazard ratio was shown for every 20 g increase in daily bean consumption [206]. Epidemiological data subjected to factor analysis revealed that beans are part of a food pattern associated with reduced BMI and waist circumference [167]. Since BMI and waist circumference are associated with many chronic diseases, it is possible that beans could be beneficial in their prevention [207].

Beans contain secondary metabolites, which have been shown *in vitro* and in animal studies to reduce obesity, inflammation, oxidative stress, and to be chemopreventive [180, 185, 208]. *In vitro*, dietary polyphenolics have been shown to have anti-inflammatory and anti-obesity effects. Human dietary intervention trials can elucidate whether health benefits of beans observed *in vitro* can be translated to health benefits in humans.

This systematic review attempts to answer the question as to whether diets including the common bean (*Phaseolus vulgaris*) reduce the risk for obesity, type-2 diabetes, heart disease and cancer in humans.

Methods

Two databases, Pubmed and Agricola, were searched for relevant articles. The search was limited to human studies available in English, clinical trials, observational studies or randomized controlled trials. Keywords searched were: *Beans (NOT lima, soybean, soy, peas, fava beans),* or *Phaseolus vulgaris* in combination with *chronic disease, cancer, heart disease, obesity,* and *diabetes*. **See Figures 2.1 and 2.2**

Inclusion criteria were randomized controlled trials, observational cohort studies and clinical trials in which dietary interventions were performed or risk assessment observed using *Phaseolus vulgaris*. Studies were included if the dietary intervention contained common beans as a part of a whole dietary intervention, provided the article was listed under the search criteria or identified from reference sections of articles obtained. All biomarkers related to the four chronic diseases covered were considered as outcome variables.

Results

Twenty-two articles fit the identified parameters for review **See Table 2.1.** This number would be vastly larger with the inclusion of soybeans, however soybeans contain higher percentages of certain isoflavonoids and lignins not reported in common beans in

substantial amounts [209], and therefore health benefits of soy may differ from those of the common bean.

Discussion

Obesity

Three intervention studies and 1 observational epidemiological study investigated effects of bean intake on weight loss [210-213]. Intervention studies were primarily investigating water-soluble extracts from white beans. White bean extracts contain α-amylase inhibitors, which may prevent the digestion and absorption of carbohydrates. In order for complex carbohydrates to be digested into absorbable monosaccharides they must first be broken down into oligosaccharides by pancreatic or salivary amylases [210]. Rat studies have shown α-amylase inhibitors from *Phaselous vulgaris* inhibit carbohydrate absorption [210]. All three studies used the same proprietary fractionated white bean extract (Phase 2) [210, 212, 213], although each study administered different amounts. Phase 2 contains other dietary supplements, which makes the results difficult to interpret and applicability to standard dietary bean consumers questionable. For example, Phase 2 contains Silymarin extract, which has been shown in clinical trials to affect glucose metabolism [214, 215].

All three intervention studies were randomized, double-blinded and placebo-controlled trials. The placebo was a pill of equal appearance, smell and taste to Phase 2. Cellano *et al.* performed a 30 day trial in Italy with a sample size of 59 and observed greater weight and adiposity loss in subjects who were given white bean extract [210]. The two other trials had a small sample size (N=27, N=25) and were conducted in the United States, and although one of these studies was of longer duration than the Italian study by

Cellano *et al.*, no weight loss was observed with the intervention [212, 213]. A major difference between the Italian study and the American studies was that the Italian study used subjects that were overweight but not obese and they performed an initial 2-week compliance study to see if they would adhere to a prescribed diet [210]. They were given a menu of exactly what to eat during the trial and which meal to take the pill with, whereas in the American studies participants were simple told to take their pills with their daily meal that was highest in carbohydrates [210, 212]. Since compliance is important in studies where subjects are asked to alter their dietary behavior in an uncontrolled setting, the addition of an initial compliance test by Celleno *et al.* added the likeliness of compliance in the trial. In one of the American studies, Udani *et al.* observed significant differences in weight loss when results were stratified into groups retrospectively according to carbohydrate consumption [213]. The higher the carbohydrate intake, the more likely they were to observe weight loss with the addition of the Phase 2 supplement [213]. Additional studies are needed to determine whether alpha amylase inhibitors in white bean extract contribute to weight loss in obese and overweight persons.

An observational study, which used data from NHANES 1999-2002, based on one 24-hour dietary recall, reported lower BMI, weight, and waist circumference in consumers of beans than in non-consumers [211]. Papanikolaou *et al.* stratified their analysis into three groups, variety bean consumers (VB), baked bean consumers (BB) and variety bean + baked bean consumers (VBBB). The study was funded by the Bush Bean Company, a leading producer of canned baked beans, which might account for creation of an individualized baked bean category. In the combined VBBB group, a 22% reduction in obesity risk was observed compared with non bean consumers, however,

bean consumption was also associated with a pattern of eating that was lower in total fat and saturated fat and added sugars, and higher in carbohydrates and dietary fiber [211]. The logistic regression model used in their analysis adjusted for age, race, gender, ethnicity and energy intake, but did not adjust for macronutrient intake. It is possible that patterns of eating associated with bean intake and not bean consumption account for reductions in obesity risk. Baked bean consumers had lower systolic blood pressure than non-consumers, even though their sodium intake was higher. The authors suggest that high potassium content in beans may directly cause sodium excretion, effect vasoconstriction or suppress the renin-angiotension system causing a reduction in blood volume [211]. As is the case with observational studies, it is possible that environmental differences shaped by cultural influences, which drive people to become consumers of baked beans, are what caused the correlation between reduced blood pressure and baked bean consumption. While this study is based on 24-hour recall data of a large sampling from a nation-wide pool, additional similar studies are needed to confirm conclusions about bean intake and obesity risk. Although adjustments were made in the statistical model, a correlation in this type of study may be confounded by other correlated factors and does not demonstrate that bean intake causes weight loss.

Jimenez-Cruz *et al.* performed a dietary intervention using glycemic index to generate two diets for type-2 diabetics [216]. The high glycemic index group consumed significantly less fiber and no beans. The low glycemic index group was associated with pinto bean consumption, higher fiber intake and a greater reduction in BMI. [216] These diets were not significantly different in energy, but the high glycemic diet was higher in sugar and carbohydrates. It is not possible from this research to determine whether the

fiber from beans specifically contributed to changes in BMI since the focus of the study was on GI and the diets differed in ways other than the addition of beans.

One possible mechanism for bean consumption contributing to weight loss is decreased fat absorption. Birketvedt *et al.* fed 24 subjects white kidney bean powder daily for 9 months [217]. They measured fat excretion in feces of 4 of their subjects. Two weeks after the bean trial was completed, they measured fat content in the feces again and found that bean consumption significantly increased fat excretion [217]. Studies of larger sample sizes are needed, but it is possible that bean consumption interferes with fat absorption in the diet.

Phaseolus vulgaris could contribute to weight loss due to high fiber content and increased satiety. Bourdon *et al.* measured the postprandial rise in CCK in response to a test meal with either bean flakes (60 additional calories) or instant rice and milk powder (168 additional calories) [218]. The bean meal resulted in higher increases of CCK for a longer duration. CCK is associated with increased satiety [218] It is possible that decreases in nutrient absorption due to fiber content of the beans cause increased exposure of the intestinal tract to nutrients prolonging the CCK response [218]. Bourdon *et al.* speculate that low levels of trypsin inhibitors (TI) found in beans may be responsible for increased satiety as TI cause CCK release [218]. Consumption of meals that stimulate prolonged increases in CCK release might contribute to overall reductions in body weight over time by reducing desire for food intake. Bourdon *et al*., however, measured the response to CCK after one meal only and did not measure differences in CCK and weight loss over time. It is possible that the body adapts to CCK release triggered by bean intake over time. More research is needed in this area.

Type-2 Diabetes

Two of the studies identified by the search parameters of this review were prospective, population-based studies where subjects were normal glucose responsive at baseline and incidence of type-2 diabetes was the outcome of interest [165, 219]. Liese *et al.* used reduced rank regression to identify food patterns associated with incidence of type-2 diabetes [165], while Villegas *et al.* used Cox proportional hazards to determine if legume consumption (excluding soy and peanuts) was associated with risk of type-2 diabetes development [219]. The two studies had opposing results. Liese *et al.* observed an increased risk of type-2 diabetes associated with dried bean consumption while Villegas *et al.* observed a significant decrease in risk. One possible reason for this discrepancy is the differences in populations studied. Villegas *et al.* were sampling Chinese women living in Shanghai, whereas Liese *et al.* sampled from an ethnically diverse USA population. Culture often determines eating patterns and it is likely that associations between diabetes risk and bean consumption reflect population risk independent of dietary practices. Liese *et al.* determined a dietary pattern associated with diabetes, and many of the other foods indicated in the pattern (low fiber foods, red meat, cheese, fried foods low intakes of wine) could be driving increases in diabetes risk, with bean consumption as an independent associated factor. More studies of this type are needed to determine whether beans have a preventative or contributing effect on risk for type-2 diabetes.

Three studies investigating effects of bean consumption on glucose response in type-2 diabetics fit the review parameters. Jimenez-Cruz *et al.* [216] used a crossover

design to test the effects of a high glycemic meal compared with a low glycemic meal incorporating ½ cup of pinto beans daily for 6 weeks. Total fiber content of other foods in the diet were significantly higher during the low glycemic index (GI) phase, which makes it difficult to attribute results to bean consumption alone. No significant differences were seen between the two trial periods in fasting glucose. However a significant reduction in both BMI and HbA1C (a long term measure of blood glucose concentration) was observed during the low GI phase [216].

In previous clinical trials using normal glucose responsive volunteers, Jenkins *et al.* observed a reduction in postprandial glucose elevation with the consumption of beans as compared to other starchy foods, leading to an eventual introduction of the Glycemic Index concept [220, 221]. Fiber and protein content contribute to slowing of gastric emptying and therefore may also lower glycemic response. Tappy *et al.* investigated postprandial glucose and insulin response to a high starch meal prepared with bean flakes compared with a potato flake meal containing fiber and protein from beans [222]. The preparation of protein and fiber components of beans, which were added to the potato flakes, involved washing several times with ethanol [222]. This would remove a large portion of the polyphenolics from the protein and fiber component of the beans. Thus, the bean flake meal likely contained polyphenolics, while the potato flake meal likely did not. Tappy *et al*. observed a reduction in the rise of plasma glucose, insulin and glucose oxidation following the bean flake meal relative to the potato flake meal [222]. Only four subjects with type-2 diabetes were included in the study, however, and 6 healthy volunteers [222]. Despite the small number of type 2 diabetics, significant differences were observed between the 2 meals, suggesting something in the bean structure other than

protein and fiber content are at the root of postprandial attenuation of hyperglycemia associated with beans [222].

Bornet *et al.* studied postprandial glucose and insulin response after dried kidney bean consumption by type-2 diabetics [220]. Beans were fed to subjects both alone and in a mixed meal and results were compared with those of 5 other starchy foods [220]. The mixed meal was iso-glucido-lipido-protidic for each of the 5 starch meals [220]. In the context of a mixed meal, bean consumption resulted in the lowest rise in postprandial glucose and insulin compared with bread, potato, spaghetti, rice and lentils [220].

Seven of the studies included in this review listed fasting glucose, HbA1c or insulin response as outcome variables, although study participants were not type-2 diabetics [211-213, 218, 223-225]. All of these studies were randomized, placebo controlled trials. Only one of them reported significant reduction in fasting blood glucose associated with beans. Jang *et al.* observed that a whole grain diet plus bean intervention significantly decreased fasting glucose, insulin resistance, and AUC for glucose and insulin. However these differences were observed only in subjects with type-2 diabetes [223]. Based on the identified studies investigated in this review, it appears that a diet high in beans does not affect glucose control over time in normal glucose responsive persons, but may help type-2 diabetics maintain glucose control.

Heart Disease

Coronary heart disease (CHD), where coronary arteries buildup plaque causing them to become narrowed and hardened (called atherosclerosis), is the most common form of heart disease [172]. CHD can lead to myocardial infarction due to decreases in the

blood supply to the heart from hardened veins [172]. The NIH considers total cholesterol (TC), HDL, and LDL cholesterol to be indicators of cardiovascular disease risk [226]. Eleven studies (with intentions of investigating effects of dietary bean intake on heart disease) included cholesterol or blood lipid parameters as an outcome variable [211-213, 216-218, 223-227]. Studies suggest that fiber in beans bind to intestinal cholesterol and bile acids, promoting excretion and forcing the body to utilize its supply of precursors in the production of new cholesterol, resulting in serum cholesterol reduction [225].

Results were variable in studies investigating effects of beans on cholesterol. Birketvedt *et al.* found a reduction in serum cholesterol after 3 months of supplementation with white bean powder, with no further reduction after 9 months. They also observed a significant reduction in LDL cholesterol and in the LDL/HDL ratio [217]. However, two similar studies performed by Udani *et al.* found no effect of white bean extract on serum cholesterol [212, 213]. The Udani *et al.* studies were both shorter in duration, and had smaller sample sizes than the study by Birketvedt *et al.*, which may account for discrepancies in results.

Using NHANES 24-hour recall data, Papanikolaou *et al.* found no differences in LDL or HDL cholesterol between bean consumers and non-consumers in 8229 subjects, however, significantly greater fiber consumption was observed in all bean groups [211]. Jimenez-Cruz *et al.* found no differences in HDL, LDL or total cholesterol during their 6-week dietary intervention using ½ cup pinto beans even though the placebo diet was a low fiber high glycemic index diet [216]. This might lead one to conclude that fiber is not responsible for cholesterol lowering effects attributed to intake of beans, and some other components of specific beans are responsible instead. However, Birketvedt *et al.*

observed cholesterol lowering effects of encapsulated white bean extract [217], whereas Udani *et al.* observed no effects on cholesterol with intakes of white bean extract [212, 213] Statistical power, study duration and population differences might also contribute to differences observed. More research is needed to test the effect of bean fiber versus water-soluble extracts on serum cholesterol.

In a 12 week long study using ½ cup of pinto beans *vs* isocaloric chicken soup as a dietary intervention on people with pre-metabolic syndrome, significant reductions in LDL, HDL and total cholesterol were observed [226]. In one study using a crossover design with intervention periods of 8 weeks, Winham *et al.* found baked pinto beans significantly lowered total cholesterol (TC) and the ratio of TC/HDL. LDL cholesterol was lowered, but not significantly [224]. The same investigators did a study using 3x3 crossover design with pinto beans, black-eyed peas or carrots for eight weeks and found a significant reduction in LDL and TC in the pinto bean group [225]. Descriptions of the populations were very similar between the two Winham et al. studies, and dietary intakes recorded were similar between the pinto bean group and the baked bean groups between studies. It is possible that genetic polymorphisms account for variability in cholesterol lowering effects of beans observed in some groups but not others.

Two cohort studies were identified investigating legume intake and incidence of cardio vascular disease (CVD) or coronary heart disease (CHD) [228], or non-fatal myocardial infarction (MI) [227]. Both studies found a reduction in heart disease risk associated with increased legume intake [227] [228]. Kabagambe *et al.* performed a retrospective case-control study in Costa Rica, where survivors of MI were matched with controls and then frequency of bean consumption was analyzed [227]. Kabagame *et al.*

observed a 38% reduction in non-fatal MI risk associated with moderate bean consumption [227].

Bazzano *et al.* found that people who consumed beans four times per week had a lower incidence of CHD when adjusted for age, sex, race and energy intake, despite being more likely to smoke and consume higher levels of saturated fat [228]. They also reported that people who consumed beans had lower BMI, less hypertension, lower systolic blood pressure, lower levels of cholesterol and hypercholesterolemia, and less diabetes [228]. After adjusting for alcohol consumption, cigarette smoking, physical activity, education and history of diabetes, Bazzano *et al.* reported a 20% reduction in risk for CHD and an 11% reduction in risk for CVD for people who consume beans 4 times per week [228]. While large observational studies can elucidate associations between food intake and disease risk, they cannot definitively link bean intake to the lowering of disease. One major flaw of studies like this is they rely on self-reported dietary intake. A person's education about nutrition, or cultural conceptions about food, may influence answers to questions about food frequency.

Many of the studies investigating heart disease and bean intake used cholesterol as an outcome variable. The results were mixed and therefore inconclusive. Two large population based studies identified bean intake as being associated with a decreased risk of CVD, CHD [228] or MI [227]. Perhaps the longer duration of these population based studies produced benefits was important to the reduction in disease risk observed.

Cancer

Five different studies investigated effects of bean intake on cancer risk in humans [7, 8, 229-231]. Two of these studies were concerned with prostate cancer risk and one with incidence of colon adenomas, one with breast cancer risk, and one with incidence of endometrial cancer. Both studies concerned with prostate cancer were retrospective case-control studies where cases of either adenocarcinoma [7]or prostate cancer [8] were matched with controls and food frequency questionnaires (FFQ) were given. Using logistic regression it was determined that bean consumption was associated with a decreased risk of adenocarcinoma [7] and a decreased risk for prostate cancer (both with and without soybeans included in the model) [8]. Jain *et al.* used subjects from various regions of Canada, whereas Kolonel *et al.* used ethnically diverse subjects from Hawaii, Los Angeles, San Fransisco, Ontariao and British Colombia. Both studies matched subjects for age as well as ethnicity [7, 8]. One major flaw of these two studies is the possibility of recall bias. Subjects were asked to recall dietary intakes in the year before diagnosis [7] or over the previous 5 years [8]. It is unlikely that subjects will accurately remember their diets over that length of time, and if the subject believes certain foods to be "good" or "bad" they might be inclined to over-report the "bad" foods in light of their diagnosis.

In a prospective cohort study using 1905 subjects with previous confirmed colon adenomas, a dietary intervention was performed [229]. Subjects were men and women from various areas of the United States who participated in the polyp prevention trial.

They were given guidelines to eat a diet that was high in fiber, fruit, and vegetables and included regular nutrition counseling sessions [229]. Every year for the four years of the intervention participants were given a FFQ [229]. Dietary intakes of the intervention group were then compared with a control group, who had no intervention but were given advise about healthy eating [229]. The intervention resulted in decreases in adenoma recurrence as compared with controls [229]. The reduction in colon adenoma recurrence was positively associated with dried bean intake [229].

Levi *et al.* matched cases of patients with histologically confirmed endometrial cancer with control subjects who were admitted to the hospital for acute nongynecological disorders [230]. The study was conducted cooperatively at hospitals in regions of Italy and Switzerland [230]. FFQ's with 50 food items were given to each individual and they were asked to recall how many times a week they consumed each food previous to onset of symptoms for endometrial cancer [230]. Levi *et al.* observed a greater endometrial cancer risk associated with higher frequency of bean consumption, even after adjusting for total energy intake in their statistical model [230]. One major limitation of this study was the limited number of food groups on the questionnaire. Phytoestrogen containing soybeans were included with *Phaseolus vulgaris* in the bean category, which confound the results of this study. The authors neglected to report the duration of time they measured dietary recall (did they ask the patients to recall the previous year or five years?).

In a case control study conducted in three centers in the United States, Washington, Georgia, and New Jersey, Potischman *et al.* analyzed 100 item FFQ's of subjects with or without localized breast cancer [231]. The subjects without cancer were

matched for age and region with the cases of breast cancer patients [231]. Bean intake was associated with a slight decrease in risk for breast cancer, but no trend for decreasing risk with increased intake were observed [231]. Subjects were asked to recall their dietary intake over the previous year from the time of the interview [231]. It is possible that subjects changed their dietary patterns upon receiving diagnosis of breast cancer, such that the FFQ would not be reflective of dietary patterns leading to breast cancer risk.

The majority of cancer studies identified were large cohort studies using food frequency questionnaires. Based on the data presented in these studies, it appears that bean intake is associated with a reduced risk for prostate adenocarcinoma [7], colon adenomas [229], prostate cancer [8] and breast cancer [231], but was associated with an increased risk for endometrial cancer [230]. Of note, the endometrial cancer study included high phytoestrogen soybeans in their bean category, which might contribute to increases in endometrial cancer risk observed [230].

Summary/conclusions

Dried beans are a staple food widely consumed around the world. When excluding soybeans from the search, 22 observational studies or clinical trials investigating effects of dried beans on chronic disease risk or biomarkers of chronic disease risk were identified. This number is surprisingly small given the widespread consumption of beans by persons of varied ethnicities and socioeconomic status.

Identifying foods that might help reduce obesity risk is pertinent to our culture for whom obesity is a growing concern. A population based study suggests bean intake is associated with lower BMI and reduced risk of obesity [211]. Intervention studies

investigating effects of beans on weight loss used alpha amylase inhibitors from white bean extract and had variable results. The white bean extract used in the trials had additional components in it, which makes it difficult to decipher whether results can be contributed to intake of beans. Few studies were available examining effects of *Phaseolus vulgaris* intake on fat excretion and CCK induced satiety, however these areas are recent and seem promising.

There is insufficient evidence to indicate whether bean intake prevents the onset of type-2 diabetes. Based on seven studies, which measured glucose control in normal subjects after bean intervention, beans appear not to affect fasting blood glucose in non-type-2 diabetics. However, beans appear to contribute to glycemic control in persons with type-2 diabetes. Compared with other starches in the context of a mixed meal, beans elicit a more controlled glucose and insulin response [220]. Observed reductions in postprandial glycemia associated with bean intake were likely not due to bean fiber or protein content, but probably some other component of the beans. [222]. More research is needed to investigate whether bean secondary metabolites contribute to the observed improvements in glycemic control.

Many of the studies investigated markers associated with heart disease, and two studies measured compared bean consumption with incidence of heart disease. Eleven studies measured blood cholesterol in response to bean intake. Results varied, and studies of about 3 months seemed more likely to have cholesterol-lowering activity. In regards to blood pressure, one study reported beans lowered systolic blood pressure and another reported a lowering of diastolic blood pressure. More studies are needed investigating effects of beans on blood pressure in hypertensive individuals, as bean consumption

would be an inexpensive way to treat hypertension. Two large population based studies found bean intake to be associated with reduced risk of MI or CVD and CHD. The risk reduction is quite large. It is difficult to study effects of bean consumption on heart disease due to the length of study duration necessary to achieve a disease endpoint like MI, CVD or CHD in humans.

Five studies investigated bean consumption and cancer risk independent of soybeans. Bean consumption is associated with reduced risk for prostate cancer [7, 8] and colon cancer [229]. Bean consumption was associated with a slight reduction in risk for breast cancer, however no trends were observed with increased intake [6]. Bean consumption was associated with an increased risk of endometrial cancer [230]. Results from these studies are susceptible to potential recall bias or confounding behavioral or dietary practices associated with bean consumption.

One concern not addressed in the literature is the possibility that beans vary in their components which may contribute to varying results observed in population based studies. Are the components of the beans grown in South America the same as the components of beans grown in areas of the US? Secondary metabolites were not measured in the beans used in any of the human studies. It is impossible to determine from this research whether polyphenolics in beans are beneficial in the treatment or prevention of chronic disease. Based on the large cohort studies, it appears that beans are associated with reduction in risk for MI, CVD and CHD, and some cancers, but not in the prevention of type-2 diabetes. Future research including analysis of the biochemical composition of the food available to the population, is needed.

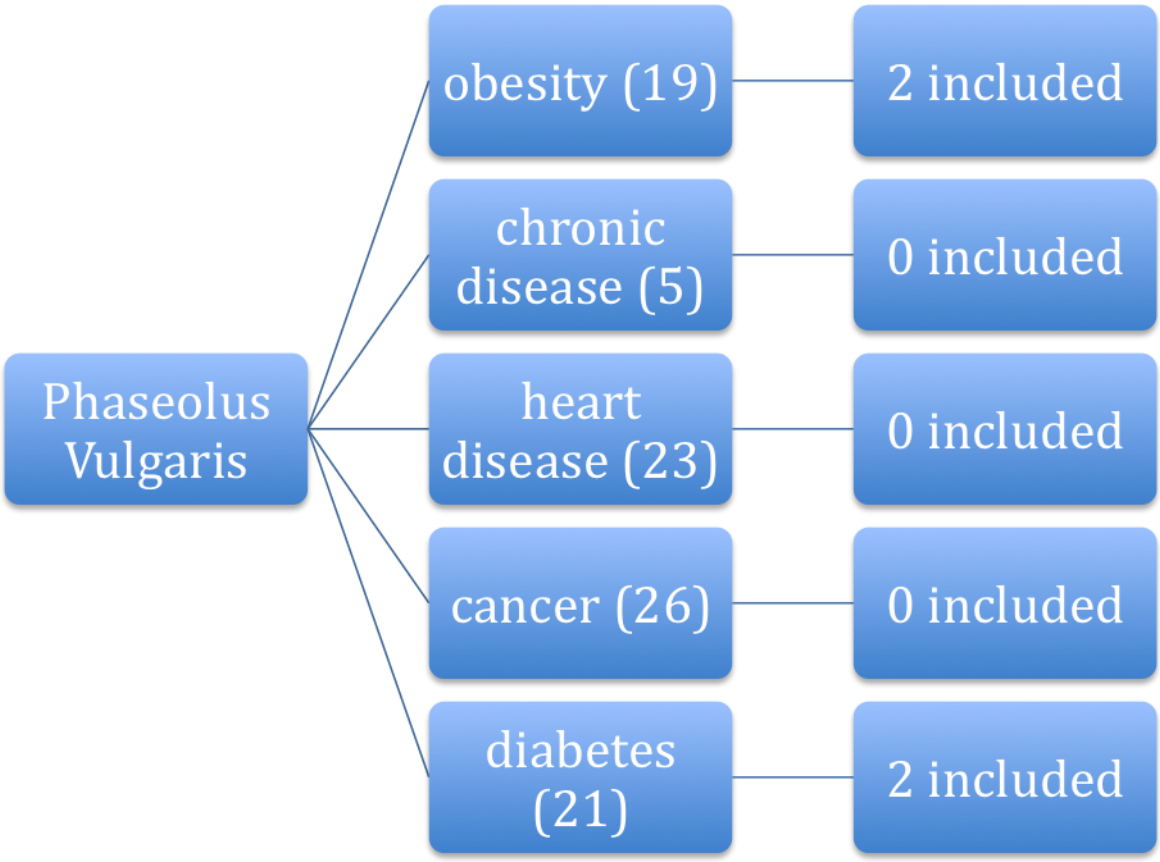


Figure 2.1 Study selection process for the search word "Phaseolus vulgaris" and "obesity", "chronic disease", "heart disease", "cancer", or "diabetes, type 2 diabetes".

Articles were excluded if they were repeats of articles already obtained from another search section, if they did not investigate one of the parameters included in this review, if they were review articles, or if they were not in English.

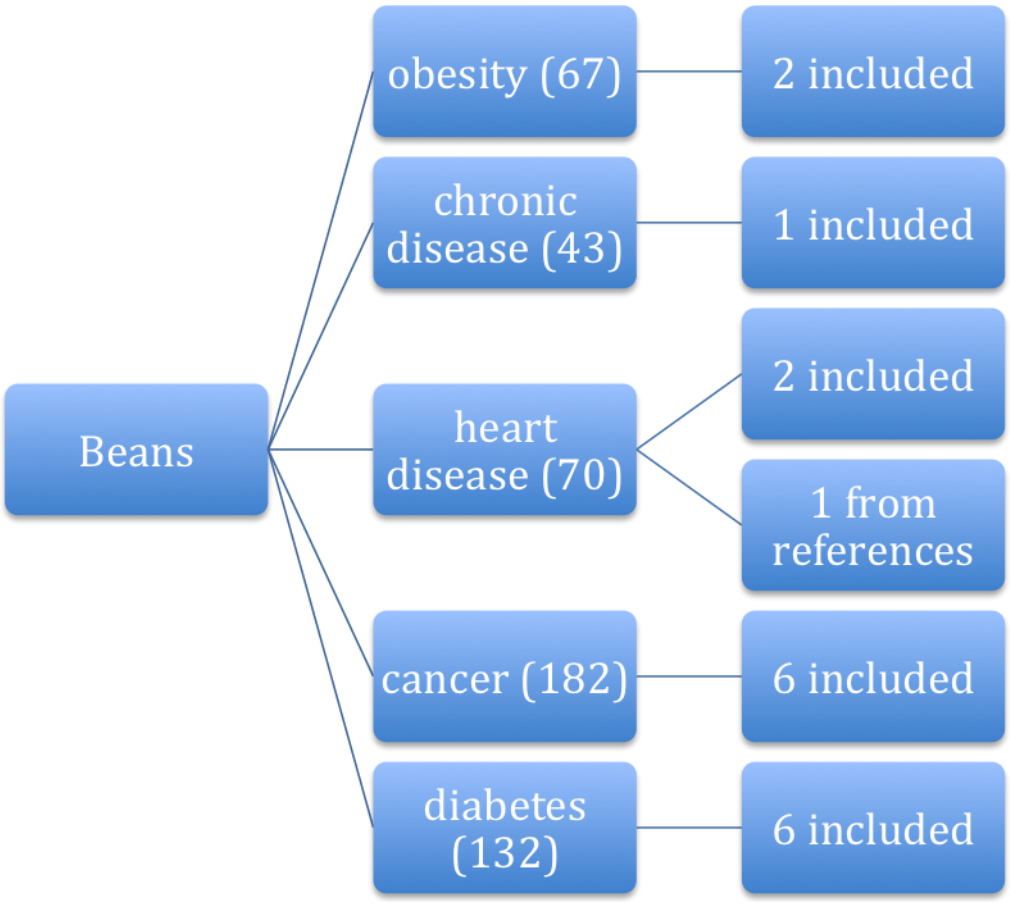


Figure 2.2 Study selection process for the search word "Beans" and "obesity", "chronic disease", "heart disease", "cancer", or "diabetes, type 2 diabetes".

Articles were excluded if they were repeats of articles already obtained from another search section, if they did not investigate one of the parameters included in this review, if the beans used in the study were not phaseolus vulgaris, if they were review articles, or if they were not in English.

Table 2-1
Effect of Phaseolus vulgaris on chronic disease incidence, risk, and biomarkers

Study	Population	Design	Intervention	Outcome	Measures	Results
Obesity						
Celleno *et al.* Italy (2007) [210]	N=59 30 intervention 29 placebo 20w, 8m age 20-45 BMI~26	Randomized Double blind Placebo controlled	LF Diet (2000-2200 kcal/day) and 1 capsule white kidney bean extract (445 mg) vs 1 placebo capsule (pills taken with high carb meal) daily 30 days	Body weight BMI Fat mass Adipose tissue thickness Waist/hip/thigh circumferences	Baseline 30 days	Intervention group had significantly greater reduction in body weight, BMI, fat mass, adipose tissue thickness and waist/hip/thigh circumferences compared with placebo group.
Udani *et al.* USA (2004) [212]	N=27 14 intervention 13 placebo (mostly w) age 20-66 BMI 30-43	Randomized Double blind Placebo controlled	HF/LF Diet and 1500 mg Phase 2 white kidney bean extract vs placebo pill (pills taken with high carb meals) Daily 8 wks	Body weight TG Body fat Energy level Appetite control Hunger HbA1C TC Waist/ hip circumferences	Baseline 2 wks 4 wks 6 wks 8 wks	Trends toward greater weight loss and greater reduction in triglycerides in intervention group with no statistically significant results.

Udani *et al.* USA (2007) [213]	N=25 13 intervention 12 placebo Age 18-40 BMI 23-31	Randomized Double blind Placebo controlled	Diet and exercise with either 1000 mg white bean extract 2x daily (4 capsules) vs identical looking placebo pills 4 wks	Body weight TG Body fat Energy level Appetite control Hunger Fasting glucose TC Waist/ hip circumferences Nutrient intakes	Blood draws at baseline and after 4 weeks. All other measures taken weekly	No significant differences between groups Both groups had significant weight loss and significant reduction in waist circumference When stratified by nutrient intake the high carbohydrate eaters lost more weight in intervention group compared with placebo group.

Papanikolaou *et al.* USA (2008) [211]	N=8229 4,076w, 4,153m age >20	Retrospective Statistical survey analysis	NHANES 24-hr recall data from 1999-2002 used. Divided into BB (n=168) VB (n=750) and VBBB (n=915) vs non-bean consumers (n=6,396) LR used, adjusted for age, gender, ethnicity, energy intake	BP LDL TG Fasting glucose Waist size HDL Overweight/ obese Nutrient profiles	One 24-hr recall	Body weight, waist circumference and BMI lower in VB and VBBB consumers. Bean consumers had 22% reduced risk of being obese. VB, VBBB and BB consumers had higher fiber, potassium, magnesium, copper, iron and energy intake. Baked bean consumers had lower systolic blood pressure but higher salt intakes.
Type-2 Diabetes						
Jimenez-Cruz *et al.* Mexico (2003) [216]	N=14 T2D 8w, 6m age 44-75 BMI~30	Randomized Controlled Crossover design	6 wk high GI (no beans), 6 wk washout, 6 wk low GI (with pinto beans)	Fasting glucose HbA1C BMI Body weight TG TC HDL LDL	Baseline 6 wks 12 wks 18 wks	Dietary fiber significantly lower during high GI period. BMI and HbA1C significantly lower in low GI group. No other significant differences

Liese *et al.* USA (2009) [165]	N=880 Non-T2D Ethnically diverse	Multicenter Prospective cohort	Non-T2D at baseline, after 5 years 144 T2D. Given FFQ Used reduced rank regression	Food patterns T2D PAI-1 and fibrinogen	Baseline 5 yrs	Higher intakes of red meat, low fiber bread and cereal, dried beans, fried potatoes, tomatoes, eggs, cheese, cottage cheese and lower intakes of wine associated with T2D. PAI-1 and fibrinogen associated with food score.
Villegas *et al.*China (2008) [219]	N=64,191 Non-T2D Chinese w Age 40-70	Prospective Population-based	Non-T2D at baseline, After 4.6 yr diets (FFQ) analyzed. Grouped by T2D status Used Cox proportional hazards	Food patterns Self-reported T2D diagnosis (fasting glucose >/= 7mmol/L, OGTT>/= 11.1 mmol/L, use of hypoglycemic medication)	Baseline ~4.6 yrs	Total legume consumption and legume consumption excluding peanuts and soy associated with decreased risk for T2D.

Tappy *et al.* Switzerland (1986) [222]	N=4 T2D obese, 3m, 1w Age 48-61	Placebo-controlled Clinical trial	T2D patients not on medication received test meal of bean flakes one day and potato flakes with bean protein and fiber added the next.	Indirect calorimetry (Glucose) Plasma Insulin GIP	Baseline, 30, 60, 90, 120, 150, 180, 210, 240 min	Glucose and insulin elevation was less in bean flake group than in potato. Differences were not due to fiber content
Bornet *et al.* France (1987) [220]	N=18 T2D 6w, 12m	Randomized clinical trial 3 factor experiment	T2D received 3 test meals on 3 consecutive days of starch, starch in mixed meal, or OGTT reference (glucose). 6 starches were tested, rice, spaghetti, lentils, dried kidney beans, white bread, potato. 3 individuals were given each starch.	Plasma Glucose + Insulin	Baseline, 30, 60, 90, 120, 150, 180 min	Beans produced the lowest postprandial rise in blood glucose both alone and in mixed meal. Beans also resulted in lowest rise in postprandial insulin in context of mixed meal.
Heart Disease						

Birketvedt *et al.* Norway (2002) [217]	N= 62 31 intervention 31 placebo 41w, 21m age 22-66 BMI >25 kg/m2	Randomized Double blind Placebo controlled	1) 2 capsules bean extract (150 mg each northern white kidney) daily 3 months vs placebo capsule 2) 24 subjects 9 month open label randomized (2 groups) 2 or 4 capsules 3x daily	HDL LDL TC TG Nutritional parameters Fat excretion	Baseline 3 mo 12 mo	Intervention group had reduction in serum cholesterol after 3 mo After 9 mo no further reduction observed in serum cholesterol. Significant reduction in LDL observed and LDL/HDL ratio. Bean extract increased fat excretion in feces. Serum B12 and folic acid reduced in intervention group, but remained within normal range.
Bourdon *et al.* USA (2001) [218]	N=8 Healthy Age 21-45 BMI 22-29	Randomized Crossover	2 test meals given with 7 day pre-washout. Test meal contained 60 g white bean flakes vs instant rice and skim milk powder	PPG insulin CCK TG TRL Cholesterol Apo B100 Apo B48	Baseline, 30, 45, 60, 120, 180, 240, 300, 360 min after start of each test meal	Bean meal resulted in increased CCK for longer duration and increases in Apo B48. No other significant differences.

Finley *et al.* USA (2007) [226]	N=80 40 pre-MetSyn 40 controls Age 18-55	Randomized 2x2 Factorial with/without pre-Met Syn	Adults assigned to consume ½ cup pinto or isocaloric chicken soup entrée daily 12 wks 4 wk initial equilibration	In vitro fecal SCFA production using various substrates Fecal bacterial population change TG HDL LDL TC	Baseline 12 wks 3 day diet records taken at baseline and 12 wks	Propionic acid and total FA production higher in bean group when bean used as substrate No total effect of bean consumption on SCFA Bean group had post intervention increase in *Eubacterium limosum* Bean group had lower post intervention LDL, HDL and TC with pre-MetSyn group having lesser reduction in HDL by 4%

Jang *et al.* Korea (2001) [223]	N=76 Male CAD Age~55 BMI~25	Randomized Double blind Placebo controlled	16 week supplement of whole grain/ bean powder compared with standard Korean diet	TG TC HDL LDL BP Fasting glucose/insulin AUC glucose/insulin HOMA Select vitamins	Baseline 16 wks	In intervention group: diastolic BP decreased, HDL cholesterol increased, vitamin E and protein intake increased, carbohydrate intake decreased, serum glucose decreased. Decreases in malondialdehyde, 8-epi-prostaglandin F (lipid peroxidation products), and homocysteine. Subjects subdivided into T2D category showed decreases in fasting glucose, AUC for glucose and insulin and decreased insulin resistance in intervention group.

Winham *et al.* USA (2007) [225]	N=16 9w, 7m Age 22-63 BMI~27 Fasting insulin >15 uU/ml	Randomized Crossover 3x3 block design	3, 8 wk trials of either pinto beans, black-eyed peas, or carrots (1/2 cup per day) in addition to regular diet. 2 wk washout periods in between.	TC LDL HDL TG Hs-CRP Glucose Insulin HOMA HbA1C Nutrient data	Baseline 8 wks 10 wks 18 wks 20 wks 28 wks 24 hr diet records at 2 wk intervals.	Pinto beans significantly lowered serum total cholesterol and LDL cholesterol.
Winham *et al.* USA (2007) [224]	N= 23 13w, 10m Age 22-70 TC 200-260	Unblinded 2x2 Randomized Crossover	2, 8 wk trials of either baked beans or carrots (1/2 cup per day) in addition to regular diet with 2 wk washout period in between.	TC LDL HDL TG Hs-CRP Glucose Insulin HOMA HbA1C BMI Nutrient data	Baseline 8 wks 10 wks 18 wks 24 hour diet records at 2 wk intervals.	Nutrient intakes remained consistent except for significant increase in fiber during bean trial. TC and LDL significantly decreased during bean trial.

Kabagambe *et al.* USA, Costa Rica (2005) [227]	N=4238 2119 MI cases 2119 controls Costa Ricans 27% w Age ~60	Retrospective Case-control Matched	Home visit Sampled blood, adipose tissue gave FFQ Used stepwise multivariate linear regression	Sociodemographic characteristic Self reported diabetes/ hypertension TG TC HDL LDL Fatty acids Bean frequency	1 measure	Moderate consumption of dried mature beans associated with 38% reduction in risk of non-fatal MI in Costa Rica.

Bazzano *et al*. USA (2001) [228]	N=9632 Age 25-74 NHANES Low income, women 25-44 and elderly oversampled	Prospective cohort	FFQ for 3 months prior to initial interview (no portions) one 24 hour recall (with portions) a 19 year follow-up used ANOVA, chi squared, cox proportional hazard	Legume intake (1-4 times per week) Documented CVD or CHD	Interview at baseline and after ~19 yrs	People who consumed legumes were significantly less likely to have CHD even though they were more likely to smoke and eat saturated fat. They remained less likely to have CHD when adjusted for confounders (those consuming 4x per wk had 20% risk reduction for CHD and 11% reduction for CVD compared with 1x per wk)
Cancer						
Jain *et al*. Canada (1999) [7]	N=1253 617 cases 636 controls age~70 m with adenocarcinoma of prostate or controls without	Retrospective Case-control Recruited from Quebec, BC and Ontario	Cases recalled diet in year before diagnosis, controls in year before call. Logistic regression performed.	Foods eaten Nutrient intakes Lifestyle factors Prostate cancer	One face to face interview	Beans associated with decreased risk for prostate adenocarcinoma. (OR .69)

Kolonel *et al.* USA (2000) [8]	N=3237 1619 cases 1618 controls African American, white, Japanese and Chinese m with confirmed prostate cancer or controls without.	Retrospective Case-control	In home interviews. 147 food items FFQ over 5 years. Logistic regression	Food frequency Nutrient intakes Prostate cancer	One face to face interview	Beans associated with decreased risk for prostate cancer (with or without soybeans) (OR .62)
Lanza *et al.* USA (2006) [229]	N=1905 686w, 1219m 1 or more confirmed adenomas Age~61 BMI~27	Prospective Multicenter Randomized Clinical trial	High fiber 4.3 g/MJ, high F&V 5-8 servings/d, low fat 20% diet vs information about healthy eating with no follow up Logistic Regression	Food frequency Colon adenomas	Baseline Annually for 4 yrs Colonoscopies at baseline, 1 yr and end of trial	Significant reduction in adenoma recurrence with dried bean intake independent of green bean intake. Still significant when adjusted for meat or processed meat reduction associated with increases in dried bean calories.

Potischman *et al.* USA (1999) [231]	N=2019 568 cases 1451 controls w age 20-44 *in situ* and localized breast cancer patients or controls without cancer	Multicenter Retrospective Case-control	In home and telephone interviews. FFQ over previous one year with 100 food items. Logistic regression	Food frequency, *in situ* and localized breast cancer	One interview either over the phone or mail in FFQ	Beans associated with slight reduction in risk for breast cancer (OR.87), however no trends for decreasing risk with increased intake were observed.
Levi *et al.* Switzerland, Italy (1993) [230]	N=846 274 cases 572 controls w age 30-75 with endometrial cancer or controls without	Multicenter Retrospective Case-control	In hospital interviews. FFQ with 50 food items. Weekly frequency previous to diagnosis was recorded. Logistic Regression	Food frequency, confirmed endometrial cancer	One face to face interview	Beans and peas food group associated with increased risk for endometrial cancer OR 1.98 in highest tertile)

Abbreviations are: CAD: Coronary Artery Disease, CHD: Coronary Heart Disease, CVD: Cardiovascular Disease, FFQ: Food Frequency Questionnaire, HbA1C: glycosylated hemoglobin, IGT: Impaired glucose tolerance, m: male, OGTT: oral glucose tolerance test, PAI-1: plasminogen activator inhibitor-1,PPG: post-prandial glucose, T2D: diabetes type-2, TC: Total Cholesterol, TG: Triglycerides, TRL: triglyceride rich lipoprotein

Chapter 3 Comparison of Antioxidant Activity, Polyphenolics, and Flavonoids in Commercial and Heirloom Variety Beans

Abstract

Common beans (*Phaseolus vulgaris*) are a culturally significant food for Native Americans. Seeds have been passed down for generations (heirloom varieties) and may differ from commercial varieties in their composition. Polyphenolics are a good source of antioxidants and may aid in the treatment of chronic disease. This study compared Native American heirloom varieties of beans with commercial varieties with regards to antioxidant potential, total polyphenolics, and flavonoid content. Three different heirloom varieties of beans were grown in shared plots. Beans were matched for color and size with two commercially available varieties. Total polyphenolics and flavonoid content were measured and antioxidant activity was quantified using a radical scavenging assessment. No differences in total polyphenolics, flavonoids, or antioxidant activity were found between matched varieties of beans. Total polyphenolics, flavonoid content and antioxidant activity were highly correlated. A cultural view of Native American nutrition indicates that a relationship with food during the growing process is essential to health of people. Western concepts of nutrition emphasize molecular composition as essential to the health of the consumer. Both cultural perspectives are examined in the present study with regards to bean consumption and health.

Introduction

Beans are a dietary source of plant secondary metabolites including polyphenolics and a subclass of polyphenolics, flavonoids. There is considerable interest in polyphenolics as they may confer health benefits on consumers. Some potential health

benefits include antioxidant (polyphenolics are responsible for the majority of the antioxidant activity in water-soluble extracts of beans), anti-inflammatory and anti-obesity affects [132]. Incidence of chronic diseases such as diabetes, heart disease, obesity and hypertension are associated with oxidative stress and inflammation. These forms of chronic disease have climbed in the Native American community over the past few decades [232]. The mortality for American Indian type-2 diabetics is almost 2 ½ times higher than the U.S. all race rate [232]. It has been suggested that increased rates of type-2 diabetes and obesity in the American Indian community is related to dietary changes in the latter part of the 20th century [233].

The traditional Native American diet is high in beans, corn, and squash. So valued are these foods that they are referred to as the three sisters crops. However, the beans that are commercially available today may not be the same as the beans that were consumed by Native Americans in the early part of the 20th century. In the United States, the food supply and farming practices have changed since World War II [36]. Food can be shipped long distances and stored for longer periods of time. The use of pesticides, herbicides and large-scale mono-crop farming alters plant secondary metabolites [36]. Selection for low tannin bean varieties may have affected the polyphenolic content in market varieties of beans [49].Though it is unknown whether this type of selection occurred, it is quite possible, because condensed tannins (proanthocyanidins) can affect palatability, decrease protein and phosphorus absorption, reduce cooking quality and affect appearance of beans (particularly during storage) [234]. Plant breeders, in selecting for desirable nutrients, could inadvertently breed out other components with health benefits [235]. For example, in an attempt to improve zinc or iron content of their beans, plant breeders

might be inclined to select for bean varieties that produce lower amounts of phytate and polyphenols [235]. Conversely, high polyphenol varieties might have been selected for over time due to increased resistance of high polyphenolic plants to pathogenic invasion. It is therefore worth investigating the traditional Native American beans differ from the commercially available varieties.

Seeds that have been saved by Native Americans and passed down through generations were gifted to the Dream of Wild Health organization in Hugo, MN. Indigenous knowledge and wisdom from the Elders says these heirloom varieties of beans are "good beans", and that they contribute to the health of those who eat them. The objective of this study was to compare secondary metabolites and overall antioxidant activity of traditional heirloom varieties of beans with similar market varieties.

Methods

Planting and Harvesting

Beans were planted at the end of May, 2008. Since the beans were from Native Americans and the farm was part of a Native American organization I was instructed to use Native wisdom in the growing of the beans. I was told that it was important that I grow the beans myself so that I might cultivate a relationship with them. The beans were allowed to dry on the plant and were collected by hand and stored in their pods, and analyzed within 4 months.

There were four rows of beans in each plot. Two rows of the same variety were planted on one side of the plot, and two rows of the compared market variety planted on

the other. Five total plots were used. The layout of the plots was the following: two Black Bean Dream of Wild Health (DWH) variety and Jordan Farms (JF) variety, two Navy Bean JF variety and Long White Good (DWH), and one A Bean (DWH) variety. A selection of about 7-10 pods from each plant was gathered. The 7-10 pods representing one plant were stored together in one sealed plastic bag, labeled, and all plant samples stored in a brown grocery bag to prevent light from penetrating. Samples were stored at room temperature until September when they were transferred to a refrigeration unit at 4°C.

Extractions

Samples were extracted using acetone/water/acetic acid (70:29.5:0.5) according to methods by Xu *et. al*, modified slightly [120]. On each day of extraction, collected beans from one plant of each variety were taken from the refrigerator (one A, DWH Black Bean, JF Black Bean, DWH Long White Good (LWG), JF Navy). Pods were emptied and gathered seeds were pooled together by plant just prior to grinding. Seeds were ground using a cyclone sample mill (UD Corporation, Boulder, CO), and seed powder was sifted through a 60-mesh sieve. The powder was collected into a glass container and set aside while other plants were ground. Each day a different bean type was ground first, to avoid one bean variety being exposed to the air longer than the others. In between each plant grind the machine was "washed" with the grinding of the same variety of grocery store black bean, and then cleaned out thoroughly to insure minimal contamination of samples. The beans were then immediately weighed into test tubes in differing amounts, as described below.

Amounts between 0.0625 and 0.5g for the different varieties of Black Beans and A beans, and 0.125-0.5g for the LWG and Navy beans were used to analyze for total polyphenolics, flavonoid content, and antioxidant activity. These amounts were determined experimentally. Five ml of acetone/water/acetic (70:29.5:0.5) acid solvent was added to each tube. The tubes were then capped and placed into a Lab-Line Orbit Environ-Shaker. The shaker was covered with cloth to prevent light penetration, and set to 300 RPM for 3 hours.

After 3 hours, tubes were placed in a rack and allowed to sit overnight in the dark at 4°C. The following morning the sample tubes were centrifuged at 2000 g for 10 minutes and supernatants collected. The pellet was washed with an additional 5 ml of acetone/water/acetic (70:29.5:0.5) acid, vortexed for 1 min, and then centrifuged as before. The supernatants collected were combined with the first. The extracts were capped and placed in the dark at 4°C for assay within two days.

DPPH Free Radical Scavenging Assay

DPPH radical scavenging was determined according to the method of Xu *et. al* [120], modified slightly. Briefly, DPPH solution was prepared using 101 µM DPPH (Sigma Aldrich Inc., Saint Louis, MO) in 100% ethanol. A standard of Trolox (4mM) (Sigma Aldrich Inc., Saint Louis, MO) was prepared once a month and stored in a dark wrapped tube at 4°C. After each use the tube was flooded with nitrogen gas to displace oxygen.

Solutions for the standard curve were prepared between 0-48 mol/L. Bean samples were analyzed in duplicate. Two hundred µL of the bean extracts were added to

3.8 mL of DPPH solution. Standards and samples were then vortexed and incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm against an ethanol blank.

Calculations for Trolox equivalency were performed according to methods adopted from Miller *et al*. [236]. Briefly, trolox quivalents were calculated using the following calculation:

{(Trolox standard curve zero intercept/2)-sample zero intercept} / slope

For expression of Trolox Equivalents (TE) in µM/100 g, this number was divided by 0.50 mM. TE are expressed as µM/g for presentation of results.

Folin-Ciocalteau Assay for Total Polyphenolics

The assay for total polyphenolics was performed according to methods outlined by Xu *et al.* [120], modified slightly. A gallic acid (Sigma Aldrich Inc., Saint Louis, MO) standard was made using 1 mg/mL gallic acid in acetone/water/acetic acid (70:29.5:0.5). The gallic standard was prepared once a month and stored in a dark wrapped tube at 4°C. After each use the tube was flooded with nitrogen gas to displace oxygen. Gallic acid standards were prepared in concentrations ranging from 0-1000 µg/ mL.

A preparation of 3 mL deionized water (DI), 250 µL Folin Ciocalteau reagent and 750 µL 7% $NaCO_3$ was combined with 0-50 uL gallic acid standard or 50 uL of bean extract. The tubes were vortexed and set in the dark at room temperature for 8 minutes. Total volume was brought to 6 mL using DI water. The tubes were incubated in the dark

for 3 hours at room temperature. Absorbance was measured at 765 nm. The zero standard solution was used as a blank.

Colorimetric Assay for Total Flavonoids

The assay for total flavonoids was performed according to methods outlined by Xu *et al.* [120], and slightly modified. Briefly, catechin stock solution (Sigma Aldrich Inc.) was prepared by dissolving +/- catechin in acetone/water/acetic acid (70:29.5:0.5) solution. The +-catechin standard was prepared once a month and stored in a dark wrapped tube at 4°C. After each use the tube was flooded with nitrogen gas to displace oxygen. Catechin standards were prepared in a range of 0-1000 µg/mL.

To 1.25 mL DI water 75 µL of 5% $NaNO_2$ was added. Preparations were made by adding 0- 250 µL catechin, or 250 µL of bean extract to this solution. After 6 min 150 µL of 10% $AlCl_3$-$6H_2O$ was added to each tube. After 5 min 500 µL 1M NaOH was added. The mixtures were brought to 2.5 mL volume with DI water. Absorbance was measured immediately at 510 nm.

Results

The Dream of Wild Health (DWH) black beans and Jordan Farms (JF) black beans differed in size and shape but not color. The DWH A beans were red and white speckled, whereas the JF Navy and DWH Long White Good (LWG) were very light in color.

The DWH black beans and JF black beans had the greatest free radical scavenging activity, but did not differ significantly from each other. The DWH A beans had significantly less scavenging activity than the DWH and JF black beans but greater scavenging activity than the JF Navy and DWH LWG, which did not differ from each other **(Figure 3.1).** The pattern for total polyphenolics **(Figure 3.2)** and flavonoids **(Figure 3.3)** was the same as for free radical scavenging acitivity. Thus, the heirloom varieties did not differ from the commercial varieties in any of the three measured parameters. There was no significant effect of plot for beans of the same variety, indicating that differences in the measured parameters were due solely to the beans themselves and not environmental differences within the growing area.

Antioxidant capacity, total polyphenolics, and flavonoid concentrations were all highly correlated with each other ($p<0.001$, $r = 0.83$-0.91) (Table 3.1).

Discussion

Our objective was to compare traditional heirloom varieties of beans with similar market varieties, to answer the question of whether the beans traditionally grown by Native Americans were different in secondary metabolites and overall antioxidant activity compared with beans commercially available today. We found no differences between the heirloom and market varieties of beans when similar types were compared (e.g. black beans), suggesting that the heirloom varieties of beans studied were not inherently different from comparable commercially varieties available today in terms of secondary metabolites.

The commercial varieties of beans used in this study were obtained from a local farm and tested within months of harvest. These beans may not be representative of commercial beans produced on a larger scale, which are available to the majority of American consumers, because beans purchased from a grocery store are often stored in clear packaging and exposed to UV light, which can oxidize polyphenolics and reduce antioxidant activity. A more accurate representation of differences between beans commercially available and heirloom varieties would have been observed had we measured beans bought directly from the grocery store. However, comparing store bought beans with farm grown beans would introduce environment as a confounding factor. Growing our beans in the same environment allowed us to compare characteristics intrinsic to the bean.

Several other studies of secondary metabolites in beanshave been reported. Xu *et al.* reported that the total flavonoid content of common beans ranged between 0.92 and 4.24 mg catechin equivalents (CAE)/g [120]. They measured black beans, red beans and navy beans [120]. Heimler *et al.* reported a smaller range of flavonoids in common beans (0.02 to 1.43 mg CAE/g) [123], which Xu *et al.* attribute to different growing conditions [120]. However, the differences are more likely due to differences in extraction methods than growing conditions, since Heimler *et al*. extracted secondary metabolites using ethanol [123] and Xu *et al*. used an acetone/water/acetic acid (70:29.5:0.5) mixture [120]. In another study by Xu *et al*., three-fold differences were observed in flavonoid content between ethanol extracted black beans and acetone/water/acetic acid (70:29.5:0.5) extracted[42]. In the present study, we used the acetone/water/acetic acid (70:29.5:0.5)

method of extraction, so it is not surprising that our results were similar to those reported by Xu *et al*.

Total phenolic content ranged from about 0.5 mg gallic acid equivalents (GAE)/g in white beans to about 3.8-4.0 GAE/g in black beans (Figure 3.2). This is similar to what has been reported by Xu *et al.*[120], who observed a range in total polyphenolics between 0.57 and 6.99 mg GAE/g. We found a high correlation between total polyphenolics and antioxidant activity. However, Heimler *et al.* did not observe a correlation between antioxidant activity and total polyphenolics [123], suggestingt that polyphenolic content does not relate to radical scavenging capacity in all varieties of beans. However, given that they used an extraction method that has been shown give an incomplete extraction, their failure to find a correlation must be viewed with caution.

Differences of 70-fold have been reported between the DPPH radical scavenging abilities of different landraces of *Phaseolus vulgaris* [123]. Xu *et al*. reported DPPH values of 18.95 and 1.48 μmol trolox equivalents (TE)/g for black eclipse and navy beans respectively [120]. We report trolox equivalents ranging from 14-18 μmol TE/g for black beans and 0-1.5 μmol TE/g for white beans. Our results were similar to those of Xu *et al.* [120], which is not unexpected given the extraction methods used were essentially the same. In both the present study and that by Xu *et al.* [120] beans for analysis were obtained from a local seed saving project. Although our white beans and black beans compared similarly to those of Xu *et al.* [120], our red beans had less antioxidant activity than theirs. Xu *et al.* [120] report DPPH values in the range of 13.5-17.5 μmol TE/g for red bean varieties. We report a trolox equivalence of 5.3 μmol TE/g for the Dream of Wild Health

"A" bean. This could be related to the mottling of white in the "A" bean, and is further indicative that bean color is related to antioxidant activity.

Caldas *et al*. performed genotyping of different varieties of beans and observed that many varieties of common beans had flavonoid concentrations which correlated with seed coat color [28]. Additionally, studies have shown that the majority of scavenging activity can be attributed to tannins found in the seed coat of beans [180]. Caldas *et al.* [28] observed that white beans often had the lowest tannin content compared with colored beans [28].The association between bean color and secondary metabolite activity is characteristic of beans in the Middle American gene pool [29].

The present study indicates that, within similar varieties of beans, there appears to be no differences in the concentration of secondary metabolites between commercial varieties and heirloom varieties of beans. However a question that remains is whether the beans obtained by the consumer at a local brocer are different in secondary metabolite concentrations from the beans grown and consumed by Native people, due to differences in growing and storage practices. I did not attempt to measure this, but I feel it would be a next logical step. As mentioned above, the beans sampled in this study exhibit a correlation between color and polyphenolics, which is characteristic of the Middle American gene pool. We did not perform a genotype analysis of the beans, which would be necessary to confirm that the beans are in fact from this gene pool. It is difficult to compare the results of the present study with those of others aside from Xu *et al.*, because different extraction solvents were used and different techniques for analysis. A standardized set of techniques for quantifying antioxidant activity in beans appears to be emerging which will make comparisons between different research groups possible. The

acceptance and widespread use of standard methods for extraction and analysis of secondary metabolites will allow confirmation of my results, and facilitate further studies of varietal differences in bean secondary metabolite concentrations. Such information would likely be useful to consumers who desire to maximize these compounds in their diet because of their potential health benefits.

A Cultural Perspective

Discrepancies exist between Native and Western views of health, which I will discuss presently. Western nutrition science tries to approach the question "are there molecular components in this food which might be of benefit to human health? Is there some processing method which might increase the benefits?" My decision to profile scavenging ability and secondary metabolites in beans as a method of determining differences between heirloom varieties and conventional varieties is an appropriate question within the paradigm of nutrition science. However, the lens of nutrition scientist is only one cultural paradigm relevant to the work done around these particular beans. The heirloom variety of beans analyzed in this research were Native American in origin, and Native Americans possess another cultural paradigm within which nutrition science is performed. Storytelling is historically a form of Native education, so I will use my story to illustrate my understanding of the Native American paradigm.

After making arrangements to use some of their plots for my research at the small, Native-owned farm in Hugo, MN, the director of the organization (elder Sally Auger) came to me and implored me to use my own hands in the growing of the beans. Having no prior experience with farming, and knowing that they had already hired a professional

farmer to tend to their farm, I was surprised. I had been hired to teach cooking classes and to develop bean soup recipes for their organization, not to work in the garden. Sally felt that since I was going to be cooking with the beans it was important that I have a relationship with the beans as well. In Native culture, all practices are spiritual because all of nature has a spirit [237]. According to Gregory Cajete "Native science incorporates a spiritual process: no division exists between science and spirituality. Every act, element, plant animal and natural process is considered to have a moving spirit with which humans continually communicate." [238] Sally described the beans as "spirit people".

Traditional Native cooking, and therefore Native nutrition, is filled with ritual. As part of my experience with the farm I learned to work with traditional foods such as hominy, beans, bison, goose, elk, swamp tea, wild rice, and fresh produce. Cooking one simple soup from start to finish was a process requiring many hands and many days. The foods that are eaten and the method of preparation used comes from cultural knowledge passed down from the Elders. Hominy is made by boiling corn in wood-ash. Western scientists have since identified that this form of processing improves the bioavailability of amino acids and the B vitamin niacin. This form of processing, however, had been used for thousands of years before vitamins were discovered. While scientific knowledge is valuable, experiential knowledge based on traditional practices carries the wisdom of years of experience and is also valuable.

Mark Bellcourt states that according to Native science, "Nature will always possess unfathomable mysteries" [237]. In Native science, mystery is accepted and not attempted to resolve, whereas western culture attempts to solve mystery using scientific methods. In a class at the University of Minnesota, a professor drew the word "TRUTH"

on a board and said that in science the truth is what we are attempting discover. She then drew a circle around the word and said that when we do a study, we develop the understanding that the truth lies somewhere within the circle. We do another study (she drew a circle to overlap the first, encapsulating the word truth), and we narrow the scope on the pulse of the truth. She continued on in this way. This is the dogma of western science; that there is a truth to be found, which is stagnant, and all studies are geared in an effort to narrow our scope around this truth. "Truth in science is determined by the empirical feedback of success in one's predictions..(this) sets science apart from other disciplines such as philosophy and religion" [239]. Scientific knowledge is presented in literature as tentative because some of the most secure truths uncovered in science have been refuted [239], despite the teachings of Newton who described the universe as being in perfect order [237].The Native paradigm, which views each living creature as having a spirit accepts unexplainable twists of natural order which are characterized through storytelling by a persona known as the "trickster" [237]. The type of wisdom governing decisions around what to eat is not based on empirical evidence derived using the scientific method, but rather on cultural wisdom, which relies on spirituality and keeping in harmony with nature [237].

As discussed in this thesis, plant metabolites are variable and subject to change in accordance with a changing environment. People do not eat single nutrients, they eat food and foods interact creating a different delivery of chemistry with every dining experience. Differences in plant genetics and disease exposure create unique individual exposures and reactions to components in food, making it difficult to predict health effects of food exposure. According to Western scientific thought, each mystery of nature will one day

be unlocked by the human intellect if we break nature down into parts and strive to better understand each individual part [237].

According to Cajete, science is both a cultural as well as an individual journey of thought [240]. Albert Einstein said "The whole of science is nothing more than a refinement of everyday thinking". Culture shapes both the content and the type of question the scientist asks as well as the tools used to approach the question. My decision to study quantities of antioxidant activity, polyphenols and flavonoids in beans came from a cultural belief that antioxidants, which can be extracted from a plant, are healing; the greater the amount of antioxidants, the greater the healing potential of that plant. I developed this belief system from western culture, which values physiochemical composition and believes that healing medicine is chemical substance, which can be extracted physically from a plant. While empirical evidence proving the healing powers of antioxidants is weak, and research testing the health benefits of eating beans is limited, Native nutrition does not rely on the production of empirical evidence to validate their food choices. The wisdom of the Elder who gave the organization seeds says that they are "good beans", and therefore Native science says they are healthy.

In an era where we know increasingly more about food chemistry, why is it that we are experiencing a rise of chronic disease which we believe to be associated with nutrition? Michael Pollan describes this as the "American Paradox: the more we worry about nutrition the less healthy we seem to become" [241]. I argue that there is still a place for nutrition and nutrition science. Perhaps nutrition science needs to expand our belief system and start asking different types of questions. Food chemistry concerns itself with the "what" of food, and nutritionists with "how much". Perhaps we need to start asking

questions such as why, where and how. How are humans experiencing food today? In what context are they experiencing food. What is their relationship with food? It is difficult to make room for questions like these in the realm of science where empirical evidence is a hallmark of valuable knowledge, and yet the need for new reasoning is outlined by the recent rise in nutrition associated chronic disease.

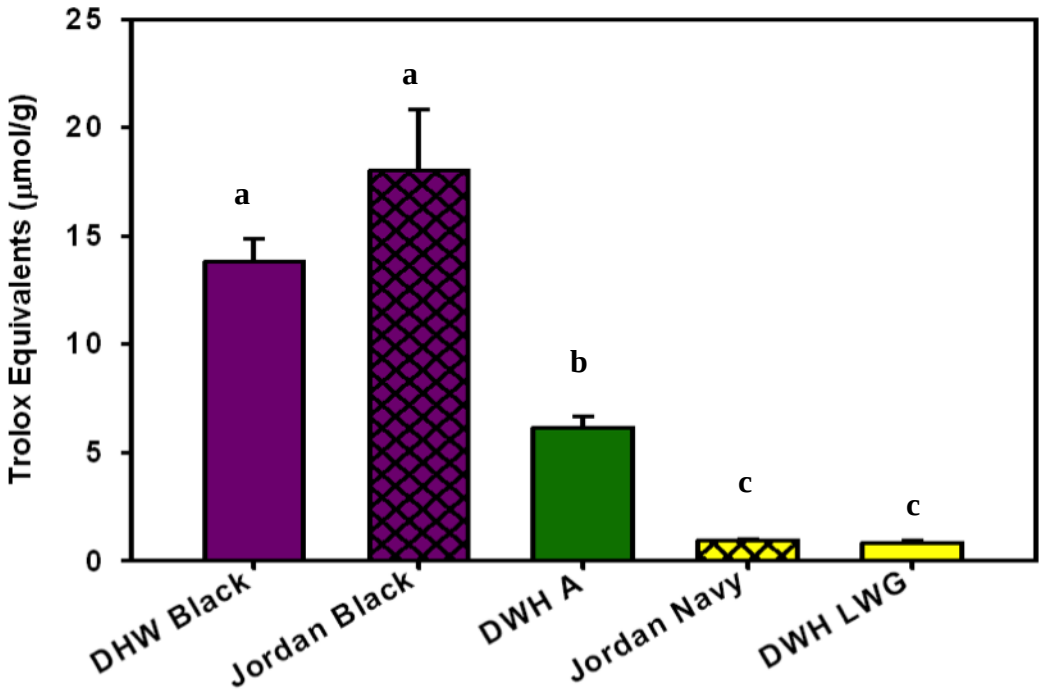


Figure 3.1 Free radical scavenging activity of black beans (purple bars), red and white "A" bean (green bar), and white beans (yellow bars) as determined by DPPH radical scavenging ability.

DWH: Dream of Wild Health heirloom variety, Jordan: Jordan Farms conventional variety, LWG:Long White Good beans. Values that do not share a letter are significantly different ($p < 0.05$). N=15 for LWG, Navy, and Jordan black, n=14 for DWH Black, n=5 for DWH A beans.

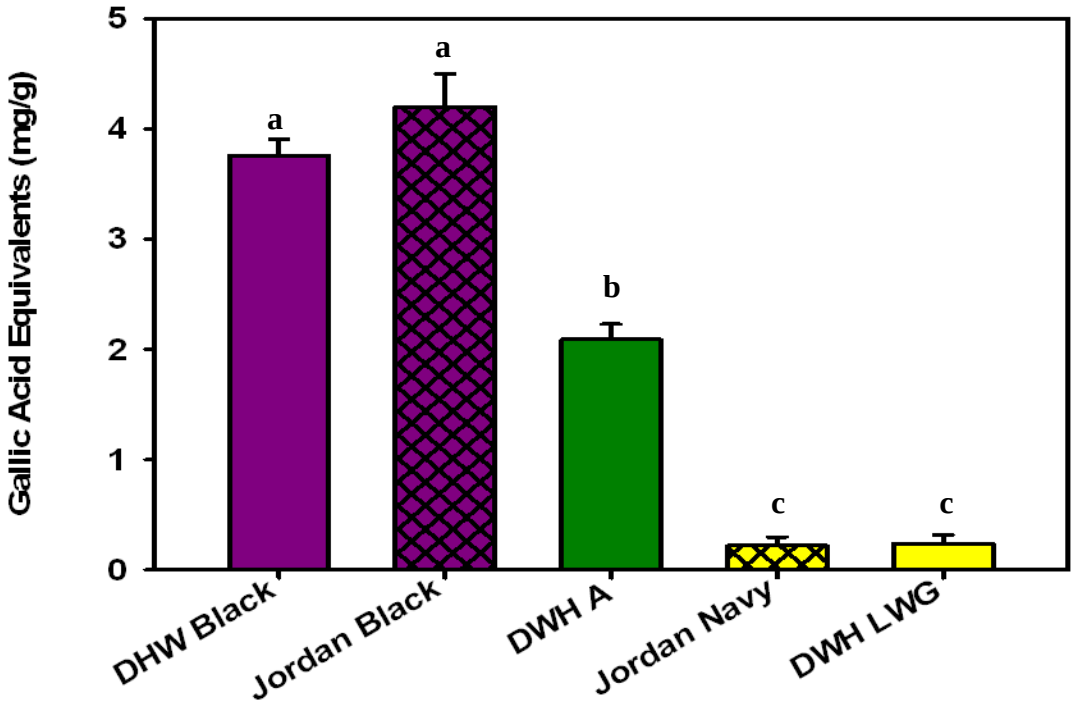


Figure 3.2 Total polyphenolics concentration of black beans (purple bars), red and white "A" bean (green bar), and white beans (yellow bars) as determined by Folin-Ciocalteu method.

DWH: Dream of Wild Health heirloom variety, Jordan: Jordan Farms conventional variety, LWG:Long White Good beans. Values that do not share a letter are significantly different ($p < 0.05$). N=15 for LWG, Navy, and Jordan black, n=14 for DWH Black, n=5 for DWH A beans.

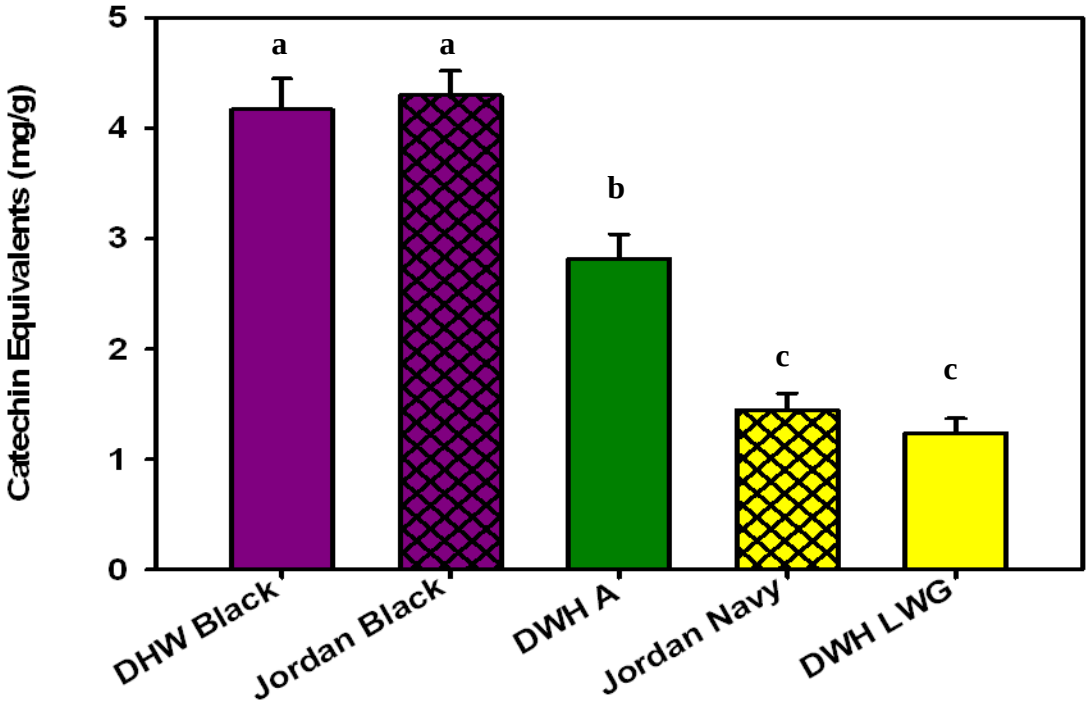


Figure 3.3 Total flavonoid concentration of black beans (purple bars), red and white "A" bean (green bar), and white beans (yellow bars) as determined by the aluminum chloride assay for total flavonoids.

DWH: Dream of Wild Health heirloom variety, Jordan: Jordan Farms conventional variety, LWG: Long White Good beans. Values that do not share a letter are significantly different ($p < 0.05$). N=15 for LWG, Navy, and Jordan black, n=14 for DWH Black, n=5 for DWH A beans.

Table 3.1
Pearson Correlation Coefficients

	Trolox eq μmol/g	Catechin eq mg/g	Gallic acid eq mg/g
Trolox eq μmol/g	1.00	0.83 (<0.0001)	0.88 (<0.0001)
Catechin eq mg/g	0.83 (<0.0001)	1.00	0.91 (<0.0001)
Gallic acid eq mg/g	0.88 (<0.0001)	0.91 (<0.0001)	1.00

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Appendices

Appendix A Methods and Assays

Appendix A-1

Method for Extraction of Water Soluble Antioxidants from Beans

Equipment and Materials

1) Whole bean seeds, completely dried, maintained in pods , and stored at 4C
2) Cyclone mill
3) 60-mesh sieve
4) Scale
5) Shaker
6) Vortex
7) Dark cloth
8) Centrifuge
9) Glass pipets
10) Test tubes
11) Beakers

Solutions

1) 1 Liter acetone/water/acetic acid (70:29.5:0.5)

    a. Acetone 700 mL
    b. Distilled H2O 295 mL
    c. Acetic acid (glacial) 50 mL

Procedures

1) Empty beans from their pods into a clean beaker, keeping the seeds from each plant separated.
2) Grind beans by running them through a thoroughly cleaned cyclone mill, collecting bean dust into a clean, labeled beaker. Clean mill in between each grind, and vaccum out dust.
3) Sift each sample using a 60-mesh sieve, collecting the bean powder in clean, labeled beakers.
4) Weight bean powders into labeled test tubes (in duplicate) the following amounts:

    a. Jordan Farms Black beans (62.5, 125, 250, 500 mg)
    b. Dream of Wild Health Black beans (62.5, 125, 250, 500 mg)
    c. Dream of Wild Health A beans (62.5, 125, 250, 500 mg)
    d. Jordan Farms Navy beans (125, 250, 500 mg)
    e. Dream of Wild Health Long White Good beans (125, 250, 500 mg)

5) Pipet 5 mL acetone/water/acetic acid (70:29.5:0.5) into each test tube.
6) Cap tubes and place in an orbital shaker at room temperature for 3 hours at 300 rpm. Cover the shaker with dark cloth before turning on.
7) Place tubes in a dark refrigerator at 4C for 12 hours.
8) Centrifuge extracts at 3000 RPM for 10 min at room temperature.
9) Remove supernatents using clean glass pipettes, being careful not to disturb the pellet, and place supernatents into clean, labeled test tubes.
10) Add 5 mL acetone/water/acetic acid (70:29.5:0.5) to the pellet and vortex for 3 min on high.
11) Centrifuge extracts at 3000 RPM for 10 min at room temperature.
12) Remove supernatents using clean glass pipettes, being careful not to disturb the pellet, and add this to the previous supernatent extract.
13) Store in the dark at 4C for use within 48 hours.

Appendix A-2

Method for DPPH Radical Scavenging Activity

Reference: Xu BJ, Yuan SH, Chang SK. (2007) Comparative analyses of phenolic composition, antioxidant capacity, and color of cool season legumes and other selected food legumes. J Food Sci 72(2): S167-77.

Solutions

1) 4mM Trolox standard solution
   a. In a test tube, dissolve 10 mg of Trolox powder in 10 mL acetone/water/acetic acid (70:29.5:0.5) (see appendix A-1).
   b. Vortex for 3 min, then displace oxygen in the tube using nitrogen gas.
   c. Wrap tube in aluminum foil and store at 4C until ready for use. Make fresh every two weeks.
2) 0.1mM DPPH solution
   a. Dissolve 12 mg of 2,2-Diphenyl-1-picryhydrazyl (DPPH) in 300 mL ethanol.
   b. Place on a stir plate and mix with a magnetic stir rod for 30 min, until all DPPH is completely dissolved. Make fresh daily.

Procedures

1) Preparation of standards
   a. Prepare 7 test tubes, each containing 12.5 mL of DPPH solution.
   b. Add Trolox solution in the following concentrations to the tubes, resulting in a standard series: 0, 25, 50, 75, 100, 125 and 150 μL.
   c. Vortex each tube for 1 min and then place tubes in the dark at room temperature for 30 min.
2) Sample preparation
   a. Prepare and label clean tubes for each bean concentration extracted in appendix A-1.
   b. Pipette 3.8 mL DPPH solution into each tube and 200 μL of the appropriate bean extract.
   c. Vortex each tube for 1 min and then place tubes in the dark at room temperature for 30 min.
3) Using a UV spectrophotometer, measure absorbance of standards and samples at 517 nm against an ethanol blank.
4) Calculations
   Reference: Miller HE, Rigelhof F, Marquart L, Prakesh A, Kanter M. (2000) Antioxidant content of whole grain breakfast cereals, fruits, and vegetables. J Am Coll Nutr 19: 312-19.

a. Using the Trolox absorbance readings, plot mg Trolox vs. absorbance and determine the zero intercept of the standard curve.
b. From the zero intercept, subtract the absorbance at 517 nm of each of your samples.
c. Plot weight in mg vs absorbance of each sample and determine the linear or logarithmic equation for each one.
d. Use this equation to calculate the weight needed to reduce the zero intercept by 50% ((Trolox Std. Curve Zero Intercept/2)-sample zero intercept)/slope.
e. Divide 200, 000 (0.50 mM) by the weight in mg calculated in the last step. This equals Trolox Units μM/100g.

Appendix A-3

Method for Determination of Total Phenolic Content

Reference: Xu BJ, Yuan SH, Chang SK. (2007) Comparative analyses of phenolic composition, antioxidant capacity, and color of cool season legumes and other selected food legumes. J Food Sci 72(2): S167-77.

Solutions

1) Gallic acid standard solution
   a. In a test tube, dissolve 10 mg of gallic acid powder in 10 mL acetone/water/acetic acid (70:29.5:0.5) (see appendix A-1).
   b. Vortex for 3 min, then displace oxygen in the tube using nitrogen gas.
   c. Wrap tube in aluminum foil and store at 4C until ready for use. Make fresh every two weeks.
2) Distilled water
3) Folin-Ciocalteu (FC) reagent (purchase prepared)
4) 7% NaCO3

Procedures

1) Preparation of standards
   a. Prepare 7 test tubes, each containing: 3 mL distilled water, 250 μL FC reagent, and 750 μL 7% NaCO3.
   b. Add gallic acid standard solution in the following concentrations to the tubes, resulting in a series: 0, 2.5, 5, 12.5, 25, 37.5 and 50 μL.
   c. Vortex each tube and allow to incubate for 8 min at room temperature in the dark.
   d. Bring each tube to 5 mL by adding distilled water.
   e. Vortex for 1 min and then place tubes in the dark at room temperature for 2 hours.
2) Sample preparation
   a. Prepare test tubes for each sample containing: 3 mL distilled water, 250 μL FC reagent, and 750 μL 7% NaCO3.
   b. Add 50 μL bean extract
   c. Vortex and allow to incubate for 8 min at room temperature in the dark.
   d. Add 950 μL distilled water to all tubes.
   e. Vortex for 1 min and incubate tubes in the dark at room temperature for 2 hours.
3) Using a UV spectrophotometer, measure absorbance of standards and samples at 765 nm against a reagent blank, which was composed of the same reagents except 50 μL distilled water was used instead of bean extract.

Appendix A-4

Method for Determination of Total Flavonoid Content

Reference: Xu BJ, Yuan SH, Chang SK. (2007) Comparative analyses of phenolic composition, antioxidant capacity, and color of cool season legumes and other selected food legumes. J Food Sci 72(2): S167-77.

Solutions

1) Catechin standard solution
   a. In a test tube, dissolve 10 mg of catechin powder in 10 mL acetone/water/acetic acid (70:29.5:0.5) (see appendix A-1).
   b. Vortex for 3 min, then displace oxygen in the tube using nitrogen gas.
   c. Wrap tube in aluminum foil and store at 4C until ready for use. Make fresh every two weeks.
2) 5% $NaNO_2$
3) 10% $AlCl_3 \bullet 6H_2O$
4) 1 M NaOH
5) Distilled water

Procedures

1) Preparation of standards
   a. Prepare 7 test tubes, each containing: 1250 μL distilled water, and 75 μL 5% $NaNO_2$.
   b. Add catechin standard solution in the following concentrations to the tubes, resulting in a series: 0, 2.5, 25, 62.5, 125, 187.5 and 250 μL.
   c. Wait 6 min.
   d. Add 150 μL 10% $AlCl_3 \bullet 6H_2O$ to each tube.
   e. Wait 5 min
   f. Add 500 μL 1M NaOH to each tube and bring total volume 2.5 mL using distilled water.
2) Preparation of samples
   a. Prepare a test tube for each sample containing 1250 μL distilled water, and 75 μL 5% $NaNO_2$.
   b. Add 250 μL bean extract.
   c. Wait 6 min
   d. Add 150 μL 10% $AlCl_3 \bullet 6H_2O$ to each tube.
   e. Wait 5 min.
   f. Add 500 μL 1M NaOH to each tube and bring total volume 2.5 mL using distilled water.

3) Using a UV spectrophotometer, measure absorbance of standards and samples at 510 nm against a reagent blank, which was composed of the same reagents except 250 μL distilled water was used instead of bean extract.

Appendix B Data Sets and SAS Codes

Appendix B-1

Individual Data

---------- type of bean measured=DWH Black ----------

Obs	Plot	Plant	DPPH	Flavonoids	Polyphenolics
1	2	3	14.24	3.69	3.96
2	1	4	7.96	3.02	3.28
3	1	5	8.88	4.28	3.63
4	2	6	18.70	5.46	4.82
5	2	7	12.15	3.39	3.59
6	1	8	10.93	5.77	4.39
7	2	9	8.53	3.96	2.78
8	2	10	13.39	2.53	4.24
9	1	11	12.33	3.20	3.14
10	2	12	16.44	4.78	3.87
11	2	13	12.93	4.33	3.42
12	1	14	16.27	3.25	3.31
13	1	15	21.00	5.49	3.99
14	1	16	19.15	5.16	4.17
15	2	17	.	.	.

---------- type of bean measured=Jordan Farms Black ----------

Obs	Plot	Plant	DPPH	Flavonoids	Polyphenolics
16	2	3	35.39	5.33	6.28
17	1	4	13.90	3.06	4.29
18	1	5	8.92	4.22	3.95
19	2	6	16.47	4.72	5.94
20	2	7	11.04	4.18	3.29
21	1	8	12.85	4.57	3.98
22	2	9	8.46	4.19	3.27
23	2	10	19.32	3.38	5.42
24	1	11	20.09	4.35	4.21
25	2	12	48.67	6.34	5.85
26	2	13	13.10	4.50	3.50
27	1	14	9.14	3.05	2.48
28	1	15	12.26	3.86	2.88
29	1	16	17.44	4.94	4.12
30	2	17	23.20	3.81	3.47

---------- type of bean measured=Jordan Farms Navy ----------

Obs	Plot	Plant	DPPH	Flavonoids	Polyphenolics
31	2	3	1.60	1.01	0.000
32	1	4	1.14	1.11	0.303
33	1	5	1.11	1.89	0.830
34	2	6	0.46	1.17	0.130
35	2	7	0.92	0.85	0.000
36	1	8	1.12	2.20	0.000

37	2	9	1.13	1.95	0.000
38	2	10	1.12	1.17	0.060

---------- type of bean measured=Jordan Farms Navy ----------
(continued)

Obs	Plot	Plant	DPPH	Flavonoids	Polyphenolics
39	1	11	1.16	0.94	0.02
40	2	12	0.75	2.17	0.14
41	2	13	0.79	1.91	0.63
42	1	14	0.80	0.84	0.33
43	1	15	0.42	1.77	0.00
44	1	16	1.10	2.28	0.92
45	2	17	0.39	0.48	0.00

---------- type of bean measured=Long White Good ----------

Obs	Plot	Plant	DPPH	Flavonoids	Polyphenolics
46	2	3	1.87	1.44	0.24
47	1	4	0.78	1.01	0.39
48	1	5	0.74	1.48	0.83
49	2	6	0.93	0.52	0.09
50	2	7	0.92	0.59	0.00
51	1	8	1.13	1.83	0.00
52	2	9	0.38	1.13	0.00
53	2	10	1.15	0.78	0.12
54	1	11	1.21	1.14	0.10
55	2	12	1.18	2.16	0.21
56	2	13	0.40	1.89	0.68
57	1	14	0.26	0.81	0.17
58	1	15	0.75	1.37	0.00
59	1	16	0.22	1.67	0.80
60	2	17	0.77	0.76	0.00

---------- type of bean measured=DWH A ----------

Obs	Plot	Plant	DPPH	Flavonoids	Polyphenolics
61	3	3	7.33	3.60	2.25
62	3	4	5.50	2.66	2.01
63	3	5	5.00	2.98	2.54
64	3	6	5.37	2.53	1.83
65	2	7	.	.	.
66	3	8	.	.	.
67	3	9	.	.	.
68	3	10	.	.	.
69	3	11	.	.	.
70	3	12	.	.	.
71	3	13	.	.	.

72	3	14	.	.	.
73	3	15	.	.	.
74	3	16	.	.	.
75	3	17	7.47	2.35	1.82

DESCRIPTIVE STATISTICS

---------- type of bean measured=DWH Black ----------

The MEANS Procedure

Variable	Label	N	Mean	Std Error	Std Dev
DPPH	trolox equivalence in umol/g	14	13.7785714	1.0940910	4.0937135
Flavonoids	catechin equivalence in mg/g	14	4.1650000	0.2777297	1.0391694
Polyphenolics	gallic acid equivalence in mg/g	14	3.7564286	0.1475612	0.5521233

Variable	Label	Minimum	Maximum
DPPH	trolox equivalence in umol/g	7.9600000	21.0000000
Flavonoids	catechin equivalence in mg/g	2.5300000	5.7700000
Polyphenolics	gallic acid equivalence in mg/g	2.7800000	4.8200000

---------- type of bean measured=Jordan Farms Black ----------

Variable	Label	N	Mean	Std Error	Std Dev
DPPH	trolox equivalence in umol/g	15	18.0166667	2.8280492	10.9529876
Flavonoids	catechin equivalence in mg/g	15	4.3000000	0.2218644	0.8592771
Polyphenolics	gallic acid equivalence in mg/g	15	4.1953333	0.3016366	1.1682335

Variable	Label	Minimum	Maximum
DPPH	trolox equivalence in umol/g	8.4600000	48.6700000
Flavonoids	catechin equivalence in mg/g	3.0500000	6.3400000
Polyphenolics	gallic acid equivalence in mg/g	2.4800000	6.2800000

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---------- type of bean measured=Jordan Farms Navy ----------

Variable	Label	N	Mean	Std Error	Std Dev
DPPH	trolox equivalence in umol/g	15	0.9340000	0.0862102	0.3338905
Flavonoids	catechin equivalence in mg/g	15	1.4493333	0.1531649	0.5932052
Polyphenolics	gallic acid equivalence in mg/g	15	0.2242000	0.0822141	0.3184140

Variable	Label	Minimum	Maximum
DPPH	trolox equivalence in umol/g	0.3900000	1.6000000
Flavonoids	catechin equivalence in mg/g	0.4800000	2.2800000
Polyphenolics	gallic acid equivalence in mg/g	0	0.9200000

DESCRIPTIVE STATISTICS

---------- type of bean measured=Long White Good ----------

The MEANS Procedure

Variable	Label	N	Mean	Std Error	Std Dev
DPPH	trolox equivalence in umol/g	15	0.8460000	0.1125497	0.4359030
Flavonoids	catechin equivalence in mg/g	15	1.2386667	0.1301862	0.5042090
Polyphenolics	gallic acid equivalence in mg/g	15	0.2420000	0.0764710	0.2961708

Variable	Label	Minimum	Maximum
DPPH	trolox equivalence in umol/g	0.2200000	1.8700000
Flavonoids	catechin equivalence in mg/g	0.5200000	2.1600000
Polyphenolics	gallic acid equivalence in mg/g	0	0.8300000

---------- type of bean measured=DWH A ----------

Variable	Label	N	Mean	Std Error	Std Dev
DPPH	trolox equivalence in umol/g	5	6.1340000	0.5237805	1.1712088
Flavonoids	catechin equivalence in mg/g	5	2.8240000	0.2196042	0.4910499
Polyphenolics	gallic acid equivalence in mg/g	5	2.0900000	0.1369306	0.3061862

Variable	Label	Minimum	Maximum
DPPH	trolox equivalence in umol/g	5.0000000	7.4700000
Flavonoids	catechin equivalence in mg/g	2.3500000	3.6000000
Polyphenolics	gallic acid equivalence in mg/g	1.8200000	2.5400000

Twoway Analysis of Variance

The GLM Procedure

Class Level Information

Class	Levels	Values
Beantype	5	DWH A DWH Black Jordan Farms Black Jordan Farms Navy Long White Good
Plot	3	1 2 3

Number of Observations Read 75
Number of Observations Used 64

Twoway Analysis of Variance

The GLM Procedure

Dependent Variable: DPPH trolox equivalence in umol/g

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	3574.902045	714.980409	22.57	<.0001
Error	58	1837.604530	31.682837		

Corrected Total	63	5412.506575

R-Square	Coeff Var	Root MSE	DPPH Mean
0.660489	69.20773	5.628751	8.133125

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Beantype	4	3505.387230	876.346808	27.66	<.0001
Plot	1	69.514815	69.514815	2.19	0.1440

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Beantype	3	3494.428810	1164.809603	36.76	<.0001
Plot	1	69.514815	69.514815	2.19	0.1440

Twoway Analysis of Variance

The GLM Procedure

Dependent Variable: Flavonoids catechin equivalence in mg/g

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	123.7258160	24.7451632	42.45	<.0001
Error	58	33.8092699	0.5829184		
Corrected Total	63	157.5350859			

R-Square	Coeff Var	Root MSE	Flavonoids Mean
0.785386	27.56752	0.763491	2.769531

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Beantype	4	123.7095493	30.9273873	53.06	<.0001
Plot	1	0.0162667	0.0162667	0.03	0.8679

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Beantype	3	123.6210197	41.2070066	70.69	<.0001
Plot	1	0.0162667	0.0162667	0.03	0.8679

Twoway Analysis of Variance

The GLM Procedure

Dependent Variable: Polyphenolics gallic acid equivalence in mg/g

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	209.1949903	41.8389981	94.44	<.0001
Error	58	25.6961536	0.4430371		
Corrected Total	63	234.8911439			

R-Square	Coeff Var	Root MSE	Polyphenolics Mean
0.890604	32.03828	0.665610	2.077547

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Beantype	4	208.7989847	52.1997462	117.82	<.0001
Plot	1	0.3960056	0.3960056	0.89	0.3484

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Beantype	3	209.0138275	69.6712758	157.26	<.0001
Plot	1	0.3960056	0.3960056	0.89	0.3484

The GLM Procedure

Duncan's Multiple Range Test for DPPH

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	58
Error Mean Square	31.68284
Harmonic Mean of Cell Sizes	10.60606

NOTE: Cell sizes are not equal.

Number of Means	2	3	4	5
Critical Range	4.893	5.147	5.314	5.436

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	Beantype

A	18.017	15	Jordan Farms Black
A			
A	13.779	14	DWH Black
B	6.134	5	DWH A
C	0.934	15	Jordan Farms Navy
C			
C	0.846	15	Long White Good

Twoway Analysis of Variance

The GLM Procedure

Duncan's Multiple Range Test for Flavonoids

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	58
Error Mean Square	0.582918
Harmonic Mean of Cell Sizes	10.60606

NOTE: Cell sizes are not equal.

Number of Means	2	3	4	5
Critical Range	.6637	.6981	.7209	.7374

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	Beantype
A	4.3000	15	Jordan Farms Black
A			
A	4.1650	14	DWH Black
B	2.8240	5	DWH A
C	1.4493	15	Jordan Farms Navy
C			
C	1.2387	15	Long White Good

Twoway Analysis of Variance

The GLM Procedure

Duncan's Multiple Range Test for Polyphenolics

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha 0.05
Error Degrees of Freedom 58
Error Mean Square 0.443037
Harmonic Mean of Cell Sizes 10.60606

NOTE: Cell sizes are not equal.

Number of Means	2	3	4	5
Critical Range	.5786	.6086	.6284	.6428

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	Beantype
A	4.1953	15	Jordan Farms Black
A			
A	3.7564	14	DWH Black
B	2.0900	5	DWH A
C	0.2420	15	Long White Good
C			
C	0.2242	15	Jordan Farms Navy

Twoway Analysis of Variance

The GLM Procedure

Duncan's Multiple Range Test for DPPH

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha 0.05
Error Degrees of Freedom 58
Error Mean Square 31.68284
Harmonic Mean of Cell Sizes 11.19518

NOTE: Cell sizes are not equal.

Number of Means	2	3
Critical Range	4.762	5.010

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	Plot
A	9.251	31	2
A			

A	7.252	28	1
A			
A	6.134	5	3

Twoway Analysis of Variance

The GLM Procedure

Duncan's Multiple Range Test for Flavonoids

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	58
Error Mean Square	0.582918
Harmonic Mean of Cell Sizes	11.19518

NOTE: Cell sizes are not equal.

Number of Means	2	3
Critical Range	.6460	.6795

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	Plot
A	2.8240	5	3
A			
A	2.8057	28	1
A			
A	2.7281	31	2

Twoway Analysis of Variance

The GLM Procedure

Duncan's Multiple Range Test for Polyphenolics

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	58
Error Mean Square	0.443037
Harmonic Mean of Cell Sizes	11.19518

NOTE: Cell sizes are not equal.

Number of Means	2	3
Critical Range	.5632	.5924

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	Plot
A	2.1290	31	2
A			
A	2.0900	5	3
A			
A	2.0183	28	1

Correlation between antioxidant polyphenolics and flavonoids

The CORR Procedure

3 Variables: DPPH Flavonoids Polyphenolics

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
DPPH	64	8.13313	9.26892	520.52000	0.22000	48.67000
Flavonoids	64	2.76953	1.58131	177.25000	0.48000	6.34000
Polyphenolics	64	2.07755	1.93091	132.96300	0	6.28000

Simple Statistics

Variable	Label
DPPH	trolox equivalence in umol/g
Flavonoids	catechin equivalence in mg/g
Polyphenolics	gallic acid equivalence in mg/g

Pearson Correlation Coefficients, N = 64
Prob > |r| under H0: Rho=0

	DPPH	Flavonoids	Polyphenolics
DPPH	1.00000	0.83212	0.87739
trolox equivalence in umol/g		<.0001	<.0001
Flavonoids	0.83212	1.00000	0.90952
catechin equivalence in mg/g	<.0001		<.0001
Polyphenolics	0.87739	0.90952	1.00000
gallic acid equivalence in mg/g	<.0001	<.0001	