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The Effects of Processing on Hydrophilic Antioxidant Capacity of Black Beans

Elizabeth Roberson McGee
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Elizabeth Roberson McGee entitled "The Effects of Processing on Hydrophilic Antioxidant Capacity of Black Beans." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

John Mount, Major Professor

We have read this thesis and recommend its acceptance:

P. Michael Davidson, Lana Zivanovic

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Anne Mayhew
Vice Chancellor and
Dean of Graduate Studies

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The Effects of Processing on Hydrophilic Antioxidant Capacity of Black Beans

A Thesis Presented
for the Master of Science Degree
The University of Tennessee, Knoxville

Elizabeth R. McGee
December, 2006

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ABSTRACT

MCGEE, ELIZABETH R. The Effects of Processing on Hydrophilic Antioxidant Capacity of Black Beans. (Under the direction of Dr. John Mount.)

Dry, uncooked black beans have been found to contain a significantly high amount of antioxidants compared to many other foods. There is little information available on the influence of thermal processing on antioxidant capacity of foods. The antioxidant capacity, reported in μmol Trolox Equivalents per gram of uncooked, blanched, or retorted black beans was evaluated using the oxygen radical absorbance capacity (ORAC) assay. A boiling water blanch of black beans for 30, 60 or 90 sec resulted in a 33% reduction in antioxidant capacity compared to uncooked beans. There was no significant difference among antioxidant capacity of beans blanched for the varying times. There was a significant effect of thermal processing black beans at 110° C for 10 and 30 min. Compared to uncooked beans (229.18 μmol TE/g), there was a decrease of 91% in antioxidant capacity for cooked beans (19.13 μmol TE/g). The 30 min cook time gave a significantly lower antioxidant capacity than the 10 min ($p<0.01$). When antioxidant capacity of black beans was calculated on a dry weight basis, it was shown that the difference in antioxidant capacity was due to an increased moisture uptake during the longer cook time. Increased water absorption decreased the amount of antioxidants per gram. Containers to be retorted were each filled with 100g of black beans and 150, 186 or 233 ml of water. Black beans filled with less water had significantly less reduction in antioxidant capacity than the other two fill ratio samples. This was determined to be due to loss of materials from beans that resulted in lower antioxidant capacities. Two commercial samples of black beans, one processed in a retort

without agitation and one processed in an agitating retort at a higher temperature but for a shorter time were found to contain similar antioxidant capacity. Samples were cooked to similar moisture contents.

Black beans that are thermally processed contain reduced antioxidant capacity per 100g due to water absorption during blanching. There is continued moisture increase during retorting but there is also up to 80% antioxidant capacity lost from the solid material in black beans during the two types of thermal processing.

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I. INTRODUCTION

Black beans (*Phaseolus vulgaris* L.) are an important food staple in many countries. They are a good source of protein, rich in dietary fiber, and a low glycemic index food (Hangen and Bennink 2002). Consumption of black beans may also reduce the risk of colon cancer and heart disease (Bazzano and others 2001; Hangen and Bennink 2002), which may be attributed to the antioxidant compounds present in the beans. It has been found that the darker the bean seed coat, the higher the level of antioxidants (Choung and others 2003). Anthocyanins discovered in black beans have been shown to possess antioxidant activity (Kahkonen and Heinonen 2003; Wang and others 1997; Wu and Prior 2005).

Food antioxidants are thought to provide a defense against oxidative stress, which has been associated with the development of many diseases. Reactive oxygen species can damage biological molecules such as proteins, lipids, and DNA (Wu and others 2004b). Antioxidant capacity assays allow the analysis of a food based on its ability to prevent reactive oxygen species damage. Antioxidant capacity has been defined as the ability of a compound to reduce pro-oxidants (Prior and Cao 1999). The oxygen radical absorbance capacity (ORAC) assay measures the antioxidant capacity of food samples in a free radical generating system. ORAC is the only assay that takes reactive species to completion and uses an area under the curve (AUC) technique (Cao and others 1995). Numerous foods have been analyzed using the ORAC assay, and the results may be used for comparisons between foods (Ou and others 2002; Prior and others 2005).

In a commercial canning operation, black beans must be blanched to remove intercellular gasses. This is usually accomplished through a water blanch. Beans can be thermally processed either in an agitating or non-agitating retort. Unfortunately, there is little information on the effect of thermal processing on retention of antioxidants or antioxidant capacity in canned black beans. Using the ORAC assay to study antioxidants provides a better understanding of the antioxidant capacity of black beans. This research utilizes the hydrophilic oxygen radical antioxidant capacity (H-ORAC) assay to analyze the antioxidant capacity of processed black beans. The objectives of this study are:

1. To determine the effect of water blanching on antioxidant capacity of black beans.
2. To compare the effect of differing solid to liquid fill ratios on the antioxidant capacity of thermally processed black beans.
3. To determine the effect of cooking at 110^{o}C for 10 or 30 min on the antioxidant capacity of black beans.
4. To compare the effect of agitating verses non-agitating thermal processing on the antioxidant capacity of commercially canned black beans.

II. REVIEW OF LITERATURE

Anthocyanin Chemistry

Anthocyanins form one of the major groups of natural pigments and are responsible for the colors of several fruits, vegetables, and flowers (Rivas-Gonzalo 2003; Strack and Wray 1994). A wide range of plant colors are attributed to anthocyanins including blue, purple, violet, magenta, red, and orange. The word anthocyanin comes from Greek *anthos*, which means flower, and *kyanos*, which means blue (Mazza and Miniati 1993). Food plants that contain anthocyanins include fruits, such as raspberries, blackberries, and blueberries, legumes, such as black beans and lentils, bulbs, such as garlic and red onions, and cereals, such as barley and wheat. Anthocyanins are common in food plants occurring in at least 27 families and 73 genera (Bridle and Timberlake 1997).

Because of their $C_6C_3C_6$ carbon skeleton, anthocyanins are considered a subclass of flavonoids (Figure 1; all figures are in the "Appendix"). The basic structure for anthocyanins is the 2-phenylbenzopyrylium of the flavylium salt. Anthocyanins are glycosides of polyhydroxy or polymethoxy derivatives of the flavylium cation (Fennema 1996). They can differ in the number of hydroxyl or methoxy groups, the nature and number of sugars attached to the molecule, the position of the attachment of the sugar, and the nature and number of aliphatic or aromatic acids attached to the sugars in the molecule. (Fennema 1996; Mazza and Miniati 1993).

Several forms of anthocyanins exist. Anthocyanosides are anthocyanins bound to sugars. Polymers of anthocyanins are referred to as "proanthocyanidins." When the sugar

portion of an anthocyanin is hydrolyzed, an aglycone called an “anthocyanidin” is formed (Macz-Pop and others 2006).

Several anthocyanidins are contained in anthocyanins found in nature. However, only six anthocyanidins are commonly found in plants: cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin (Figure 2) (Fennema 1996). Cyanidin is the most common of the anthocyanidins (Merken and Beecher 2000). These anthocyanidins are based on the same 2-phenylbenzopyrylium skeleton hydroxylated at the 3, 5, and 7 positions. They differ in the number and location of hydroxyl and methoxyl groups in the B-ring. From these anthocyanidins, a number of anthocyanins can be created, differing in the nature and number of sugars attached to the aglycone and the position of the attachment. Anthocyanins also differ in the type and amount of aliphatic or aromatic acids attached to the sugar residues (Rivas-Gonzalo 2003).

Function of Anthocyanins

The most important function of anthocyanins is to provide color to the plant in which they are present. Color is an important characteristic of food. It is used as an identification tool and to determine quality. Color also contributes to aesthetic appeal and can impact our desire for a food. Anthocyanins in plants help attract insects and animals, which results in pollination and seed dispersal (Strack and Wray 1994).

The presence of anthocyanins in plant leaves protects against ultraviolet radiation damage. Anthocyanins have also been reported to contribute to pathogen resistance in plants (Mazza and Miniati 1993). Anthocyanins along with other flavonoids, may also be important factors in the resistance of insect attack on plants (Harborne 1994).

Stability of Anthocyanins

Anthocyanins are subject to degradation. Several factors play a role in the extent of degradation of anthocyanins.

Structural Transformation

Several structures exist for anthocyanins which can significantly influence their degradation rate. Typically, increasing hydroxylation decreases stability and increasing methylation will increase stability. For example, the color of foods is less stable for those containing anthocyanins with high concentrations of cyanidin, delphinidin, or pelargonidin aglycones compared to those with high concentrations of malvidin or petunidin aglycones (Fennema 1996). Glycosylation and acylation of the sugars of anthocyanins increases stability. Therefore, diglycosides are more stable than monoglycosides (Rivas-Gonzalo 2003).

pH

Anthocyanin structure and therefore color stability is affected by pH. The effect of pH on anthocyanin stability was first investigated in the 1960s (Harper 1967; Jurd 1963). Due to their amphiprotic nature, anthocyanins can act somewhat like pH indicators (Jackman and others 1987). Below pH 3, most anthocyanin solutions display red coloration. As solution pH increases, a rapid proton loss occurs, color fades, and at pH 4 to 6 most anthocyanins appear colorless. Upon further increase in pH, solutions are purple and blue but can change from blue to yellow upon heat treatment or prolonged storage (Iacobucci and Sweeny 1983; Jackman and others 1987; Mazza and Miniati

1993). The fact that the color differs with changes in pH indicates one disadvantage when considering anthocyanins as food colorants (Timberlake 1980).

Light

Light has opposite effects on anthocyanins. It aids in biosynthesis but also increases degradation. As to the biosynthetic role, immature apples of the red varieties that are left to ripen in darkness will remain green . The adverse effect of light has been illustrated in many fruit products. For example, it was found that colorants extracted from grape pomace and formulated in a carbonated beverage showed accelerated degradation when exposed to light (Palamidis and Markakis 1975). Bokowska and others found in 2003 that cyanidin was degraded by sunlight. Loss of cyanidin-3-glucoside after 3 months of storage in the sun was about 54% more than that of the same chemical stored in the dark for the same time (Bakowska and others 2003). These results agree with those of Ochoa and others who found that samples of raspberry and sweet cherry preserves kept in the presence of light illustrated a greater decrease in the concentration of pigments than those kept in the dark (Ochoa and others 2001). The type of anthocyanin influences light degradation. In wine, acylated diglucosides were the most stable and monoglucosides were least stable. The nonacylated diglucosides were intermediate stability (Vanburen and others 1968).

Temperature

The rate of anthocyanin degradation is influenced significantly by processing and storage temperatures (Ioncheva and Tanchev 1974; Jackman and others 1987; Markakis 1982). For example, Kirca and others found that refrigerated storage (4° C) of black

carrot anthocyanins markedly reduced degradation as compared to carrots stored at 37° C (Kirca and others 2006). In contrast, Meschter found a half-life of only 1 hr for strawberry preserve pigments heated to 100° C (Meschter 1953). Markakis and others found similar results and recommended that the best process for strawberry pigment retention is a short time/high temperature process (Markakis and others 1956).

Enzymes

Enzymatic systems that have the ability to destabilize or decolorize anthocyanins have been found in the leaves, roots, and fruits of plants, and even in fungi (Cevahir and others 2004; Forsyth and Quesnel 1957; Peng and Markakis 1963; Pifferi and Cultrera 1974; Vicente and others 2006; Zhang and others 2005). The enzymatic discoloration has been attribute to glycosidases (Forsyth and Quesnel 1957; Huang 1955) and polyphenol oxidases (Peng and Markakis 1963; Siddiq and others 1994).

Glycosidases hydrolyze the anthocyanins to anthocyanidins and sugar moieties (Francis 1989; Huang 1955; Markakis 1982). Huang found that liberated anthocyanins changed to colorless derivatives. He also noted that the enzymatic de-glycosylation of anthocyanins from fungal glycosidase was found to follow first order kinetics at pH 3.95 and 30° C (Huang 1956).

Polyphenol oxidase (PPO) enzymes are detrimental to anthocyanins, although anthocyanins are not acted directly upon by PPO. PPO oxidizes ortho-hydroxyphenols such as caffeic acid to ortho-quinones, which degrade anthocyanins through oxidation or condensation reactions (Francis 1989). Kader and others found that in a model system containing PPO and blueberry anthocyanins, there was a 29% loss of color (Kader and

others 1997). PPO enzymes have a low affinity for anthocyanins but the reaction is usually pronounced when other phenolics are present. Jiang reported that the presence of phenolic extract stimulated the oxidation of pigment in lychee, and that anthocyanin-PPO-phenol reactions are responsible for browning of lychee pericarp (Jiang 2000).

Enzyme degradation of anthocyanins may be prevented. Red tart cherries that were steam-blanched for 45-60 seconds prior to freezing showed minimal anthocyanin destruction (Siegel and others 1971). Red orange fruit juice stability was increased through pasteurization (Markakis 1982). Anthocyanin degradation by enzymes also can be retarded through pH reduction or by refrigeration (3-5° C) (Kalt and others 1999).

Oxygen and Hydrogen Peroxide

It was found in the 1930s that oxygen had an effect on degradation of anthocyanins. Tressler and Pederson found that by filling grape juice bottles with no headspace, color change from purple to brown could be prevented (Tressler and Pederson 1936). Processing anthocyanin-pigmented fruit juice under nitrogen or vacuum to remove oxygen also prevents color change (Clydesdale and others 1978; Daraving.G and Cain 1965).

Hydrogen peroxide (H_2O_2) has been shown to be responsible for degradation of anthocyanins. The effect of high and low H_2O_2 concentrations on strawberry, pomegranate, and sour cherry anthocyanins was studied by Ozkan and others (Ozkan and others 2005). Degradation was first-order kinetics and at high H_2O_2 concentrations and strawberry anthocyanins showed the highest susceptibility to H_2O_2, followed by pomegranate and sour cherry anthocyanins.

Mechanical Processing

Anthocyanins are not uniformly distributed in plant tissue. The skin or peel contains high amounts of anthocyanins (Tomas-Barberan and Espin 2001). Food fractionation may result in a loss of some polyphenolic compounds (Scalbert and Williamson 2000). For example, polyphenols in wheat grain are principally contained in the outer layers (seed coat) and are lost during the refining of flour (Shahidi and Naczk 1995).

Anthocyanin Extraction

The most common method for anthocyanin extraction employs methanol or ethanol solutions acidified with different concentrations (usually 0.1-1.0%) of HCl (Rivas-Gonzalo 2003; Shahidi and Naczk 1995). Depending on the nature of the sample, water may need to be added in order to obtain complete extraction of anthocyanins (Rivas-Gonzalo 2003). Water and alcoholic solvents are useful when extracting because they are transparent in the visible region, aiding in analyses such as UV-visible spectroscopy (Brouillard 1983). It has been reported that HCl may produce hydrolysis of highly acylated anthocyanins. Therefore, more gentle acid conditions may be preferred. Some acids that may be used are acetic, tartaric, citric, or formic acids (Bakker and others 1997; Strack and Wray 1994). Cho and others used an extraction solution containing methanol/water/formic acid at a ratio of 60:37:3 by volume to extract blackberries, blueberries, and red grapes. The extracts were used to examine flavonol glycosides and hydrophilic antioxidant capacity using the hydrophilic oxygen radical absorbance capacity assay (H-ORAC). Samples were filtered through Miracloth™ and evaporated to

dryness, then re-suspended in an acetic acid solution (Cho and others 2004; Cho and others 2005). Pigment degradation may occur as formic acid is concentrated during evaporation of the methanol-formic acid solvent (Kong and others 2003). Lam and others analyzed fruit extracts including blueberries, blackberries, and wine grapes for antioxidant capacity using the H-ORAC method. Their extraction method was similar to that of Cho and others, using methanol/water/formic acid at a ratio of 60:37:3 by volume and filtering through Miracloth™, however they did not evaporate or concentrate their samples (Lam and others 2005).

Antioxidant Capacity Assays

Definition and Importance of Antioxidant Capacity Measurement

Antioxidant capacity has been defined as the ability of a compound to reduce pro-oxidants (Prior and Cao 1999). Antioxidant assays, which utilize biological free radical sources, are used to find the relative antioxidant capacity of dietary components. Genetics, harvest maturity, growing conditions, storage conditions, and processing all have an effect on antioxidant levels in foods. By measuring the antioxidant activity, it can be determined what conditions may promote or interfere with a food's health benefits that are provided by antioxidants. These assays may also be used to compare different types of foods.

Methods for measuring total antioxidant capacity of a biological sample have been classified as inhibition methods involving reactive species (Rice-Evans and Miller 1994). The three methods developed most recently to measure total antioxidant capacity include FRAP (ferric reducing ability of plasma, also known as ferric reducing

antioxidant power), TEAC (Trolox equivalent antioxidant capacity), and ORAC (oxygen radical absorbance capacity). The ORAC method, and comparisons between ORAC, TEAC, and FRAP are discussed below.

History of ORAC Assay

In 1990, Glazer published a method for analysis of reactive oxygen species. This method measures the decrease in fluorescence of B- or R-phycoerythrin (PE) in the presence of reactive species and relates a lag phase to antioxidant capacity of an antioxidant sample (Glazer 1990).

The ORAC method, based on the research of Glazer, was first developed in 1992 by Cao, Alessio, and Cutler. In this assay system, B-phycoerythrin (B-PE) was an indicator protein, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was used as a peroxyl radical generator, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble vitamin E analogue) as a control standard. Results were given as ORAC units, where 1 unit represented the net protection produced by 1 μmol Trolox (Cao and others 1993). While the method was unique because it took the oxidation reaction to completion, it lacked automation.

In 1995, Cao and others worked to semi-automate the ORAC assay. This was done by equipping the COBAS FARA II centrifugal analyzer with a fluorescence-measuring attachment. B-PE, AAPH, and Trolox were still used in the assay. This technique combines both inhibition time and inhibition percentage by antioxidants against reactive species into a single quantity. The ORAC assay takes reactive species to completion, using an area under the curve (AUC) technique (Cao and others 1995).

ORAC values were expressed as micromoles of Trolox equivalent (TE) per liter or per gram of sample (μmol TE/l or μmol TE/g).

The COBAS FARA II is no longer available because it was discontinued by the manufacturer. The method developed in 1995 has other limitations such as interactions between B-PE and antioxidants, and an inability to measure lipophilic antioxidants. Another drawback of this method is the COBAS FARA II can only analyze nine samples of a single concentration per run, so repeated runs are needed until a satisfactory result can be determined (Huang and others 2002b).

In 2001, Ou and others (Ou and others 2001)developed and validated an ORAC assay which replaced the original fluorescent probe, B-PE, with fluorescein (3',6'-dihydroxyspiro[isobenzofuran-1[3*H*],9'[9*H*]-xanthen]-3-one). Compared to B-PE, fluorescein (FL) does not interact with antioxidants, shows improved photostability, gives consistent results from lot to lot, and reduces the cost of experiments. The ORAC assay using fluoroscein is referred to as the $ORAC_{FL}$ assay.

Full automation for the $ORAC_{FL}$ assay using a multichannel liquid handling system on-line with a microplate fluorescent reader in 48 or 96-well format was described by Huang and others in 2002. It was shown that the automated platform could analyze at least 130 samples per day without repeat runs (Huang and others 2002b).

In 2002, Huang and others (Huang and others 2002a) also developed and validated an ORAC assay for lipophilic antioxidants which used randomly methylated β-cyclodextrin (RMCD) as the solubility enhancer. It was found that the improved $ORAC_{FL}$ assay (equipped with the microplate fluorescent reader) could be successfully used with lipid soluble antioxidants. The updated ORAC method could now measure lipophilic

antioxidant capacity (L-$ORAC_{FL}$) and hydrophilic antioxidant capacity (H-$ORAC_{FL}$) to determine total antioxidant capacity. To date, ORAC is still the only assay that takes reactive species to completion and uses an area under the curve (AUC) technique (Cao and others 1995).

Basic Principle of the ORAC Assay

Antioxidant capacity (AOC) is the ability of a compound to reduce or inhibit pro-oxidants (Cao and Prior 1999). The most widely used ORAC assays measure the scavenging capacity of antioxidants against the peroxyl radical, one of the most common reactive oxygen species in the human body (Bank and Schauss 2004). The H-$ORAC_{FL}$ assay measures water soluble AOC and the L-$ORAC_{FL}$ assay measures lipid soluble AOC. Combining the results of these two assays gives total antioxidant capacity.

The principle behind the ORAC assay is as follows. An extracted sample containing potential antioxidants is added to a free radical generating system. The inhibition of the free radical action is measured over time and the results are related to the antioxidant capacity of the sample. For the $ORAC_{FL}$ assay, AAPH generates the free radicals and FL is used as an indicator of free radical attack. The change of fluorescent intensity shows the degree of free radical damage. The inhibition of free radical damage by an antioxidant, shown by protection against the change of fluorescence over time in the $ORAC_{FL}$ assay, is a measure of its antioxidant capacity against the free radicals (Huang and others 2002b). The net AUC for standards and samples is calculated according to the following equation.

$$AUC = (0.5 + f_5/f_4 + f_6/f_4 + f_7/f_4 + \ldots + f_i/f_4) * CT$$

Where:

f_4 = initial fluorescence reading at cycle 4

f_i = fluorescence reading at cycle *i*

CT = the cycle time in minutes

The net AUC is found by subtracting the AUC of the blank from the AUC of the standard or sample (Prior and others 2003). Figure 3 illustrates the AUC. Final ORAC values are calculated by obtaining a standard curve, using a regression equation ($Y = a + bX$ for linear; or $Y = a + bX + cX^2$ for quadratic) between Trolox concentration (Y; μM) and the net area under the FL decay curve (X). Regressions used are in the range of 6.25-50 μM Trolox. ORAC values are found by calculating the Trolox equivalents of a sample using the standard curve. Antioxidant capacity units for this assay are expressed as TE/g or TE/l which represents μmol of Trolox equivalents (TE) per gram or liter of sample (Huang and others 2002b).

Comparison of ORAC to Other AOC Assays

Ferric reducing ability of plasma, or FRAP was introduced as a method for assessing "antioxidant power" by Benzie and Strain in 1996 (Benzie and Strain 1996). Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. The values for the FRAP assay, expressed as μmol Fe^{2+}/L, are found by comparing the absorbance change at 593 nm in test reaction mixtures with mixtures containing ferrous ions in known concentration. Benzie concluded that the FRAP assay gives fast reproducible results with plasma from healthy Chinese adults, with single antioxidants in pure solution, and with mixtures of antioxidants in aqueous solution.

The Trolox equivalent antioxidant capacity, or TEAC assay is based on antioxidant inhibition of the radical cation 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), also known as ABTS. Trolox is the standard. Antioxidant capacity is measured as the ability of test compounds to decrease color by reacting directly with the ABTS radical. This measurement is made at a fixed point in time, or can be made over a time period and is often reported in μmol Trolox equivalent per gram (μmol TE/g) or per liter (μmol TE/L). The original TEAC assay uses ABTS and H_2O_2 to generate ABTS radical cations in the presence of metmyoglobin as a peroxidase (Miller and others 1993). Re and others modified this method by generating the ABTS cation through a reaction between ABTS and potassium persulfate (Re and others 1999). The extent of ABTS cation absorbance inhibition was plotted as a function of concentration to determine TEAC, which was determined as a function of time. Since the ABTS radical cation can be dissolved aqueous and acidified ethanol medium, this assay is capable of testing the antioxidant activity of both hydrophilic and lipophilic compounds (Arnao and others 2001).

In 1998, Cao and Prior published a study which compared different analytical methods to assess total antioxidant capacity of human serum (Cao and Prior 1998). The ORAC method used was that of Cao and others in 1992, which required the COBAS FARA II spectrofluorometric analyzer and B-PE. The FRAP assay was also carried out on a COBAS FARA II spectrophotometric analyzer following the 1996 method of Benzie and Strain. The final FRAP results were converted to μmol TE/L in order to compare them to the other assays, which were also in units of μmol TE/L. A COBAS MIRA spectrophotometric analyzer was used for the TEAC assay of Rice-Evans and Miller.

There was a weak linear correlation between serum ORAC and serum FRAP. There was no correlation either between serum ORAC and serum TEAC or between serum FRAP and serum TEAC. Cao and Prior stated that the FRAP assay is simple and inexpensive but does not measure the SH-group-containing antioxidants whereas the ORAC assay is highly specific and responds to several antioxidants. In 1999, Cao and Prior concluded that the ORAC activity against peroxide radicals is more sensitive than TEAC and FRAP assays (Cao and Prior 1999). It is important to note that these results were based on studies of human serum, not food products. Also, the COBAS FARA II and R-PE were used for the ORAC assay, which was updated to the $ORAC_{FL}$ in 2002.

Ou and others (2002) published a comparative study of antioxidant activities of common vegetables using the ORAC and FRAP assays. This study was conducted on freeze-dried vegetable samples and the FRAP and ORAC results were expressed as micromoles of Trolox equivalents per gram on a dried basis (μmol TE/g, db). Cao's ORAC method from 1992 which required COBAS FARA II was used, and FL was the fluorescent probe. Both the ORAC and FRAP methods showed values for fruits and vegetables that varied depending on cultivar, locations, weather conditions, and harvest periods. The researchers attempted to rank the antioxidant activity of the vegetables but the ORAC and FRAP data covered a broad range and overlapped significantly among different vegetables and therefore, they could not draw a clear conclusion as to rank. FRAP and ORAC values did not correlate well. It was concluded that the ORAC method is chemically more relevant to chain-breaking antioxidant activity, while FRAP has some drawbacks such as interference, reaction kinetics, and quantitation method (Ou and others 2002).

Discussion of TEAC, FRAP and ORAC Findings

ORAC, TEAC, and FRAP are among the more popular methods of measuring antioxidant capacity. These assays have been covered in several studies over the years; however the methods used differed depending on when the research was conducted. In order to provide a better understanding of the comparative studies discussed earlier, this section focuses on the principles and mechanisms of the antioxidant capacity assays and relates them to the conclusions from various research studies.

Antioxidant reactions are typically chain reactions which involve initiation, propagation, branching, and termination of free radicals. From a mechanistic standpoint, there are two categories of antioxidants: preventive and chain-breaking. Preventive antioxidants inhibit formation of reactive oxygen species (initiation). Chain-breaking antioxidants scavenge oxygen radicals (propagation and branching) and thereby break radical chain sequences. The TEAC and FRAP methods are based on singlet electron transfer (SET) reaction between an oxidant and a free radical and the ORAC method is based on a hydrogen atom transfer (HAT) reaction (Ou and others 2002). Antioxidants are oxidized by the ABTS cation for the TEAC method and by Fe (III) for the FRAP method. In these SET-based reactions, a single electron is transferred from the antioxidant molecule to the oxidant, causing a change in absorbance which is used to quantify AOC. The ORAC's HAT-based method uses AAPH to generate a peroxyl radical that abstracts a hydrogen atom from an antioxidant, inhibiting reaction between the peroxyl radical and FL.

Wright and others conducted a study to try to determine which mechanism, HAT or SET, is most important in the reactions of phenolic antioxidants with free radicals.

Both HAT and SET mechanisms can occur in parallel, but at different rates so the study was to determine which had the faster rate. The procedure was based on density functional theory to calculate the bond dissociation enthalpy (BDE) and ionization potential for phenolics antioxidants. Their results illustrated that HAT will be dominant in most cases of phenolics antioxidants. Ou and others also feel the HAT process is more important because oxygen is hydrogenated and the reductants are dehydrogenated. The ORAC method, based on HAT reaction which plays a dominant role in biological reactions, is more closely related to biological functions of chain-breaking antioxidants (Ou and others 2001; Ou and others 2002). TEAC and FRAP are based on the SET mechanism, and as a result neither actually measures chain-breaking antioxidant activity or preventive antioxidant activity (Ou and others 2001).

Assays that do not consider inhibition time are not ideal because two compounds having the same inhibition percentage at one time point may reveal different inhibition percentages at another point in time. This was shown by ascorbate and urate in the TEAC assay and also by Trolox and glutathione in the ORAC assay (Cao and Prior 1999). This illustrates an advantage of the ORAC assay and the TEAC assay (when measuring inhibition over time) in that they take the reactive species to completion, combining inhibition time and percentage, rather than focusing on inhibition at one fixed point in time. The FRAP assay fixes the reaction time at 4 minutes (Benzie and Strain 1996).

The radical source used in an assay can have significant effects on the antioxidant capacity due to the response of different types of antioxidant compounds to that radical source (Cao and others 1996). Because of this variation in response, the use of radical sources that are relevant to human biology becomes important when analyzing food

products. The peroxyl radical, analyzed by ORAC, is the most common free radical in human biology (Bank and Schauss 2004). ABTS, used in the TEAC assay of food antioxidant capacity, is a radical that is foreign to biology (Wu and others 2004b) . The TEAC assay does not measure the radical scavenging activity of the food sample, just the ability of the sample to reduce the ABTS cation. The FRAP assay involves electron transfer using a ferric salt with a redox potential similar to that of ABTS (Ou and others 2002; Pellegrini and others 2003). The FRAP assay is based on the reduction of a ferric trypyridyltriazine [Fe(III)-$(TPTZ)_2$] complex by an antioxidant to the ferrous tripyridyltriazine [Fe (II)- $(TPTZ)_2$] at the non-physiological pH of 3.6. Also, there is no oxygen radical involved in either the TEAC or FRAP assay, therefore the results do not necessarily represent antioxidant activities (Ou and others 2001). ORAC activity against peroxide radicals is considered to be more sensitive than TEAC and FRAP assays because the final standard concentration required is much lower than those required in TEAC and FRAP (Cao and Prior 1999).

The total antioxidant capacity, as measured by $ORAC_{FL}$ or any other *in vitro* methods, may not accurately reflect *in vivo* antioxidant effects. Other issues such as absorption, metabolism and physicochemical properties of different antioxidants are also very important to consider (Wu and others 2004b).

Black Beans

History

Black beans, along with pinto, navy, and kidney beans are all varieties of the genus and species *Phaseolus vulgaris*. These varieties are all derived from a common

bean ancestor originating in Peru. From Peru, they were carried to Central and South America by migrating Indian tribes (Ensminger 1994; Fortin 1996).

In the 15th century, beans were brought to Europe by Spanish explorers returning from their voyages to the New World. Spanish and Portuguese traders spread beans throughout Africa and Asia (Heriteau 1978). Black beans are an important commodity in Mexico, Brazil, Guatemala, the Dominican Republic, and Cuba. Brazil is the leading producer of edible dry beans, followed by India, China, Burma, Mexico, and the United States (USDA-ERS 2006).

Nutrition and Health Implications

The value of beans for providing essential nutrients has been recognized for centuries. Edible beans have the highest protein content of all commercial seed crops (Heriteau 1978). They have been called "the meat of the poor," which illustrates their role in supplying nutrients such as protein among lower socioeconomic groups (Aguilera and Stanley 1985). Beans are rich in dietary fiber and considered to be a low glycemic index food. It has been found that the darker the bean seed coat, the higher the level of antioxidants. Black beans were found to have the highest anthocyanin content, followed in descending order by red, brown, yellow, and white beans (Choung and others 2003). Besides providing nutrition from protein, carbohydrates, vitamins, and minerals, beans have also shown to help prevent illnesses such as obesity, type 2 diabetes, cancer, and cardiovascular diseases (Hangen and Bennink 2001).

In 2002, Hangen and Bennink found that consumption of black beans reduced colon carcinogenesis in rats. Black beans had an impact on azoxymethane-induced colon

cancer in rats, decreasing total tumor incidence by 54% and adenocarcinoma incidence by 75% as compared to the control (Hangen and Bennink 2002). Singh and Fraser determined that individuals consuming legumes (including beans, lentils, and split peas) more than two times per week were 47% less likely to develop colon cancer than individuals that consumed legumes less than one time per week (Singh and Fraser 1998).

Bazzano and others found that individuals consuming legumes at least four times per week had a 22% lower risk of heart disease than those consuming legumes less than once per week (Bazzano and others 2001). It was also found that men who followed a "Prudent diet" (one that contained more legumes, whole grains, fish, and poultry) had a 30% lower risk of having heart disease than men that the "Western diet" (more red meat, refined grains, sweets, French fries, and high fat desserts) (Hu and others 2000). The consumption of canned and dry beans has been shown to reduce serum cholesterol (Anderson 1985; Anderson and others 1999; Bourdon and others 2001; Geil and Anderson 1994; Jenkins and others 1980; Shutler and others 1989; Tsuda and others 1994).

Black Bean Anthocyanins

Beans vary greatly in flavonoid content and seed coat color, depending on the cultivar. The presence of anthocyanins has only been reported in black, red, and blue-violet colored beans (Beninger and Hosfield 2003; Romani and others 2004; Tsuda and others 1994). Nine anthocyanins were detected in black beans by Wu and Prior in 2005. Three of these anthocyanins, delphinidin 3-glucoside, petunidin 3-glucoside, and malvidin 3-glucoside, had previously been found in black beans (Mazza and Miniati

1993; Wu and Prior 2005). Six other anthocyanins were found in black beans for the first time: delphinidin 3,5-diglucoside, petunidin 3,5-diglucoside, delphinidin 3-galactoside, malvidin 3,5-diglucoside, petunidin 3-galactoside, and malvidin 3-galactoside (Wu and Prior 2005).

Thermal Processing

Thermal processing of fruits and vegetables is used to decrease the number of or eliminate spoilage and pathogenic microorganisms and to inactivate enzymes responsible for reduced quality. Foods that are not adequately thermally processed for a particular use are susceptible to spoilage due to bacteria and/or have the potential to be hazardous because of the survival of pathogenic bacteria and are therefore not fit for consumption. However, foods that are over processed with heat may have decreased nutritional properties and may lose flavor or texture.

Blanching is a necessary step for many canned fruits and vegetables. This step aids in the removal of unwanted impurities such as soil, foreign material, and even some microorganisms (Eskin 1989). However, the main reasons for blanching are to inactivate enzymes that may cause deterioration, and to remove intercellular gasses. Blanching processes prevent changes in odor, color, and flavor in many processed products (Eskin 1989). Various blanching operations exist including water blanching, steam blanching, vacuum steam blanching, in-can blanching, microwave blanching, and hot-gas blanching (Selman 1994). The most commonly used system is water blanching. Although blanching has positive effects such as retarding enzymatic browning, soluble materials may be leached out, especially during water blanching (Shahidi and Naczk 1995).

Canning is defined as a method of food preservation wherein a food and its container are rendered commercially sterile by the application of heat, alone or in combination with pH and/or water activity or other chemicals (Gavin and Weddig 1995). The sterility of a food is sustained by a hermetically sealed container. If a product is commercially sterile, there will be destruction of all viable organisms of public health significance as well as microorganisms that may reproduce under normal non-refrigerated conditions. Although commercially sterile processed foods are considered "canned" foods, there are several types of packages other than metal cans that can be used (Gavin and Weddig 1995).

Determining the right temperature and time for sterilization depends on knowledge of the product, container dimensions, and thermal processing details. It is also important to understand the survival needs and thermal resistance of the microorganisms on the food (Gavin and Weddig 1995). The thermal process may also be increased to obtain the most acceptable texture for some food products.

Thermal processing of canned food products is usually accomplished through the use of retorts, which can apply both pressure and high temperatures. There are several types of retorts. Non-agitating including still and hydrostatic retorts, operate at a steady processing temperature that allows heat to be transferred to the container and the food inside by either conduction and/or convection heat currents depending on the food product and liquid inside the container. .

Agitating or rotary retorts provide product agitation and as a result offer several advantages over non-agitating retorts. Agitation of containers produces movement of the food, which causes heat transfer by convective currents. Through agitation, the heat

transfer occurs more quickly than in non-agitating retorts where heat transfers through conduction. This means that processing time can be reduced. Also, product agitation permits the use of high processing temperatures (up to 137.8° C) (Gavin and Weddig 1995). Often, product uniformity and quality are improved. Movement of the headspace plays a major part in product mixing so it is important to avoid a small headspace, which may lead to under processing.

III. MATERIALS AND METHODS

Bean Samples

Processing

About 11.35 Kg (25 lb) of raw, uncooked black beans (*Phaseolus vulgaris*) were obtained from a local commercial food processor. All samples were made from this batch. Procedures used were to simulate those steps in a commercial process. This included a short blanch process and then a thermal process with steam under pressure in a sealed container. A detailed list of equipment and supplies used for sample treatments and the ORAC assay may be found in the Appendix.

Water blanching was performed on 100 g of black beans placed in a stainless steel mesh sieve that allowed water to flow around the beans. Deionized water (16 L) was added to a 20 L Dover steam kettle, model TDC/2-20 (Dover Corp., Elk Grove Village, IL) and heated to boiling (100°C). Each been sample was blanched for 30, 60, or 90 sec with agitation to assure uniform heat and water contact. Following heating, beans were immediately placed into an ice-water bath for 10 sec and agitated to assure uniform temperature contact. All three samples of beans within each replication were blanched in the same water. Fresh deionized water was used for each of the two subsequent replications. Blanch times were rotated so each time was performed 1st, 2nd or 3rd during the three replications. Following cooling, the beans were placed into Whirl-Pak long-term sample retention bags (Nasco Modesto, CA) until extraction for ORAC analysis. Samples were stored at -20°C for up to three weeks prior to extraction.

For the thermal process treatments, samples were heated in steam under pressure in a retort in 532 ml (18 oz) Whirl-Pak long-term sample retention bags with barrier film (Nasco). All beans were blanched for 60 sec in deionized water prior to being filled into the bags. Each bag was filled with 100 g of blanched black beans and deionized water was added to give different solid to liquid fill ratios. Based on 100 g of beans per sample, the solid to liquid fill levels were as follows: low fill ratio: 150 ml water or 40% solid fill weight, medium fill ratio: 186 ml water or 35% solid fill weight and high fill ratio: 233 ml water or 30% solid fill weight. At least 15 bags (5 bags for each fill level) of beans were placed on racks in single layers within the pressure vessel (Dixie RDSW-3 still retort, Dixie Canner Co. Athens, GA). The bags of beans were then heated to 110°C as indicated in two extra bags of beans in which the temperatures were monitored using Ecklund thermocouples (Ecklund-Harrison Technologies, Inc. Fort Meyers, FL). Sample bags of black beans were heated in two batches for either 10 or 30 min after the temperature of the beans inside the bags reached 110°C. The come-up time after venting for both processing times was 10 min. The bags of beans were allowed to cool, under air pressure, to 100°C and then removed from the retort. After being removed from the retort, bags were cooled in ice water. The bags were cut open and the liquid was drained from the beans. Beans were then placed in new Whirl-Pak long-term sample retention bags (Nasco) so no more water would be absorbed into the beans after cooking. Drained samples were stored at 2.7°C (37°F) for no more than two days prior to ORAC analysis.

The following beans samples were analyzed by ORAC analysis: 7 samples of raw uncooked beans, 9 samples of blanched beans (3 blanch times x 3 replications) and 30 samples of pressure cooked beans (3 fill ratios x 5 replications at each cook time).

Commercial samples of canned black beans also were obtained. These samples of beans had been cooked using a proprietary process in either an agitating retort for ~10 min at ~125°C or in a non-agitating retort for ~30 min at ~120°C.

Extraction

All beans were extracted using methanol/water/formic acid at a ratio of 60:37:3 by volume (Cho and others 2004; Cho and others 2005; Lam and others 2005). The volume of solvent used for each extraction was determined by the form of the uncooked bean sample. Calculations for the ORAC value are based on samples extracted using 25 ml. Any dilutions and the sample weights are required in the calculation of ORAC values. All samples were measured four times at a 1:1000 dilution and the four net AUCs were averaged for each ORAC value.

Uncooked beans were ground in a Wiley mill model 3383-L10 (Thomas Scientific Swedensboro, NJ) with mesh size of 20, which produced pieces about 850 μm in size. Blanched and cooked (bags and commercial containers) beans were ground three times through a food grinder (Sunbeam-Oster Co. Schaumburg, IL). The grinder and mill were cleaned between bean samples.

A sample size of 0.5 g was used for uncooked beans and blanched beans. However, 25 ml was not enough solvent to remove all of the color (anthocyanins) so the extraction method was modified to use a total of 250 ml. Because the calculation is based on 25 ml, the 250 ml produced a 1:10 dilution. From that, serial dilutions were made to a final dilution of 1:1000. The 0.5 g sample was added to a beaker with 50 ml extraction solvent. This was placed on a stir plate, covered with parafilm (Fisher Scientific), and

stored in the dark at 2.7°C (37°F) overnight. The next day, the sample was filtered through Miracloth™ (Fisher Scientific) and extracted again in 50 ml increments until all color was removed. The final volume was brought to 250 ml before the sample was diluted.

The 10 and 30 min cooked beans and the commercial samples were extracted using 25 ml of solvent. Approximately 1g of each sample, weighed to the nearest hundredth, was placed in a 50 ml centrifuge tube. About 5 ml of extraction solvent was added and the sample homogenized with a Polytron homogenizer-Kinematica model PT10/35 (Brinkman Instruments Westbury, NY) for one minute. Then solvent was removed by filtration through Miracloth™ in a two inch funnel, into a 25 ml volumetric flask. The sample was placed back into the centrifuge tube with 5 ml of fresh extraction solvent and homogenized again. The homogenizer was sprayed with solvent also, which was filtered through the Miracloth™. These steps were repeated until a final volume of 25 ml was attained.

Moisture, Solids, and Dry Weight Basis

Ground samples were analyzed for moisture in a Napco E series model 5851 vacuum oven (Precision Scientific Chicago, IL). Weigh dishes with sample identification written on the bottom were stored in a dessicator prior to analysis, to remove any moisture. Ground samples were placed in the drying oven at 100° C for 4 hr at 20 mmHg. Three measurements were made in order to calculate the amount of moisture and solids per gram of sample: dish weight in grams (A), dish and sample prior to drying weight in grams (B), and the dish with residue weight in grams after drying (C). The

amount of moisture was calculated by: (B-C)/(B-A) and solid content was calculated by (C-A)/(B-A). These could be multiplied by 100 to express them on a percentage basis. Measurements A and B were taken prior to drying and measurement C was taken after samples were removed from the drying oven and cooled overnight in the dessicator. In order to report ORAC values on a dry weight basis, ORAC measurements for each sample were divided by their solid content.

Chemical Reagents

Extraction Solvent

The solvent used for the extraction of antioxidants from bean products was that used by Cho and others (Cho and others 2004; Cho and others 2005). The solution contained methanol (Fisher Scientific Pittsburgh, PA), water, and formic acid (Fisher Scientific) at a ratio of 60:37:3 by volume. Chemicals were added to a beaker with a magnetic stir bar under a hood. Solvent was stored in 1L amber glass bottles at room temperature.

Phosphate Buffer

The phosphate buffer used throughout this research was prepared by adding 75 mM potassium phosphate monobasic (KH_2PO_4) to 800 ml potassium phosphate dibasic (K_2HPO_4) (Fisher Scientific) until a pH of 7.4 was reached. The pH 7.4 phosphate buffer was stored in an amber glass bottle and refrigerated (4°C). This buffer was used to prepare solutions of reagents and functioned directly as a blank solution in the ORAC assay. See the appendix for step-by-step instructions on buffer preparation.

6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox)

6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) was used to make the Trolox standard (Aldrich Chemicals Milwaukee, WI). A 1 mM solution of Trolox was prepared using 25 mg of Trolox in 100 ml of the phosphate buffer, which was then diluted to 500 μM. 1.5 ml samples of the 500 μM Trolox stock solution were added to 1.8 ml Eppendorf tubes and stored at -80°C until use. Standard concentrations of 3.12 μM , 6.25 μM, 12.5 μM, and 25 μM Trolox solutions were made fresh each day ORAC assays were performed by mixing 500 μM Trolox stock solution (thawed to room temperature) with the phosphate buffer. Results from the ORAC assay are reported in μmol of Trolox equivalents (TE) per gram (μmol TE /g).

Fluorescein Solution

To prepare the stock solution, 22.5 mg fluorescein (Fisher Scientific) was added to 50 ml of working phosphate buffer and mixed well using a vortex mixer. 50 μl of this solution was added to 10 ml of working buffer (75 mM) and mixed with a vortex mixer to make the final stock solution. The stock solution was stored in 1.8 ml Eppendorf tubes at -20° C until use.

Fluorescein working solution was made fresh each day by adding 800 μl of the stock solution (thawed to room temperature) to 50 ml of the phosphate buffer. This amount was used in two ORAC assays each day and was kept at 37°C prior to use.

2, 2' –Azobis (2-amidino-propane) dihydrochloride (AAPH)

2, 2' –Azobis (2-amidino-propane) dihydrochloride (AAPH) (Wako Chemicals Richmond, VA) solution was prepared fresh before each ORAC analysis. Immediately

before the start of the assay, 0.108 g of AAPH was added to 5 ml of phosphate buffer which had been incubated in a water bath at 37°C. This produces an AAPH solution containing 79.6 μmol/ml. A 35 μl aliquot provides 2.8 μmol AAPH per well.

ORAC Assay

H-ORAC Procedures

Samples were analyzed based on the hydrophilic oxygen radical absorbance capacity (H-ORAC) method modified by Prior and others (Prior and others 2003). Each test involved analyzing the bean samples along with a blank (phosphate buffer pH 7.4, 75 mM) and different concentrations of the control standard (3.13, 6.25, 12.5, and 25 μM Trolox). Tests were performed at 37°C. The specific protocol for the FLUOstar OPTIMA fluorescence microplate reader (BMG Lab Technologies, Inc. Durham, NC) is in the Appendix.

Before beginning the assay, a water bath (HAAKE DC10-V26B refrigerated open bath with digital control, Thermo Electron Corp. Waltham, MA) and the microplate reader incubator were set to 37°C. Next, the fluorescein and Trolox stock solutions were thawed to room temperature and used to make working solutions per the methods described earlier. Extracted samples were diluted using the working buffer.

Samples were added to a 48 well polystyrene microplate (BD Falcon, Fisher Scientific) by pipette. Buffer (20 μl), Trolox standards, and bean samples were added to their designated wells. 200 μl of working fluorescein solution was added to a well to serve as a gain adjustment for the reader. The buffer was added to the outer wells of the plate because temperature differences in the external wells of the microplate may have

affected reproducibility (Lussignoli and others 1999). Any outliers (values that were ± 2* standard deviation) in the final data were thrown out. A layout of the plate is given in Figure 4.

When the wells of the plate were filled, it was placed in the microplate reader to incubate for 10 min. The AAPH solution was made according to the methods described earlier, just before the start of the assay. The fluorescein and AAPH working solutions corresponded with injectors one and two, respectively. The injectors were primed. First, 1000 µl of each solution was pumped in to a waste container, to rid the injectors of any residue or contaminants. Then, 2000 µl of each solution was sent through the injectors and back in to their respective containers in order to prime the injectors and make sure there were no bubbles present in the tubing. 200 µl of fluorescein was added to each well in the microplate at cycle 2 and 35 µl of AAPH was added to each well at cycle 4.

Each sample was measured four times, each of the four Trolox concentrations were measured twice, and the blank samples were measured 28 times to produce the AUCs. Microplates were analyzed in the FLUOstar OPTIMA fluorescence microplate reader equipped with 2 automated injectors, incubator, and FLUOstar OPTIMA version 2.0 evaluation software (BMG Lab Technologies, Inc. Durham, NC). There were 35 cycles and each cycle was 210 seconds. The plate was shaken for eight seconds before the measurement of each cycle. The entire test was two hours and one minute long.

Calculations

Calculations were based on those of Prior and others (Prior and others 2003). Final ORAC values were calculated using a regression equation ($Y = a + bX$ for linear; or

$Y = a + bX + cX^2$ for quadratic) between Trolox concentration (Y; μM) and the net area under the FL decay curve (X). Linear regression used was in the range of 3.12-25 μM Trolox. Data are reported as micromoles of Trolox equivalents (TE) per gram of sample (μmol TE/g). The area under the curve (AUC) was calculated as follows:

$$AUC = (0.5 + f_5/f_4 + f_6/f_4 + f_7/f_4 + \ldots + f_i/f_4) * CT$$

Where f_4 = initial fluorescence reading at cycle 4, f_i = fluorescence reading at cycle i, and CT = the cycle time in minutes. The net AUC was found by subtracting the AUC of the blank from the AUC of the sample.

Data Analysis

AUC and ORAC values were calculated using a Microsoft Excel (Microsoft Roselle, IL) spreadsheet. Statistical analysis was done using SAS® 9.1 statistical evaluation software (SAS 2002-2003). All models used were CRD. The model used to analyze both the fill weight and cook time had two fixed effects. The SAS programs used for analyses are in the Appendix.

IV. RESULTS AND DISCUSSION

The influence of different thermal processing treatments on the antioxidants in black beans was determined by measuring peroxyl radical scavenging activity by the H-ORAC assay. The effect of blanch time retort time and solid/liquid fill ratios were evaluated. Commercially processed black beans thermally processed in either an agitating retort or non-agitating retort were also compared

Blanch Treatments

Uncooked black beans were subjected to a blanch treatment in boiling water for 30, 60, or 90 sec and the biologically active antioxidants were measured and expressed as the ORAC value. When beans were placed in blanch water and then in a water-ice bath, a visual leaching of the anthocyanins into the water was observed as evidenced by a purple color (Figure 5). With each blanch treatment, the water became darker. Since the same blanch water was used for all treatments, it was thought that the presence of anthocyanins may lead to re-absorption by beans in subsequent blanch samples. Therefore, the order for the 30, 60, or 90 sec blanch was rotated for each replication. Percent moisture of all beans is shown in table 1 (Note: all tables are in the Appendix).

Blanch time had no effect on the reduction of antioxidant activity of black beans as measured by the H-ORAC value per g black beans (table 2). No significant difference in blanch time was observed for antioxidant capacity values either on a fresh weight ($p= 0.8956$) or dry weight ($p=0.8641$) basis. Table 3 illustrates that moisture content in the black beans increased significantly with each increase in blanch time.

These results indicate that moisture uptake, which reduced the concentration of antioxidants per 100 g sample, did not cause a significant change in antioxidant capacity among blanched samples. The loss of anthocyanins into the blanch water and ice-water bath, which was visually apparent, also did not cause a significant change in antioxidant capacity. It is possible that longer blanch times would show a reduction in antioxidant activity due to increased water absorption or loss of antioxidants to the water. However, commercially processed beans are exposed to similar blanch times and exhibit similar moisture levels after blanch treatment. Commercially, black beans are blanched for shorter times than many other dry beans by processors to minimize color loss.

The results of the effect of blanch order on antioxidant capacity in the black beans may be found in table 4. The order in which the samples were placed in to the blanch water did not produce a significant difference on antioxidant capacity either on a fresh weight ($p= 0.9201$) or dry weight ($p=0.9313$) basis. Therefore the concentration of antioxidants that leached in to the water from samples did not have an effect on the antioxidant capacity of subsequent samples. This is another indication that blanching did not influence antioxidant activity among the various treatments.

Thermal Processing Treatments

Results for the effect of retort time on the antioxidant activity as measured by H-ORAC values of 100 g samples of beans at 110° C in plastic bags is shown in table 5. Packages of black beans thermally processed under pressure in a retort for 10 min had significantly higher antioxidant capacity than those processed at 30 min on a fresh weight basis ($p= 0.0003$). However, on a dry weight basis, processing time did not significantly

influence antioxidant capacity (p=0.0836). Differences in H-ORAC values on a fresh weight basis are due to water absorption during the increased cook time. This was confirmed when the percent moisture between the two process times was compared (table 6). The moisture content of the black beans was significantly lower (p<0.0001) for beans processed for 10 min compared to those processed 30 min. The additional water absorbed into the blanched black beans during thermal processing decreased the antioxidant capacity more in the beans cooked for 30 min. This is confirmed by the fact that the H-ORAC values were not significantly different when the process times were compared on the dry weight of the beans.

Additional water allows for greater profitability for companies, however it may decrease antioxidant capacity. Canned black beans are consumed on a fresh-weight basis and therefore increased liquid fill or processing time may also result in decreased antioxidant capacity per 100 g of black beans since more water is likely to be absorbed during the process.

Solid/Liquid Fill Ratios

Based on 100 g of beans per sample, the solid to liquid fill levels were as follows: low fill ratio: 150 ml water or 40% solid fill weight, medium fill ratio: 186 ml water or 35% solid fill weight and high fill ratio: 233 ml water or 30% solid fill weight (table 7). Antioxidant capacity values for fill ratios shown in table 7 were averaged across the two thermal process times since there was no significant interaction between fill ratio and cook time (p=0.7861). There was a significant difference in antioxidant capacities among the fill ratio on both the fresh weight (p=0.0012) and dry weight (p=0.0004) basis. The

low fill ratio retained a significantly higher level of antioxidant capacity as measured by H-ORAC than either the medium or high fill ratios. The latter were not significantly different. It can be concluded that, for this experiment, based on 100 g samples of beans, there was a difference in H-ORAC values regardless of thermal process time. In order to provide the most nutritional product as far as antioxidant capacity, it would be useful in a commercial setting to utilize the least amount of brine possible. However several tests would be needed to ensure that the product met consumer expectation of flavor, color, and texture. Black bean samples were removed from excess liquid immediately after cooling the retorted packages. There may be additional moisture absorption if the black beans were stored in the solution following processing which is what occurs during commercial processing.

Statistical analysis of the fixed effects indicated that there was no interaction between fill and thermal process time either on a fresh weight ($p=0.7861$) or dry weight ($p=0.6531$) basis. Results are illustrated in table 8. For beans on a fresh weight basis at a 10 min cook time, the low fill had a significantly higher antioxidant capacity than the medium or high fills. This was also true for the 30 min cook time. These results are also illustrated in table 7 where samples were analyzed on fill weight, regardless of time. There was no significant difference in the antioxidant capacity of the black beans in the low fill weight containers processed for 30 min than those in the medium and high fill weight containers processed 10 min. Similarly, the high fill processed at 10 min was not significantly different than any of the fill weights processed for 30 min.

Antioxidant capacity of the black beans on a dry weight basis for low and medium fill processed for 10 min was not significantly different. This indicates that the difference

in antioxidant capacity found for the beans on a fresh weight basis was likely due to water uptake by the beans (table 8). The antioxidant capacity of the beans in the low and medium fill bags (dry weight) at 10 min were not significantly different than those processed in the low fill weight bags for 30 min. The low fill antioxidant capacity between times are the same and significantly different than the high fill values, but the high fill values within both times are not significantly different than the medium fill antioxidant capacity values.

In these experiments, low fill products, or those with less water in the container, typically absorbed less moisture. This will cause a higher antioxidant capacity because the beans incorporated more water, thereby diluting the amount of antioxidant materials per 100 g sample on a fresh weight basis. By looking at the antioxidant capacities on a per-gram basis of dry matter, we can see that the water uptake in the beans did play a part in the differences in ORAC values. For example, the low fill antioxidant capacity was the same for both 10 and 30 min where it was not the same on a fresh weight basis. However, the H-ORAC values also illustrate that there was another potential influence on antioxidant capacity and that is degradation or loss of the antioxidants from the beans during processing. This is illustrated by the fact that both the fresh and dry weight basis H-ORAC values were significantly lower for the medium and high fills than the low fill weight containers (tables 7 and 8). The increased amount of water may have allowed more antioxidant materials to leach out of the beans during pressure cooking.

Effect of Processing Treatment on Antioxidant Capacity of Black Beans

The effect of blanching and thermal processing on the antioxidant capacity of the black beans was determined by comparing with the H-ORAC values of uncooked beans (table 9). A natural log transformation of the H-ORAC values was used in the statistical analysis of this data to provide normality and allow for analysis of variance. According to the test of fixed effects, there was a significant difference among the antioxidant capacities of the uncooked, blanched, and pressure cooked black beans ($p<.0001$) on both a fresh and dry weight basis. On a fresh weight basis, all three treatments were significantly different (table 9). The actual means (in μmol TE/g) for fresh weight samples were 229.18 for uncooked beans, 153.24 for blanched beans, and 19.13 μmol for cooked beans. According to the dry weight results, cooked beans have significantly less antioxidant capacity than uncooked or blanched beans, but blanching does not decrease the antioxidant capacity of uncooked beans. The actual means for dry weight samples were (in μmol TE/g) 259.80 for uncooked, 243.62 for blanched beans, and 51.69 for cooked beans.

The percent loss in antioxidant capacity for the blanched and cooked beans as compared to the uncooked beans is shown in table 10. On a fresh weight basis, there was a significant difference among retained antioxidant capacity for all three treatments. According to the actual mean values, blanched beans had a 33.13% decrease in antioxidant capacity as compared to uncooked beans and cooked beans had a 91.65% decrease. On a dry weight basis, blanching caused a 6.23% decrease and thermal processing a 80.10% decrease in antioxidant capacity compared to uncooked beans.

No trend of decreasing antioxidant capacity was observed between uncooked and blanched beans as is shown by the dry weight basis results in table 9. Therefore moisture, not temperature was likely the source of loss illustrated by the fresh weight H-ORAC values for the samples analyzed in this study.

It should be noted that differences may be due to the precision of the H-ORAC assay. The actual means for fresh weight samples were 229.18 ± 101.23 μmol TE/g for uncooked beans and 153.24 ± 63.68 μmol TE/g for blanched beans. This large variation was despite the fact that all samples were taken from the same batch, extracted, and analyzed in the same manner. Uncooked/dry black bean antioxidant capacity has been reported only once in the literature as 75.93 μmol TE/g for H-ORAC value (Wu and others 2004a). It should be noted that only one sample was analyzed in that study. ORAC value precision is within ± 15% in both manual and fully automated systems (Huang and others 2002a; Ou and others 2001). Accuracy varies from 97.60 to 115.01% within individual batches and from 95.39 to 107.5% between all batches when using a fully automated system (Huang and others 2002a). Because the literature accepts such a wide range in values as being equal, and because the collected data has a wide standard deviation which may be typical for this assay, the data in table 9 suggest that there is no difference in H-ORAC values for the uncooked and blanch-treated beans due to temperature effects.

There is still the question of why beans did not exhibit a significant difference in antioxidant capacity between blanch times (table 2) or between uncooked and blanched beans on a dry weight basis (table 9) despite the loss of color (presumably primarily anthocyanins) into both the blanch water and ice bath. The amount of antioxidants lost in

the water may have been so small, that it did not have any effect on antioxidant capacity. Another theory would be that the anthocyanins lost to the water did not contribute a significant amount to overall antioxidant capacity in uncooked beans. Anthocyanins are water soluble and exhibit a range of polarities. It is a possibility that the type of anthocyanin that leached into the blanch water also exhibits a lower antioxidant capacity than the anthocyanins or any other antioxidants that remained in the beans. Black beans contain several anthocyanins including delphinidin-3-glucoside, petunidin-3-glucoside, and malvidin-3-glucoside (Wu and Prior 2005), which are listed in decreasing order of polarity (Amic and others 1993). It has been reported that antioxidant capacity of the anthocyanidins is delphinidin > petunidin > malvidin (Kahkonen and Heinonen 2003). This is also true of their respective antioxidants delphinidin-3-glucoside, petunidin-3-glucoside, and malvidin-3-glucoside (Kahkonen and Heinonen 2003; RiceEvans and others 1996; Wang and others 1997). These findings suggest that the type of anthocyanins lost to the blanch water do not necessarily contribute less to antioxidant capacity than those which remained in the beans; they were just lost in very small amounts compared to those which were retained. Further studies could be conducted which would analyze the concentration and chemical structure of those anthocyanins lost to blanch water and also measure color and H-ORAC values to see if there is a correlation.

The pressure cook treatments caused a loss in antioxidant capacity for several reasons. One being that there was an uptake of moisture as was seen with other samples. Heat treatment and the leaching of antioxidants into the fill water also caused a decrease in antioxidant capacity. As seen in tables 6-8, longer pressure cooking times and higher

water to bean ratios in sample bags contribute to changes in antioxidant capacity as measured by H-ORAC. Heat increases cell permeability and adds to other stresses such as water expansion which causes skin cells to be damaged and release their contents (Sapers and others 1985). Perhaps, the addition of pressure also causes the transfer of antioxidants out of the beans and into the water.

Jiratanan and Liu observed a loss in antioxidant capacity due to thermal processing. Green beans were thermally processed at 115° C for 10, 20, or 40 min. The total antioxidant activity (reported as μmol vitamin C equivalents per gram) decreased 9% for all time treatments which was not significantly different ($p > 0.05$). There also was a 60% decrease in flavonoid compounds at 10 min which also was not significantly influenced by process time ($p> 0.05$) (Jiratanan and Liu 2004). Kim and Padilla-Zakour found a 35% decrease in antioxidant capacity when making jam with cherries, plums, and raspberries. There was also a reduction in the concentration of anthocyanins. The authors attribute these losses to the disruption of the fruit tissue followed by heat treatment under high acid and sugar conditions (Kim and Padilla-Zakour 2004).

Beans are edible due to the cooking process, which makes them tender and also aids in improvement of palatability. Although thermal processing reduces the antioxidant capacity of beans, this step is necessary to produce acceptable shelf stable products. Lower fill levels and cook times may be considered in order to reduce this loss. Different cultivars could also be considered. Process time may be affected by genetic factors, physical structure, chemical composition, and processing (Salunkhe and Kadam 1989) so processors should take this in to consideration if attempting to design a process that allows for the highest antioxidant capacity.

Commercial Canned Black Bean Samples

H-ORAC values of commercial canned black beans cooked with and without agitation are shown in table 11. There was no significant difference in hydrophilic antioxidant capacity between the two retort treatments as reported on a fresh weight ($p=0.7723$) or dry weight ($p=0.3325$) basis. Both samples had similar moisture levels, around 70% (table 1).

Agitation allowed process time to be reduced to ~ 10 min at ~ 125° C. Non-agitating retort samples were processed for ~ 30 min at ~ 120° C. There was no more destruction of antioxidant capacity by the longer time at the lower temperature than the higher temperature and shorter time. Both samples had similar percent moisture so the moisture absorption by the beans and resulting solid content was also similar. Thermal processing with agitation allows for a shorter process time which may lead to reduced energy and labor costs for commercial processors. However, cooking black beans with agitation may result in greater break down of bean structure.

Conclusion

Initial processing treatments such as blanching are often necessary before the thermal processing of canned foods. Therefore the influence of blanching in boiling water on antioxidant capacity of black beans as measured by the H-ORAC assay was observed. Although there was no difference in antioxidant capacity between blanching for 30, 60, and 90 seconds, there was a difference between blanched samples and uncooked samples. In this study, moisture uptake is the cause for the difference in antioxidant capacity between raw and blanched samples on a fresh weight basis. However, there was a visual

leakage of anthocyanins in the blanch water and water-ice bath. If longer blanch times were observed, there may have been a significant decrease in antioxidant capacity either due to moisture uptake or prolonged exposure to boiling temperatures. However, longer blanch times are unnecessary in a commercial setting. Future studies involving analysis of the blanch water for antioxidant content and antioxidant capacity would be interesting. Also, it would be useful to conduct a study using a different blanch method, such as steam.

The effect of thermal processing on the antioxidant capacity of black beans was observed at 110° C for 10 and 30 minutes. The 10 min cook time had a significantly higher antioxidant capacity as measured by the H-ORAC method compared to the 30 minute cook time. Surprisingly, this difference was due to increased water absorption over time. The 30 min samples had more water, so there were less antioxidants present per gram than in the 10 min samples. Cooked samples had a significantly lower antioxidant capacity than uncooked samples, so in addition to moisture uptake and loss of antioxidants to the surrounding water, heat played a role in the reduction of antioxidant capacity. Further research is suggested to determine the effects of different temperatures on antioxidant capacity. It would also be interesting to measure color or texture changes at different cook times and temperatures to determine if there is a correlation with antioxidant capacity.

Samples cooked with a lower fill showed higher antioxidant capacity than those with medium or high fill. This was true on both a fresh and dry weight basis. Samples were cooked in the same retort for the same time, so the change in antioxidant capacity should not have been due to different heat exposure. Because the samples exhibited

different antioxidant capacities on a dry weight basis, the change in antioxidant capacity can not be attributed to moisture uptake in the samples with medium an high fills. However, because the medium and high fill samples contained more water, this may have allowed for more water soluble antioxidants to leach out of the beans. Future research, involving a wider range of fill weights and processing at different temperatures would be interesting.

Processing vegetables in agitating retorts with reduced cook time has been shown to aid in color and texture retention (Abbatemarco and Ramaswamy 1994). Theoretically, the reduced process time should help prevent loss in antioxidant capacity. This theory was not confirmed for black beans. There was no more destruction of antioxidant capacity by the longer time at the lower temperature than the higher temperature and shorter time. This may have been because agitation facilitated the loss of antioxidants to the brine, resulting in an antioxidant capacity similar to that of the samples which had been processed longer and without agitation. Research involving different amounts of agitation, for example in intervals, or different agitation speeds would be interesting.

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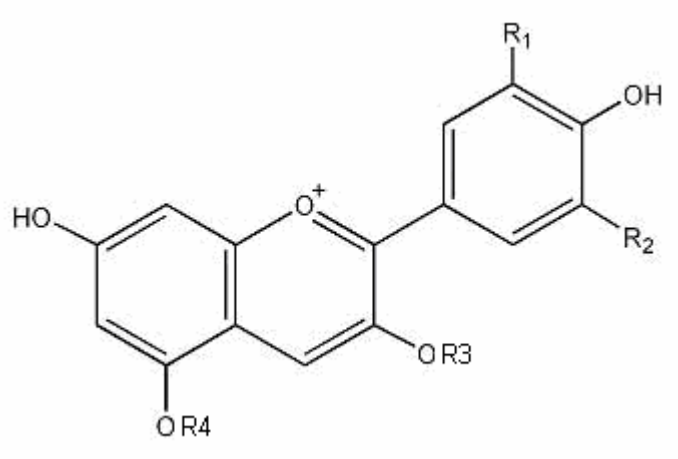
APPENDIX

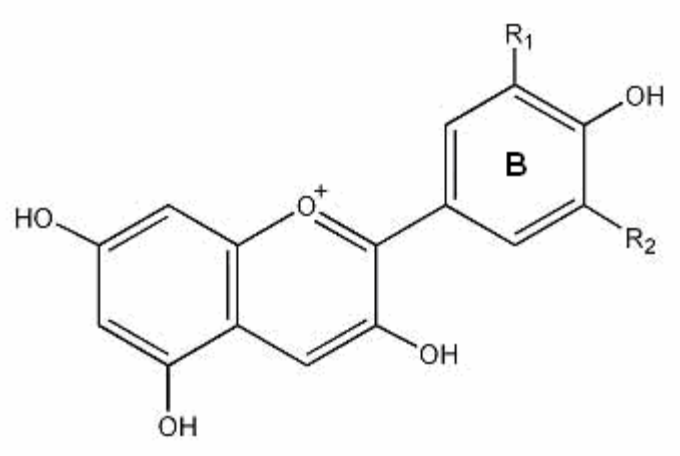
APPENDIX



R1	H, OH, or OCH_3
R2	H, OH, or OCH_3
R3	glycosyl
R4	H or glycosyl

Figure 1: Base Strucutre of Anthocyanins

Return_to_Review_of_Literature



Anthocyanidin	R1	R2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 2: Structures of Six Common Anthocyanidins

Return_to_Review_of_Literature

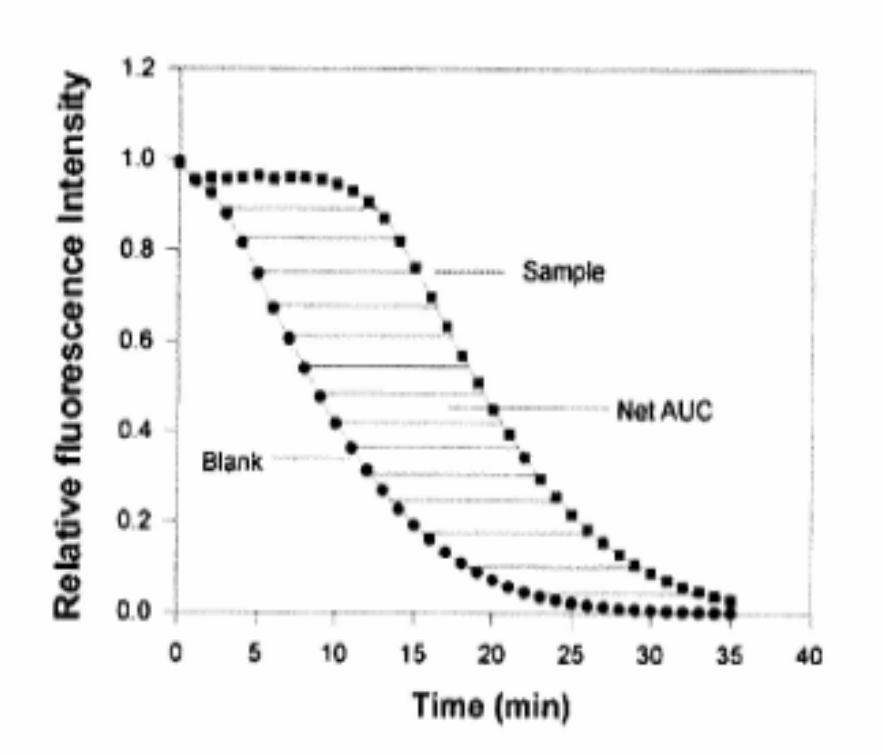


Figure 3: Area Under the Curve

Relative ORAC value= [(AUCsample – AUCblank)/(AUCTrolox – AUCblank)](molarity of Trolox/molarity of sample)

Return_to_Review_of_Literature

C1	B	B	B	B	B	B	B
B	S2	X3	X4	S3	X1	X2	B
B	X4	S4	X1	X2	S1	X3	B
B	S1	X2	X4	S2	X3	X1	B
B	X1	S3	X3	X2	S4	X4	B
B	B	B	B	B	B	B	B

C1= Gain
B= Blank

S1= 3.12 µM Trolox
S2= 6.25 µM Trolox
S3= 12.5 µM Trolox
S4= 25 µM Trolox

X1= Sample 1
X2= Sample 2
X3= Sample 3
X4= Sample 4

Figure 4: 48 Well Plate Layout

Return_to_Materials_and_Methods

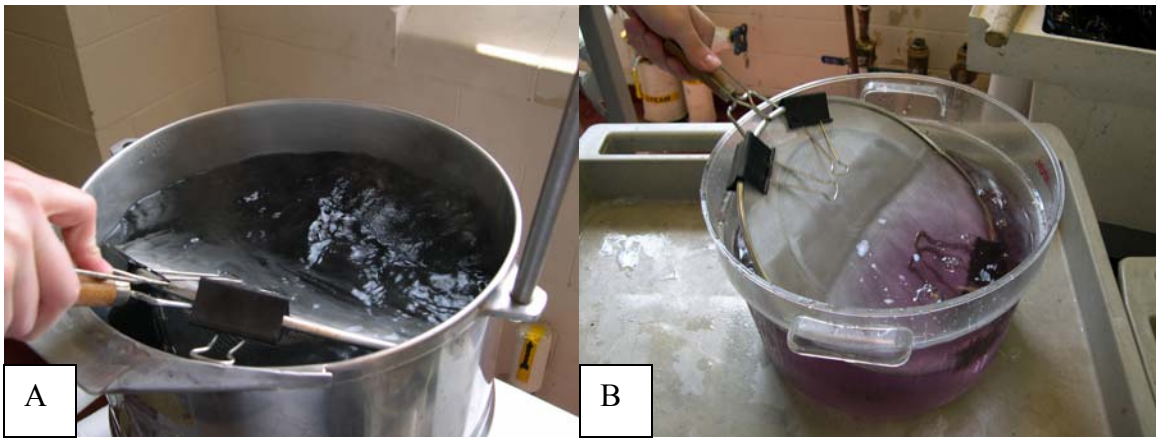


Figure 5: Transfer of Anthocyanins from Black Beans to Blanch Water (A) and Ice-water Bath (B)

Return_to_Results_and_Discussion

Table 1: Percent Moisture of Black Beans

Sample		% Moisture[a]
Dry		11.76
Blanch	30 s	33.91
	60 s	37.37
	90 s	39.68
10 min cook	low fill	60.08
	med fill	61.49
	hi fill	61.70
30 min cook	low fill	66.75
	med fill	67.07
	hi fill	62.59
Agitate cook		67.98
Non-Agitate cook		70.68

[a] Measured in triplicate. Averages are given.

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Table 2: Effect of Blanch Time on H-ORAC Value (µmol TE /g) of Black Beans

Blanch Time (s)	Mean ORAC[a] Fresh Weight Basis		Mean ORAC[a] Dry Weight Basis	
30	159.28 ± 71.57	A	240.98	A
60	137.27 ± 60.72	A	219.28	A
90	163.17 ± 82.61	A	270.59	A

n= 3 for each time treatment.

[a] Means followed by the same letter within a column are not significantly different ($p>0.05$).

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Table 3: Percent Moisture of Black Beans at Different Blanch Times

Blanch Time (s)	Mean Percent Moisture[a]	
30	33.91 ± 0.31	C
60	37.37 ± 0.58	B
90	39.68 ± 0.35	A

n= 3 for 30 and 60 seconds and n=2 for 90 second time treatment.
[a] Means followed by the same letter within a column are not significantly different ($p>0.05$).

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Table 4: Effect of Blanch Order on H-ORAC Value (µmol TE /g) of Black Beans

Blanch Order	Mean ORAC[a] Fresh Weight Basis		Mean ORAC[a] Dry Weight Basis	
1st	142.28 ± 67.02	A	229.39	A
2nd	151.12 ± 72.36	A	237.49	A
3rd	166.33 ± 77.76	A	263.97	A

n= 3 for each order treatment.

[a] Means followed by the same letter within a column are not significantly different ($p>0.05$).

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Table 5: Pressure Cook Time H-ORAC Values (µmol TE /g) of Black Beans on a Fresh and Dry Weight Basis

Time (min)	Mean ORAC[a] Fresh Weight Basis		Mean ORAC[a] Dry Weight Basis	
10	21.21 ± 3.92	A	54.44	A
30	17.05 ± 2.68	B	49.69	A

n= 15 for each time treatment.

[a] Means followed by the same letter within a column are not significantly different ($p>0.05$).

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Table 6: Percent Moisture of Black Beans at Different Cook Times

Time (min)	Mean Percent Moisture[a]	
10	61.09 ± 0.77	B
30	65.47 ± 2.17	A

n= 9 for each time treatment.

[a] Means followed by the same letter within a column are not significantly different ($p>0.05$).

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Table 7: Pressure Cook Fill Level H-ORAC Values (µmol TE /g) of Black Beans on a Fresh and Dry Weight Basis

Fill Level	Mean ORAC[a] Fresh Weight Basis		Mean ORAC[a] Dry Weight Basis	
low	22.03 ± 4.30	A	60.11	A
med	18.29 ± 3.14	B	51.01	B
hi	17.07 ± 2.50	B	45.06	B

n= 10 for each fill treatment

[a] Means followed by the same letter within a column are not significantly different ($p>0.05$).

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Table 8: Pressure Cook Time and Fill Level H-ORAC Values (µmol TE /g) of Black Beans on a Fresh and Dry Weight Basis

Time (min)	Fill Level	Mean ORAC[a] Fresh Weight Basis		Mean ORAC[a] Dry Weight Basis	
10	Low	24.28 ± 4.73	A	60.85	A
	Med	20.68 ± 2.36	B	53.71	AB
	Hi	18.67 ± 2.34	BC	48.75	BC
30	Low	19.77 ± 2.55	B	59.38	A
	Med	15.89 ± 1.52	C	48.31	BC
	Hi	15.47 ± 1.50	C	41.37	C

n= 5 for each time/fill treatment.
[a] Means followed by the same letter within a column are not significantly different ($p>0.05$).

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Table 9: Comparison of Black Bean Treatments using the Logarithmic H-ORAC Values (µmol TE /g) on a Fresh and Dry Weight Basis

Bean Sample	N	ln Mean ORAC[a] Fresh Weight Basis		ln Mean ORAC[a] Dry Weight Basis	
Uncooked	7	5.36 ± 0.42	A	5.48	A
Blanched	9	4.93 ± 0.49	B	5.40	A
Cooked	30	2.93 ± 0.20	C	3.93	B

[a] The natural log of means followed by the same letter within a column are not significantly different ($p>0.05$).

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Table 10: Percent Reduction in H-ORAC Values (µmol TE /g) for Black Bean Treatments on a Fresh and Dry Weight Basis

Bean Sample	N	Mean ORAC Fresh Weight Basis	Percent Reduction	Mean ORAC Dry Weight Basis	Percent Reduction
Uncooked	7	229.18	-	259.80	-
Blanched	9	153.24	33.13%	243.62	6.23%
Cooked	30	19.13	91.65%	51.69	80.10%

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Table 11: Cook Type H-ORAC Values (µmol TE /g) for Black Beans on a Fresh and Dry Weight Basis

Cook Type	Mean ORAC[a] Fresh Weight Basis		Mean ORAC[a] Dry Weight Basis	
Agitate Shorter time Higher temp	22.12 ± 7.19	A	69.03	A
Non-Agitate Longer time Lower temp	22.97 ± 5.72	A	78.39	A

n= 10 for each cook type treatment.
[a] Means followed by the same letter within a column are not significantly different ($p>0.05$).

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FLUOstar OPTIMA Version 2.0 Evaluation Software Protocol Description

Reader: FLUOstar OPTIMA

Plate type: BD Falcon 48 well Polystyrene

Basic Parameters:
Positioning delay: 0.3s
Number of kinetic windows: 1
Number of cycles: 35
Measurement starting time: 0.0s
Number of flashes per cycle: 15
Cycle time: Depends on the chosen layout
Filters and integration: Fluorescence Intensity
Number of multichromatics: 1
Gain: Changes with each gain adjustment
Pause before each cycle: 0
Excitation filter: 485 nm
Emission filter: 520 nm
Calculation range: Start from 1, stop at 35

Concentration/Volume/Shaking
Volume 1: 200 µL, Pump speed: 420 µL/s
Volume 2: 20 µL, Pump speed: 420 µL/s
Shaking mode: orbital
Shaking width: 4 mm
Additional Shaking: before each cycle
Shaking time: 8 s

Injection and timing
Volume group 1 injection cycle: 2
Volume group 2 injection cycle: 4
Volume group 1 injection start time: 0.0s
Volume group 2 injection start time: 0.0s

Equipment and Supplies

Following is a list of equipment and supplies used for sample treatments and the ORAC assay:

1. FLUOstar OPTIMA fluorescence microplate reader equipped with 2 automated injectors, incubator, and FLUOstar OPTIMA version 2.0 evaluation software (BMG Lab Technologies, Inc. Durham, NC)
2. Microsoft Excel (Microsoft Roselle, IL)
3. SAS® 9.1 statistical evaluation software (SAS Institute Inc. Cary, NC)
4. BD Falcon 48 well polystyrene plates (Fisher Scientific Pittsburgh, PA)
5. Miracloth filter cloth (Fisher Scientific Pittsburgh, PA)
6. Eppendorf Repeater Plus pipette (Fisher Scientific Pittsburgh, PA)
7. The HAAKE DC10-V26B refrigerated open bath with digital control (Thermo Electron Corp. Waltham, MA)
8. Fisher brand Versamix magnetic stir plate (Fisher Scientific Pittsburgh, PA)
9. Vortex Genie model K-550-G (Fisher Scientific Pittsburgh, PA)
10. Corning brand 50 ml centrifuge tubes (Fisher Scientific Pittsburgh, PA)
11. Eppendorf tubes, 1.5 ml (Fisher Scientific Pittsburgh, PA)
12. Mettler DL12 titrator and pH meter (Mettler-Toledo, Inc. Columbus, OH)
13. Mettler-Toledo AB204 analytical balance (Mettler-Toledo, Inc. Columbus, OH)
14. Napco E series model 5851 vacuum oven (Precision Scientific Chicago, IL)
15. Thompson Scientific Wiley mill model 3383-L10 (Thomas Scientific Swedensboro, NJ)
16. Polytron homogenizer-Kinematica model PT10/35 (Brinkman Instruments Westbury, NY)
17. Oster heavy duty food grinder and sausage maker (Sunbeam-Oster Co. Schaumburg, IL)
18. Dixie RDSW-3 Retort (Dixie Canner Co. Athens, GA)
19. Dover steam kettle model TDC/2-20 (Dover Corp. Elk Grove Village, IL)
20. Ecklund thermocouples (Ecklund-Harrison Technologies, Inc. Fort Meyers, FL)
21. Whirl-Pak long-term sample retention bags with barrier film, 18 oz. (Nasco Modesto, CA)
22. Other supplies included Eppendorf pipette tips, stirring rods, timers, gloves, thermometers, and various glassware (beakers, volumetric flasks, dilution tubes).

Buffer Preparation

1. To make 75 mM monopotassium phosphate, place 10.21 g of KH_2PO_4 (Fisher Scientific Pittsburgh, PA) in a 1000 ml volumetric flask.
2. Add deionized water until the volume reaches 1000 ml and stir the solution with a magnetic stir bar until the monopotassium phosphate is dissolved.
3. For the 75 mM dipotassium phosphate, place 13.06 g of K_2HPO_4 (Fisher Scientific) in a1000 ml volumetric flask.
4. Add deionized water until the volume reaches 1000 ml and stir with a magnetic stir bar until the dipotassium phosphate is dissolved.
5. To make the working buffer solution, add 800 ml of the 75 mM dipotassium phosphate solution to a 1000 ml beaker. Place a pH electrode in the beaker and add the monopotassium phosphate solution until a pH of 7.4 is reached. This requires about 200 ml of the monopotassium phosphate solution.
6. Store the buffer in an amber glass bottle at 2.7°C (37° F) until use.

[Blanch Time]

```
data one;
input time $ batch ORAC;
datalines;

%include 'C:\Danda.sas';
%mmaov (one, ORAC, class=time, fixed=time);
Run
```

[Blanch Order]

```
data one;
input order $ ORAC;
datalines;

%include 'C:\Danda.sas';
%mmaov (one, ORAC, class=order, fixed=order);
run
```

[Fill Level and Cook Time]

```
data ten;
input fill $ bag ORAC;
time=10;
datalines;

data ten_use; set ten;
source=1; *cooked;
keep source ORAC;
run;

data thirty;
input fill $ bag ORAC;
time=30;
datalines;

data thirty_use; set thirty;
source=1; *cooked;
keep source ORAC;
run;

data both;
set ten thirty;
source=1;  *cooked;
run;

%include 'C:\Danda.sas';
options ls=80 nodate nonumber;
title 'Time and Level Effects';
%mmaov (both, ORAC, class=fill time, fixed=fill|time);
run;

proc means data=both n mean std maxdec=2;
```

```
class fill time;
var ORAC;
title 'Descriptive Stats by fill and time';
types fill time fill*time;
run;
run;
```

[Uncooked vs Blanched vs Cooked Beans]

```
data blanched;
input time $ batch ORAC;
source=2;  *blanched;
datalines;

data blanched_use; set blanched;
keep source ORAC;
run;

data dry;
input source orac;
datalines;
run;

proc format;
  value sc 1='cooked'
           2='blanched'
           3='dry';
data all_ten;
set both blanched_use dry;
format source sc.;
log_orac=log(orac);
run;

title 'Ten minute cooked, vs. blanched vs. dry';
title2 'log of ORAC';
%mmaov (all_ten, log_ORAC, class=source, fixed=source);
```

VITA

The author was born and raised in Arkansas. She graduated with a B.S. in Food Science from Clemson University in May 2004. In August 2006, she completed the requirements for earning her M.S. in Food Science from the University of Tennessee.