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CHARACTERIZATION OF EXTRACTION METHODS TO RECOVER PHENOLIC-RICH EXTRACTS FROM BLACK BEANS (*PHASEOLUS VULGARIS*) THAT INHIBIT ALPHA-AMYLASE AND ALPHA-GLUCOSIDASE USING RESPONSE SURFACE APPROACHES

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by

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Black beans contain high phenolic contents that are considered potent antioxidants. Relatively little is known about their ability to inhibit the carbohydrate-hydrolyzing enzymes α-amylase and α-glucosidase from releasing glucose from starch and/or disaccharides. The objective of this project was to determine the optimum procedures for extracting total phenols (TP), total flavonoids (TF) and total condensed tannins (TCT) from black beans (*Phaseolus vulgaris)*, and then to determine the ability of the phenolic rich extracts to inhibit α-amylase and α-glucosidase. Due to their high phenolic levels, it was hypothesized that black beans would be an effective inhibitor of α-amylase and α-glucosidase, which would have potent health benefits for diabetics. The rationale for this project is that extracts obtained from black beans have the potential of inhibiting these key carbohydrate-hydrolyzing enzymes due to the high levels and different types of phenols present in black beans. The chemical diversity of the phenols extracts used affected the results, which in turn was affected by the extraction procedures. Therefore, extractions procedures were characterized using three different solvents and adjusting for solvent:water ratio, solid:solvent ratio and mixing time using a face centered cubic response surface design.

This experimental design resulted in 17 samples per solvent system, each of which were tested for total phenols (TP), total flavonoids (TF) and total condensed tannins (TCT) followed by their ability to inhibit α-amylase and α-glucosidase. The optimized factors for TP (3.82 mg/g) consisted of acetone: water ratio of 25:75, a solid:solvent ratio of 18 percent and mixing time of approximately 111 minutes. The optimal factors for TF (3.61 mg/g) were a 25:75 acetone: water, 30 percent solid:solvent ratio and a mixing time of approximately 143 minutes. Optimum levels of TCT (15.58 mg/g) were achieved with an 25:75 acetone: water ratio, a 14 percent solid:solvent and 60 minutes of mixing. The acetone solvent system produced the highest inhibition of α-amylase (36.65 % inhibition /mg of extraction) and α-glucosidase (34.10 % inhibition /mg). It should be noted that although the inhibition occurred from different extracts but but both used acetone as the solvent system. Inhibition of α-amylase decreased by ~3 fold using both ethanol and methanol at 10.29 % 10.84 % inhibition /mg extraction, respectively. However, for α-glucosidase, the inhibition by an ethanol extract was slightly higher (16.68 % inhibition/mg) than that exerted by methanol (6.16 % inhibition /mg). Yet, alpha-glucosidase inhibition for the acetone demonstrated the highest correlations with TP, TF and TCT with R values as follows: R=97, R=96, and R=91, respectively, indicating that total phenolic levels present in any given extract were responsible for the inhibitory effect. In summary, the significance of this project is that black bean extracts are capable of inhibiting key carbohydrate hydrolyzing enzymes, but this property depends on the extract and thus the extraction procedure used.

DEDICATION

For those who supported me in my education

and made it possible for me to succeed especially,

My father, Obaid,

And

My mother, Mariam.

It is also dedicated to the memory of my Uncle

who passed away before I was able to graduate

Hadi Alharbi

ACKNOWLEDGEMENTS

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A. LITERATURE REVIEW

A.1 Summary

The black bean (*Phaseolus vulgaris)* is an important food staple around the world for thousands of years. Historically, the black bean migrated from South America throughout the entire world due, in part, to its low cost and high micronutrient, protein and fiber content. More specifically, one cup of black beans provides more than 20 percent of more than ten nutrients. In addition, black bean is highly satiating while still maintaining a low glycemic score. The black bean can be easily incorporated into almost any diet either as a side dish or as a vegetarian main dish. Most importantly, the black bean has shown to be a dietary system may capable of preventing many types of chronic diseases, such as cardiovascular disease, cancer, obesity and diabetes. The black bean's high fiber-protein levels coupled with its flavonoid and phenolic content have been attributed to these health-promoting properties.

This literature review examines the history, production, and nutritional value of the black bean along with an emphasis on its phenolic content and its effects on the carbohydrate hydrolyzing enzymes involved (α-amylase and α-glucosidase) in releasing glucose from starch and/or disaccharides. Finally, this section describes the characterizing extraction processes using response surface methodology (RSM) for obtaining phenolic rich extracts capable of inhibiting the cited enzymes α-amylase and α-glucosidase, which if left unchecked can cause additional risks for diabetic or pre-diabetic individuals.

A.2 Black Bean History

Black beans are able to grow in many regions around the world, including Central America, South America, Mexico, Indonesia and the United States (Wells, 2016). As stated previously, black beans can be a valuable part of any diet because of their nutrient rich composition (Table 1) and as an alternative to meat as they contain high levels of protein (20-30%) with most of the essential amino acids present (Table 2) (Garden-Robinson & McNeal, 2013).

A.2.1 *Black Bean Production.* Brazil and India are the main producers of dried black beans, while China leads the world in green, non-dried black beans (Wells, 2016). The United States accounts approximately 12 percent of black bean production (Wells, 2016). Because dry edible beans are not subsidized by federal price support programs, the influx of beans from international sources and replacement of higher selling commodities has resulted in a decline in the U.S. production of black beans among other dry beans over the past decade (Wells, 2016). In fact, the U.S. now imports more dry edible beans than it exports, mostly from China (Wells, 2016). Outside of the U.S., the popularity of the black bean has grown because of its low cost and nutrient rich composition (Winham & Barr, 2008).

A.2.2 *Black Bean Consumption*. In the U.S., more than half of black beans are consumed in the South where approximately 14 percent of households eat beans on any given day (Wells, 2016). Although the consumption of dry edible beans has fallen from a high of 9.6 pounds per person, each year to 7.7 pounds per person per year (Wells, 2016).

Table 1: Chemical Composition of Black Beans (USDA, 2016)

Nutritional Values ½ cup cooked black beans	
Calories	113
Fat	>1 g
Saturated Fat	>1 g
Cholesterol	0 mg
Carbohydrate	20 g
Protein	8 g
Dietary Fiber	8 g
Sodium	1 mg
Thiamin	>1 mg
Folic Acid	128 mcg
Copper	>1 mg
Iron	2 g
Magnesium	60 mg
Manganese	>1 mg
Phosphorus	120mg
Potassium	306 mg

Table 2: Amino Acid Content of 1 cup of Black Beans (*essential amino acids)

INDIVIDUAL AMINO ACIDS	
nutrient	**amount**
Alanine	0.64 g
Arginine	0.94 g
Aspartic Acid	1.84 g
Cysteine	0.17 g
Glutamic Acid	2.32 g
Glycine	0.60 g
Histidine*	0.42 g
Isoleucine*	0.67 g
Leucine*	1.22 g
Lysine*	1.05 g
Methionine*	0.23 g
Phenylalanine*	0.82 g
Proline	0.65 g
Serine	0.83 g
Threonine*	0.64 g
Tryptophan*	0.18 g
Tyrosine	0.43 g
Valine*	0.80 g
The nutrient profiles were taken from The Food Processor, Version 10 12.0 ESHA Research, Salem, Oregon	

Black beans were not widely consumed prior to 1980, but its consumption is now holding steady or declining slightly (USDA, 2012). Dry edible beans are generally considered a poor man's food by many cultures, but households with higher incomes eat more black beans (Lucier et al., 2000).

In the U.S., the majority of black beans are consumed outside the home in non-fast food restaurants. As black beans can be prepared in a variety of ways, they are easily incorporated into almost any diet. For example, many recipes use black beans as a main ingredient, such as baked beans, bean salads, bean dips, various pasta dishes, but it can be used as a stand-alone side dish, such as soup. Black beans can be substituted into soups to replace cream or higher-fat ingredients. Lastly, by simply mashing or pureeing the beans to replace fat ingredients in brownies or cookies, healthier deserts have been formulated with this legume (Garden-Robinson & McNeal, 2013).

A.3 Chemical Composition of Black Beans

A.3.1 *Nutrient Content*. The nutritional values present in cooked beans provide a rich source for several nutrients (Table1). For instance, just one cup of cooked beans provides the following recommended daily allowance (RDA) of dietary components: fiber 60 percent, iron 20 percent, protein 30 percent, folate 64 percent, magnesium 38 percent, carbohydrates 18 percent, calcium 29 percent and vitamin B-6 35 percent (Winham & Barr, 2008). The protein content of black beans, combined with their high fiber content, creates a protein-plus-fiber content that is unparalleled by other foods. Black beans rate a 4.1 of 5 for their satiation factor, making them an excellent dietary choice for weight loss. In addition, they do not adversely affect blood sugar levels due to their low glycemic load (GL) (Table 1) (Thomson et al., 2012).

A.3.2 *Protein Value*. The body synthesized most amino acids; however, eight essential amino acids must be derived through diet. Considering that proteins are broken down in the gut, amino acids from protein are broken down and thereby released into circulation (Reeds & Garlick, 2003).

Chronic inflammation is currently a severe health problem for our society as this cellular stress can lead to arthritis, autoimmune diseases, diabetes, cancer and cardiovascular disease (Nordqvist 2015; Xu & Chang, 2007; Williams et al., 2003). It has been postulated that black beans aid in regulating many of these problems, because their proteins are not easily broken down by enzymes in the small intestine. Instead, they pass into the large intestine where bacteria use the protein as a food source to ferment it into short chain fatty acids, such as butyrate. Several studies have shown butyrate acts as an anti-inflammatory (Canani et al., 2011; Inan et al., 2000; Koshihara, 1984; Saemann et al., 2000). Black beans are particularly appealing as a source of protein due to their essential amino acid content (Table 2), and thus can be a meat alternative when paired with grains, such as rice (Bressani et al., 1963; Garden-Robinson & McNeal, 2013). One cup of black beans contains 60 percent of the (RDA) of protein and can be used in the body for many important functions. Research completed by Bressani et al., (1963) on rats fed uncooked black beans determined growth improved as protein in the diet increased (up to 18.5 percent of the diet). These authors demonstrated that the amino acid, methionine, was the first limiting amino acid in black beans (Bressani et al., 1963). Yet, there are several challenges associated with using protein as a therapeutic agent. For instance, proteins are limited by their molecular size, low permeability and rigid structures, which limit water molecules in the protein interior (Erickson, 2009). Still, an

epidemiological study by Beerens et al. (2003) demonstrated that protein transduction domains (PTDs) could be developed for delivering therapeutic proteins to cells and tissues. Beerens et al. (2003) described PTD's as "small peptides that are able to ferry much larger molecules into cells independent of classical endocytosis." In a latter study completed by Kim et al. (2014), the former results were confirmed in that PTD fusion proteins transduced into cells and tissues making them potentially excellent protectors against various diseases, including skin inflammation and neuronal diseases (Kim et al., 2014). Future studies are necessary to confirm the effects of the potential the black bean holds as a PTD source with high protein content.

Many other important major and micro nutrients are present in the black beans (Tables 1 and 2), but for the scope of this project, the phytochemical of interest (phenols) will be discussed in detail. Readers are directed to other resources to learn more about the other valuable components in black beans (USDA, 2012).

A.4 Phenolic Compounds and Black Beans

A.4.1 *Phenols Compounds.* Black beans are rich source of the phenolic compounds (Figure 1), such as phenolic acids (Figure 2) and flavonoids (Figure 3). Each of their position in a wider perspective of plant-based composition is shown in Figure 4. Phenols are secondary metabolites present in all plants and including some insects. These compounds protect organisms from environmental stressors (Wildman, 2006), such as drought conditions, weed and insect pressure and various diseases, due in part to their anti-oxidant properties (Hui et al., 2010). The soluble phenols are located within the plant cell vacuoles while the plant's insoluble phenols are bound to the cell walls (Stalikas, 2007).

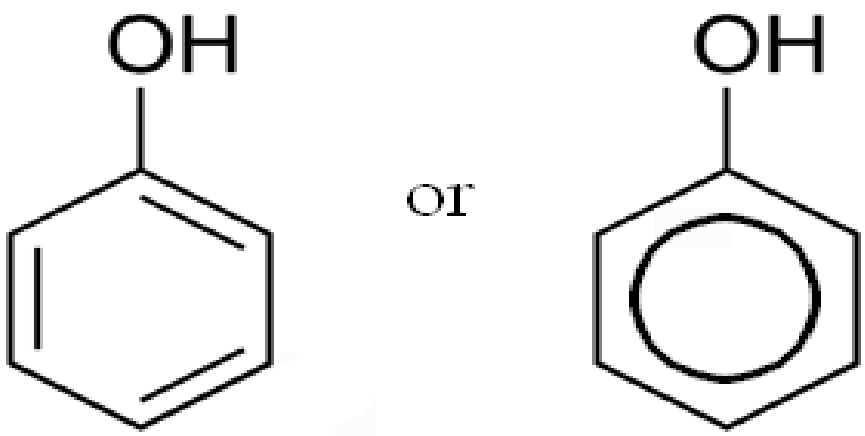

Figure 1: Basic chemical structure of phenols.

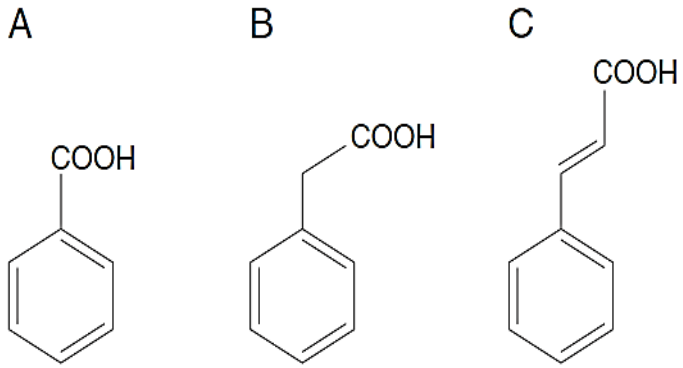

Figure 2: Basic structure of phenolic acids. (A) hydophenylacetic acid, (B, C) hydroxycinnamic acids.

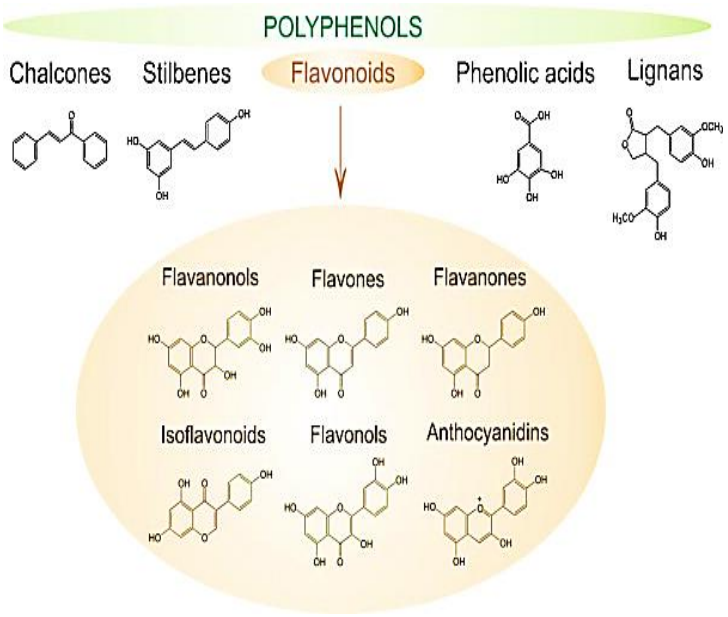

Figure 3: Polyphenols including the structures of the various flavonoid classes.

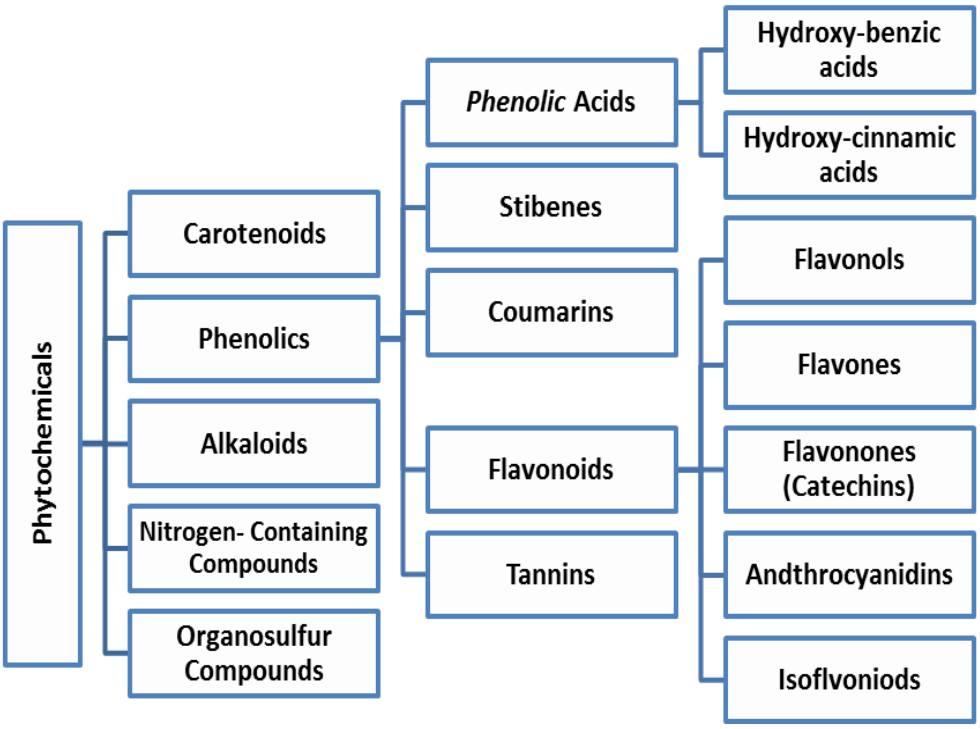

Figure 4: Classification of phytochemicals.

The majority of phenolic acids are derivatives to cellulose through ester, ether, and acetyl bonds (Stalikas, 2007).

Phenols are highly reactive because they contain one or more hydroxyl groups attached to an aromatic ring (Figure 1). One class of phenols, phenolic acids, (Figures 2-4) are further categorized into two groups, i.e., hydrophenylacetic and hydroxycinnamic acid, with the latter being the most abundant (Han et al., 2007). In particular, the phenolic acids present in dry beans include ferulic, sinapic and chlorogenic acid along with numerous triterpenoids, all of which have been shown to function as both antioxidant and an anti-inflammatory agents (Thomson et al., 2012; Han et al., 2007). As antioxidants, phenolic acids act as free radical chain-breaking agents. Their hydrogen and electron donating capacity thus have the ability to stabilize oxidizing stress that may result, if left unchecked. The antioxidants provide electrons to free radicals and prevent them from oxidizing nearby cells (Teixeira et al., 2013). Despite the potential health benefiting properties of plant polyphenols, the body does not easily absorb polyphenols (Rondini & Bennick, 2012). One accepted hypothesis that the poor bioavailability is due to active glucuronidation system of intestinal cells. When phenols are digested, the system quickly exports the phenols; they are glucuronidated and moved through the digestive system to the gut (Bennick & Rondini, 2008). The second issue is that the small amount of phenols that escape glucuronidation are filtered through the liver and then excreted as bile (Bennick & Rondini, 2008). Yet, even though low amounts of phenols are actually absorbed into the blood, it has been widely accepted that they are highly beneficial to the well-being of individuals (Bennick & Rondini, 2008; Huang et al., 1992; Jacob & Burri, 1996, Oomah et al., 2005).

A.4.2 *Flavonoid Content*. The most studied group of polyphenols is the flavonoids (Figure 3-4) (Pandey et al., 2009). Flavonoids contain two phenyl rings and a heterocyclic ring (Figure 3), which are bonded to one or more hydroxyl groups (Shahidi & Naczk, 1995). The position of these hydroxyl groups on the ring aid in further categorizing the flavonoids into different categories (Figure 3) (Shahidi & Naczk, 1995). Flavonoids are typically glycosylated or acetylated, which in turn affects both their chemical and physical properties, (such as solubility). Flavonoids not conjugated to any other molecule, besides hydroxyl groups are called alglycones (Luthria et. al., 2011).

The antioxidant properties of flavonoids are beneficial in protecting human health from various diseases (Bhattacharya et al., 2010; Xu & Chang, 2008; Hui et al., 2010). When ingested by humans, these flavonoid-rich nutrients act by chelating oxidizing metals, i.e., iron and copper, modulating mammalian enzyme activity, enhancing intracellular signaling, strengthening membranes and binding receptor sites along with their antioxidant/free radical scavenging abilities (Lila, 2007; Rice-Evans et al., 1996; Schroeter et al., 2002).

A.4.5 *Tannin Content*. Tannins are classified as polyphenols but differ in their structure due by forming their oxidative linkage with other phenols (Figure 5) (Dai, et al., 2010). Tannins can be further classified as hydrolysable and non-hydrolysable (also called condensed tannins) (Robbins, 2003). The hydrolysable tannins are compounds containing a central core of glucose or another poly esterified gallic acid (known as gallotannin) or hexahydroxydiphenic acid (known as ellagitannins) (Figure 5) (Dai et al., 2010). The condensed tannins are also known as pro-anthocyanins, and along with anthocyanins, provide color and taste (Gomez-Plaza et al., 2011).

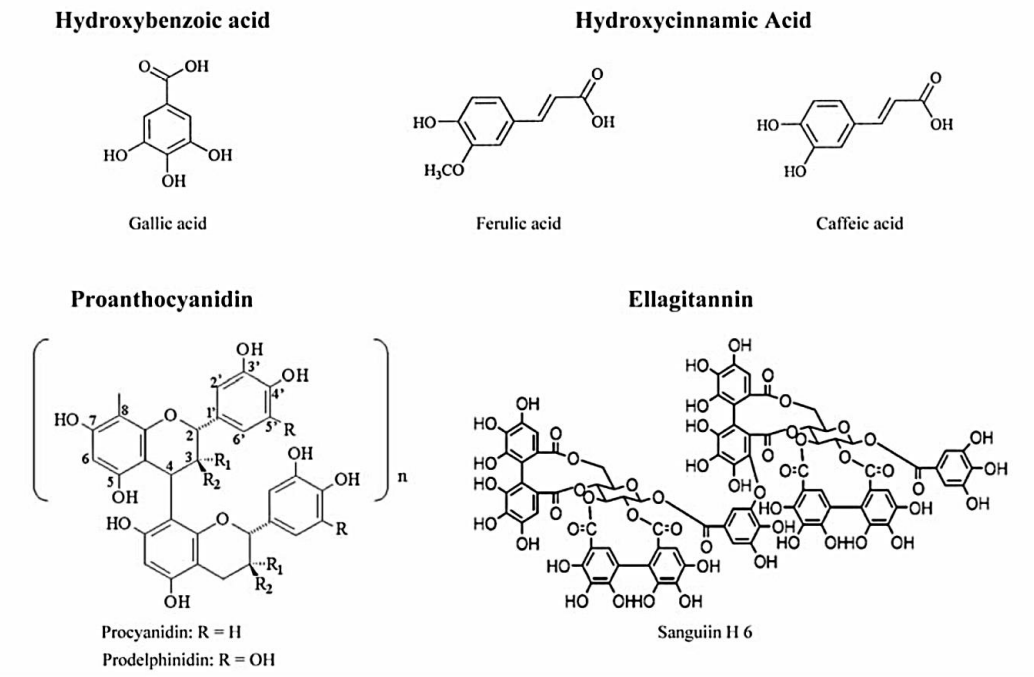

Figure 5. Structures of phenolic acids and tannins.

Condensed tannins are the second most abundant natural phenols after lignins (phenolic acids bonded to each other as one polymer) and have generated intense interest because of their antioxidant capacity and possible protective effects on human health (Gu et al., 2004; Matilla et al., 2000). Tannins bind to protein by various digestive enzymes thereby blocking their digestion and absorption (Carmona et al., 1996; Sahoo, 2011). The tannins in beans have been shown to inhibit pancreatic trypsin, chymotrypsin and α-amylase (Carmona et al., 1991).

A.4.4 *Dry Beans and Phenols*. A study completed by Lin et al. (2008) of phenolic acids in twenty four edible dry bean market classes (alubia, black, cranberry, dark red kidney, great northern, light red kidney, navy, pink, pinto, and small red, plus eight varieties, belonging to four market classes, pinto (Buster and Othello), black (Jaguar and T-39), navy (Seahawk and Vista) and great northern (Matterhorn and Weihing), and 7 generic off-the-shelf items (purchased from the local grocery store) showed that main phenolic acid structures in all of the market classes were the hydroxycinnamic acids with the flavonoid components being less prominent (Lin et al., 2008).

The researchers demonstrated that all three black bean cultivars contained the flavonoid, anthocyanins, which are known for its high antioxidant properties (Lin et al., 2008). In yet another study, black bean consumption was able to protect against cardiovascular disease and breast cancer (Xu et al., 2007), which was attributed to its high antioxidative capacity. It must be noted that other epidemiological studies have also shown a correlation between the consumption of foods with antioxidant values and a reduced incidence of several degenerative diseases (Galli et al., 2006; Garcia-Lafuente et al., 2009; Tracey, 2002).

A study conducted at the Beijing Normal University in Hong Kong investigated two types of common beans (pinto and black beans) and two types of soybeans (yellow and black) and determined that uncooked raw beans exhibited higher cellular antioxidant activities (CAA) as lower malondialdehyde (MDA) production, i.e., a byproduct of lipid peroxidation occurred in the liver of mice fed these beans. In particular, black soybeans exhibited the greatest CAA followed by black beans (Xu & Chang, 2011). In a similar study, Lin et al. (2001) examined the inhibitory effect of different combinations of hot water extracts (HWEL) obtained from each bean type using the same model as described above. Their results showed that legumes (mung beans, adzuki beans, black beans, and rice beans) protected against damage to cell membranes by reducing the level of lipid peroxides. Yet, treatment with HWEL extracted black bean exhibited the most protective properties as evidenced by the production of lower lipid peroixdes compared with mung beans, adzuki beans, and rice beans (Lin et al., 2001).

Xu and Chang (2009) studied the phenol content black beans and discovered that black beans had five anthocyanins namely, delphinidin-3-glucoside, malvidin-3, 5-diglucoside, petunidin-3-glucoside, malvidin-3-galactoside, and malvidin-3-glucoside. Research by Carmona et al. (1996) reported that the condensed tannins found in black beans formed a tannin-protein complex that impaired macronutrient utilization in the small intestine (Carmona et al., 1996).

A.5 Extraction of Phenols from Natural Systems

Characterization of extraction methods targeting plant phenols from any natural systems requires sample preparation, separation, detection and identification. With respect to only the extraction method, traditional methods have evolved over the past two

decades. Current methods are far more advanced with the most common techniques involving liquid:liquid and liquid:solid methods (Krygier et al., 1982). Solvents used to extract phenols from various natural systems include methanol, ethanol, acetone, diethyl ether and/or ethyl acetate (Altuner et al., 2012; Liazid et al., 2007; Santana et al., 2009). Moreover, enzymatic reactions have been used to release phenolic acids from cell walls or from other derivatives (Altuner et al., 2012; Liazid et al., 2007; Santana et al., 2009). In addion, such factors, as pH, temperature, sample-to-solvent volume ratio and the number and time intervals of individual extraction steps can effect extraction (Krygier et al., 1982). These factors change for different naturals systems, phenolic compounds are especially targeted due to their chemical diversity. Other phenolic based extractions have been be completed by supercritical fluid extraction, pressure fluid extraction, microwave assisted extraction or through mechanical means such as vortexing followed by centrifugation, mechanical stirring and continuous rotary extraction, which is the most common approach (Lin et al., 1999; Perry, 1999; Pineiro et al., 2004; Song et al., 2007).

In many of these cases, one variable has been changed to characterize the extraction method, but this approach is time consuming and costly. Other studies have simply used previously cited literature without any additional characterization. This strategy provides only a fraction of the information concerning amount or types of phenols present in any natural system. Therefore, the extraction method used for each natural system must be characterized to better understand the effects on phenol recovery (type and levels). For this study, a response surface methodology (RSM) approach was used to determine levels of phenols only, as explained below.

A.5.1 *Response Surface Methodology*. The origin of (RSM) and the use of response curves can be traced back to the 1930's when Box and Wilson developed experimental designs for fitting linear response surface methods to maximize high yield and purity of compounds as means to lower costs (Dean & Voss, 1999). As a result, first-order and second-order models were established to optimize chemical reactions in the industry's manufacturing process (Dean & Voss, 1999). This approach consisted of using sequential experimentation that consisted of multiple variables instead of just one. The study showed that the "response is a quantitative continuous variable (e.g., yield, purity, cost), and the mean response is a smooth but unknown function of the levels of p factors (e.g., temperature, pressure), which are real-values and accurately controllable" (Dean & Voss, 1999).

Response surface methods have since provided researchers a means to collect information from experiments where multiple but combined independent variables are used and then analyze their influence on a given response. In brief, RSM uses statistics to identify the optimum conditions for the desired outcome. For example, if a researcher is studying the growth of a seedling, the independent variables (*x)* could be water and light. These variables have a continuous range of values and adjusting either independent variable can change the dependent variable *(y*). The dependent variable in this example is growth of the seedling. Growth is affected by different combinations of the independent variables, i.e., $x_{1,}$ $x_{2,}$ for light and water, respectively.

As stated previously, two types of modeling are used for RSM, i.e., first order and second order. For the purpose of our experiment, a second-order model was applied, which is the most frequently used model due as it is flexible yet highly structured and can

be used to approximate the optimum point (Shukla & Nishkam, 2014). This experiment design can capture a curvature in the response surface and approximate the interaction of 2 variables (Bradley, 2007). The model used for a second-order modeling was:

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2}^{k} b_{ji} X_i X_j$$

Several designs used for second-order models with the most common being the 3k factorial design, Doehlert, Box-Behnken and central composite designs (CCDs). For this project, the more popular second-order design, central composite design (CCD), was used. The CCD, reported by Box and Wilson in 1951, "combines a two-level full or fractional factorial design with additional start points, and at least one point at the center of the experimental region" (Shukla & Nishkam, 2014). Central composite designs consist of first-order design with factorial points, center points and axial points (Dean & Voss, 1999). Khuri (2006) explained that the standard CCD, also called a face-centered cube design (FCCD), is less burdensome to implement because the center of the design region allows for the estimation of the quadratic terms. For the FCCD design, 15 points can be used to generate a CCD when three factors and three variables are used as was the approach for this project (refer to Materials and Methods). When more center points are added, the power of the method increases. The FCCD design with three center points was thus applied to characterize the extraction of phenolic rich extracts from black beans capable of inhibiting α-amylase and α-glucosidase.

A.6 <u>Diabetes Mellitus</u>

The World Health Organization (2016) describes diabetes as a chronic disease that occurs when the pancreas does not produce enough of the hormone, insulin, to regulate blood sugar (Type 1 diabetes) or when the body cannot effectively use the

insulin it does produce (Type 2 diabetes) (WHO, 2016). Several factors lead to the dysregulation of blood absorption of glucose. For example, glucose uptake in the intestines is inhibited the body can control blood glucose levels after a meal by promoting glucose disposal in tissues (Thilagam et al., 2012). The raised blood sugar can damage then progress to damaging the body's nerves and blood cells. Over time, these risk factors can lead to heart disease, stroke, reduced blood flow with nerve damage in the feet; blindness, kidney failure, and an overall risk of death that is double that of peers without diabetes (WHO, 2016).

A.6.1 *Prevalence of Diabetes*. Type 2 diabetes is a growing problem with 12 percent of the global health expenditure spent on diabetes (International Diabetes Foundation, 2016)**.** Globally, there are currently more than 415 million diabetics, and that number is expected to grow exceed 642 million by the year 2040 (Tabak et al., 2012) International Diabetes Foundation, 2016). More than a half million children in the U.S. have type 1 diabetes, and 1 in 7 births are affected by gestational diabetes (International Diabetes Foundation, 2016). For U.S. adults, the problem is even more dramatic with approximately 1 in 3 adults being pre-diabetic, and 9 in 10 of these people being unaware of their condition (Danael et al., 2011). Among the young, more than 86 million people under the age of twenty and 51 percent of adults of over the age of 65 are currently pre-diabetic (Danael et al., 2011). Pre-diabetes predisposes people to greater risk of heart disease, stroke, nerve damage, kidney failure, and eye problems (Lee et al., 2014; Plantinga et al., 2010)

A.6.2 *Role of α-glucosidase and α-amylase*. Plant-derived foods have a complex mixture of interacting natural chemicals (Sreerama et al., 2012). Among these

compounds, the flavonoid class of phytochemicals, or bioflavonoids, have shown to be effective in combating type II diabetes or noninsulin-dependent diabetes mellitus (NIDDM) by acting (directly or indirectly) on key enzymes involved in glucose absorption, triglyceride absorption and pancreatic lipase (Sreerama et al., 2012). Inhibition of these enzymes could be a key strategy in controlling diabetes (Sreerama et al., 2012). In particular, inhibition of α-glucosidase and α-amylase (located in the mouth, pancreas, and small intestine, respectively) are possible therapeutic options, because these enzymes inhibit the breakdown of starch and disaccharides thereby reducing the rate of glucose entering the bloodstream (Torpy, 2006) (Figure 6 and 7).
A diabetic is thus better able to regulate their blood sugar with effective inhibitors of both these enzymes (Adisakwattana et al., 2009).

Many dietary components have been shown to inhibit α-glucosidase and α-amylase, including the phenols, as stated previously. For example, Nile and Park (2014) determined that phenols in maize (such as phenolics, flavonoids and carotenoids) had a strong inhibitory effect against intestinal α-glucosidase. A New Zealand study of blueberries showed that the Highbush blueberries were able to inhibit both carbohydrate hydrolyzing enzymes, α-amylase and α-glucosidase (Pranprawit et al., 2014). Another study completed by Oboh et al. (2014) examined the effect of phenols and other antioxidants in several commercial teas (one green tea (GT), two black teas (BT), and a special herb tea of white tea, hawthorn berry and Chinese yam fragrant Solomon seal rhizome) on inhibiting α-amylase and α-glucosidase.

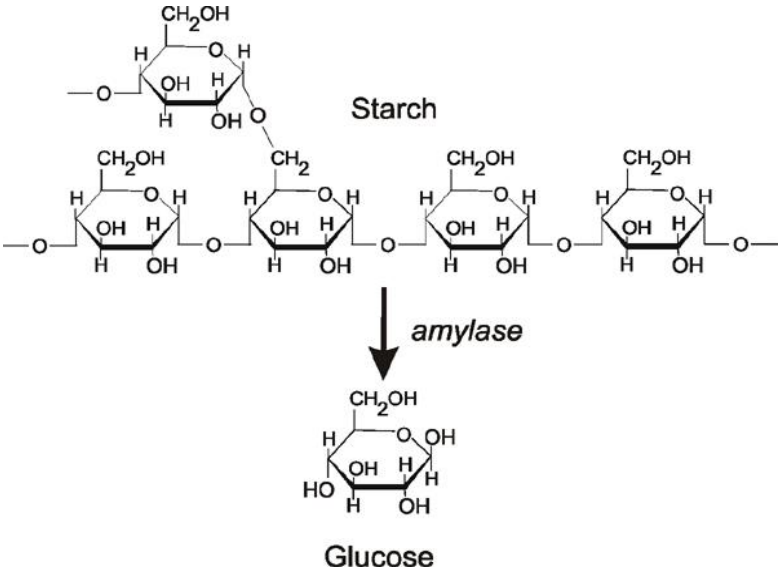

Figure 6a: Amylase breaking down starch to glucose.

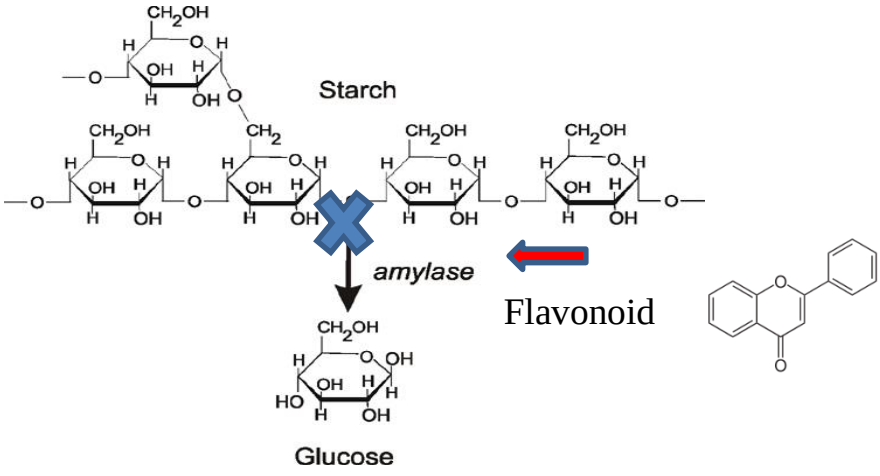

Figure 6b: A flavonoid inhibiting the amylase and thus the immediate breakdown of starch into glucose.

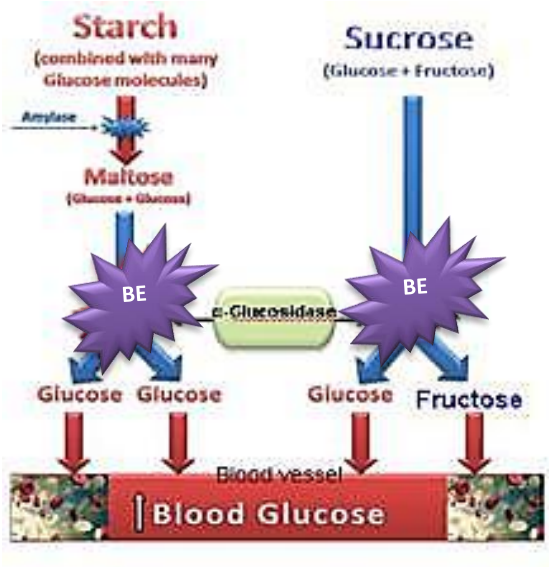

Figure 7a: Enzymatic digestion of starch and sucrose to glucose.

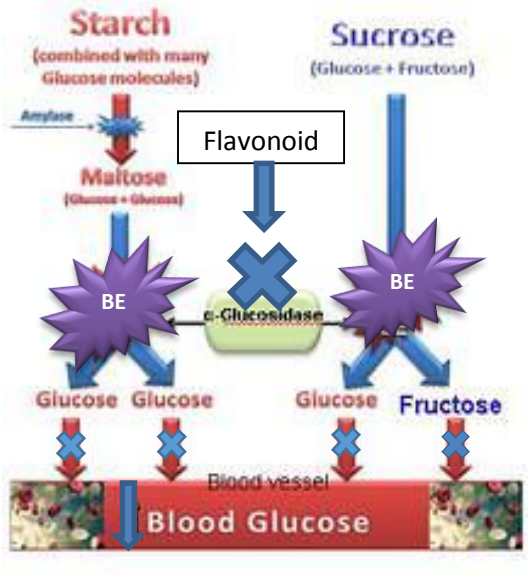

Figure 7b: Examples of flavonoid inhibiting α-glucosidase thereby blocking glucose production.

Figure 7: Enzymatic digestion of dietary carbohydrate (starch, maltose, and sucrose) in biological systems and the inhibition of intestinal α-glucosidase and subsequent decrease or delay in blood sugar levels after eating a meal

The researchers reported that GT had the highest phenolic and antioxidant content but did not outperform the other teas in their anti-diabetic potential (Oboh et al., 2014) as all inhibited pancreatic α-amylase and the intestinal α-glucosidase enzymes (Oboh et al., 2014). Tormo et al. (2004) researched the ability of legumes, specifically white kidney beans, to release glucose in the by inhibiting α-amylase. The researchers reported the hypoglycemic activity of bean α-amylase inhibition. McCarty (2005) suggested that further studies are warranted into the effectiveness of legumes in inhibiting carbohydrate hydrolytic enzymes to aid in reducing diabetes.

B. OBJECTIVE and SPECIFIC AIMS

The *objective of this project* was to characterize the extraction methods for extracting phenolic-rich extracts using response surface methodologies (RSM) from black beans capable of inhibiting α-amylase and α-glucosidase. This research is tied to black beans due to their high phenolic quantities. Moreover, consumption is increasing in the United States as well as in other parts of the world. Despite this slow but steady increase, studies have yet to be completed on characterizing the extraction methods to obtain the phenols from black beans, which limits our ability to study the bean as whole dietary system or compare it to other results reported in the literature. Therefore, prior to analyzing the ability of black beans to inhibit the cited enzymes, extraction methods for TP, TF and TCT were characterized. The objective of this project was thus satisfied by completing the following specific aims.

B.1 <u>Specific Aim #1</u>: To characterize the extraction procedures using RSM in terms of solvent (methanol, ethanol and acetone), solvent polarity, mixing time and solid:solvent ratio for obtaining extracts for obtaining phenolic rich black bean extracts. Use this data

to construct a model and formulate an extraction equation for determining the optimal methods for obtaining TP, TF and TCT. This approach was expected to aid in the evaluation of the effects of the different factors relative to one another in recovering phenolic rich extracts.

B.2 Specific Aim #2: To determine if the phenolic rich extracts obtained from each RSM point are able to inhibit α-amylase and α-glycosidase inhibition. In addition, to determine a given extraction procedures was more effective in inhibiting a given enzyme relative to the other, and if this was related to the amount of phenols or type of phenols present.

C. MATERIALS and METHODS

C.1 Chemicals and Reagents

C.1.1 *Reagent and Standard Information.* Extraction solvents for Aim 1 including methanol, ethanol, and acetone, were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Three other reagents used for the study, sodium carbonate, sodium nitrite and hydrochloric acid, were also provided by Fisher Scientific. Other reagents were purchased from various vendors, including Folin-Ciocalteu, (MP Biomedical Inc., Solon, OH), aluminum chloride, vanillin (Acros Organics Inc., Fair Lawn, NJ), and sodium hydroxide (Beckton Dickinson Co., West Chester, PA). The standards used for the phenolic tests, (gallic acid), flavonoid (catechins) and tannin (catechins), were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Additional reagents used in Aim 2 included sodium phosphate monobasic monohydrate, sodium chloride and sodium potassium tartrate, which were provided by Fisher Scientific. Starch was provided by J.T. Baker (Center Valley, PA), whereas sodium hydroxide, dinitrosalicylic acid color reagent porcine amylase enzyme,

nitrophenyl B-D-glucopyranoside and rat intestinal powder was provided by Sigma Aldrich Chemical Co. Lastly, acarbose solution was purchased from LKT Laboratories, Inc. (St. Paul, MN).

C.1.2 *Sample Information.* The black beans (*Phaseolus vulgaris)* used in the experiment were grown in 2014 by Dr. Carlos Urrea (Department of Agronomy and Horticulture at the University of Nebraska Panhandle Research and Extension Center). Upon arrival, the beans were maintained at -20 ^{o}C until prepared for analysis.

C.2 Specific Aim 1

C.2.1 *Response Surface Methodology (RSM) to Phenolic Extractions***.** The bean samples were prepared for analysis by initially grinding to a fine powder using an electric grinder. To ensure consistent particle sizes, the powder was passed through a 9 mesh sieve to remove large solids (a corresponding opening size of 2.00 mm). The flour that passed through the sieve was maintained for the further analysis, while the flour that did not was subjected to addition grinding and passed through the sieve again.

A RSM approach (three-factor-three-level face-centered cube design) (Table 3) was used to extract the phenols, flavonoids and tannins from the beans by using three solvents (methanol, ethanol and acetone). Three factors were adjusted for each solvent that included polarity ratios (75:25, 50:50, and 25:75), solid:solvent ratios (30%, 20%, and 10%) over varied mix times (60, 120, and 180 minutes). Therefore, this three factor, three level design resulted in 17 different samples extractions with three center points. (The coded and non-coded values are shown in Table 4). More specifically, the solid amounts were adjusted accordingly to maintain a 3-5 ml final extraction volume.

Table 3: Levels of independent variables for extraction process based on central composite face centered design

Independent Variable	Units	Factor	Coded Levels		
			-1	0	+ 1
*Organic Solvent : Water	(v:v)	X1	25:75	50:50	75:25
Solid : Volume	(%)	X2	10%	20%	30%
Time	(min)	X3	60	120	180

***Methanol, ethanol, or acetone:water ratio**

Table 4: Three factor, three-level face-centered cube design with three center points used for RSM, (coded and uncoded) parameters

Standard Order	Factor X_1	Factor X_2	Factor X_3	Solvent Ratio	Solid:Vol (%)	Time (min)
1	0	1	0	50-50	30	120
2	0	0	1	50-50	20	180
3	-1	1	-1	25-75	30	60
4	0	0	-1	50-50	20	60
5	1	0	0	75-25	20	120
6	-1	0	0	25-75	20	120
7	-1	-1	1	25-75	10	180
8	0	0	0	50-50	20	120
9	1	-1	1	75-25	10	180
10	1	1	-1	75-25	30	60
11	-1	-1	-1	25-75	10	60
12	1	-1	-1	75-25	10	60
13	0	0	0	50-50	20	120
14	0	-1	0	50-50	10	120
15	0	0	0	50-50	20	120
16	1	1	1	75-25	30	180
17	-1	1	1	25-75	30	180

The suspension was mixed horizontally under steady rocking for the designated time period at room temperature. The samples were then centrifuged at a temperature of 25 ºC for at least 10 minutes. The supernatant was collected and analyzed for TP, TF and TCT content, as described below. Each extraction was performed in triplicate.

C.2.2 *Phenolic Analysis of Sample Extracts.*

C.2.2.a Total Phenolic Assay: The Folin-Ciocalteu method was used to determine TP levels in the collected supernatant as described by Singleton and Rossi (1965) at room temperature with intermittent shaking. Detection of the phenols was achieved with a UV-Vis spectrometer (Beckman Coulter, Brea, CA) at a wavelength of 760 nm. A standard calibration curve using gallic acid was plotted to calculate the results. Total phenols were thus expressed in mg gallic acid / g black bean powder in mean ± standard deviation.

C.2.2.b Total Flavonoid Assay: Quantification of flavonoids was accomplished by using the aluminum chloride assay as described by Adom and Liu (2002). The sample supernatant (125 µL) obtained from centrifuging the RSM extracts was combined with 37.5 µL of 5 percent (w/v) sodium nitrite and 0.625 mL of nanopure water. After allowing the reagent to react with the sample for 4-6 minutes at room temperature, 75 µL of 10 percent (w/v) aluminum chloride was added to each sample, followed by 0.25 mL of 1.0 M sodium hydroxide. Nanopure water (0.4 mL) was combined with the samples and mixed for 5-7 minutes. After vortexing the mixture, an aliquot was measured at wavelength 510 nm. Total flavonoids were expressed as mg catechin / g black bean powder in mean ± standard deviation.

C.2.2.c Total Condensed Tannin Assay: To quantify TCT, 250 µL of the prepared sample supernatant was combined with 1.00 ml 4 percent vanillin, 250 µL of catechin

and 0.500 ml concentrated HCl, which was based on the procedure cited be Bhat et al. (2007). After mixing, each sample was incubated at room temperature in the dark. A 4 percent vanillin solution (1 ml) was added to each sample (250 µL supernatant + 250 µL Catechin) and mixed. Concentrated hydrochloric acid was then added and the samples were mixed again, and then the sample's absorbance was monitored at a wavelength of 500 nm. Total tannins were expressed as mg catechin / black bean powder in mean ± standard deviation.

C.2.2.d RSM Analysis and Regression Equations: The behavior of each the extraction parameters relative to extracting the given components was analyzed by a second degree polynomial equation, as shown below:

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2}^{k} b_{ji} X_i X_j$$

Where Y is the response, b_o is the constant coefficient, b_i are the linear coefficients, b_{ii} are the quadratic coefficients, b_{ij} are the interaction coefficients, and X_i and X_j are the coded values of the independent variables. To perform this operation, Stats Graphic, Centerium, (version 26, Warrenton, VA) was used to develop a regression equation between extraction variables and TP, TF and TCT.

C.2.3 *Statistical Analysis and Verification of the Model.* All determinations were completed in triplicate and the experimental results were expressed as the mean ± SD. The statistical analyses were performed using Stats Graph Centerium (version 26, Warrenton, VA). The RSM experimental data were analyzed by multiple regression analysis through the least squares method. The model and the regression coefficients involved in the model and their effect were analyzed by Pareto ANOVA charts and were considered significant at $p < 0.1$. The fitness of the regression curve was further evaluated

by determining the correlation coefficient for the model R^2 (>75), whereas the ability of the model to fit the experimental data was assessed by a lack of fit test ($p>0.05$). Regression equations were formulated based on whether the data obtained from each solvent system complied with the criteria stated in this section.

C.3 Specific Aim 2

C.3.1 *Sample Preparation*. For Aim 2, the supernatants (prepared from Aim 1) were placed in pre-weighed test tubes at 2 ml aliquots and dried to a residue at room temperature using an Isotemp-Vacuum Oven, Model 280A (manufactured by Fischer Scientific) for a period of 7-10 days with daily monitoring. The samples were removed before they were completely dry, and the drying process was completed by purging gently with nitrogen. The samples plus the tests tubes were then weighed again to determine final weight of the residue. Samples were stored for up to two weeks under nitrogen in the freezer until analyzed as described below.

C.3.2 *Enzymatic Analysis of Sample Extracts*.

C.3.2.a Alpha-Amylase Assay: This procedure was conducted by prepared five different solutions; 1) a *control blank* 2) a *blank* 3) a *test blank* 4) an *experimental sample* group and 5) a *known inhibitor/positive control group (acarbose).* (Each was prepared as shown in Table 5.) Dimethyl sulfide was added to each test tube because the extract residue would not completely dissolve into an aqueous based solution with this compound. Therefore, both the enzyme and the positive control contained DMSO to ensure that it did not affect the assay. (Note: Dimethyl sulfide was added to the test sample at this point, not during storage, and the assay was completed immediately to avoid oxidation by DMSO). Each solution was mixed and pre-incubated at room

Table 5: Preparation of samples and blanks for α-amylase test

Solution	Control Blank	Blank	Experimental Blank*	Experimental Sample*	Positive Control
Amylase	0.150 mL			0.150 mL	0.150 mL
Exp Sample*			0.150 mL	0.150 mL	
DMSO **	0.150 mL	0.150 mL			
20 mM phosphate		0.150 mL	0.150 mL		
Acarbose(1 mM)					0.150 mL

**Experimental samples of extracts/compounds being* tested *for inhibitory activity*

*** 1% solution of Dimethyl Sulfoxide (DMSO)*

temperature for 10 ± 1 minute. The reaction was initiated by transferring 0.150 mM of 0.5 percent starch solution to each sample followed by mixing and incubating at room temperature for an additional 30±1 min. Next, the reaction was stopped by adding 0.300 ml of DSN color reactant to each sample (Table 5) with gentle mixing. The samples were then placed in a bath of boiling water for 10 ± 0.5 minutes and cooled to room temperature. Each sample was diluted with 2 ml of nanopure water and the absorbance was determined at a wavelength of 540 nm. The effectiveness of the sample extract was then calculated by the following equations:

$$ExpB = A540Exp - A540ExpBlank$$

The percentage of α-amylase inhibition for each sample was then determined by the following equation:

$$\% \ Inhibition = \frac{A540Exp - A540ExpB}{A540 \ control} \times 100$$

C.3.2.b Alpha-Glucosidase Assay: Similar to the above assay, five different solutions were initially established for this test as Shown in Table 6. Each solution was mixed and pre-incubated at 37 ^{o}C temperature for 30±0.5min. The reaction was initiated by adding 300 µl of 1 mM 4-nitrophenyl β-D-glucopyranoside (PNPG) solution to each sample and incubated at 37 ^{o}C for 20±0.5 min. Next, the reaction was stopped by placing samples in a bath of boiling water for 10±0.5min. After allowing the samples to cool to room temperature, each were diluted in 1.5 ml nanopure water and then measured at a wavelength of 400 nm absorbance.

Table 6. Preparation of samples and blanks for α-glucosidase test

Solution	Control Blank	Blank	Experimental Blank*	Experimental Sample*	Positive Control
Rat Intestinal	100 µL			100 µL	100 µL
Exper Sample*			100 µL	100 µL	
DMSO**	100 µL	200 µL	100 µL		
Acarbose (1mM)					100 µL

** Experimental samples of extracts/compounds being tested for inhibitory activity*

*** 1 % solution of Dimethyl Sulfoxide (DMSO)*

The following equation was used to determine each extract's inhibition ability.

$$ExpB = A400Exp - A400ExpB$$

Determining the α-glucosidase inhibition in the control group was calculated by subtracting the absorbance of the control blank from the absorbance of the control group. The calculation for the *control test blank* was:

$$ContB = A400Con - A400ConB$$

The percentage of α-amylase inhibition for each sample was determined by the following equation:

$$\% \ Inhibition = \frac{A400ConB - A400ExpB}{A400Con} \times 100$$

C.3.3 *Statistical Analysis.* All methods and procedures were completed in triplicate and the experimental results were expressed as a mean ± SD after the results were normalized to percent Inhibition/mg of residues. In addition, TP, TF and TCT were calculated in each of the residue based on the original extract amounts obtained from the RSM values but normalized and expressed as μg / mg of residue. The statistical analysis was performed using StatsGraph Centerium (version 26, Warrenton, VA). The experimental data used regression analysis, and compared the inhibition of α-amylase and α-glucosidase with TP, TF and TCT.

D. RESULTS AND DISCUSSION

D.1 *Specific Aim 1*: *Response Surface Approach to Phenolic Extractions*

D1.1 *RSM Characterization: Selection of Independent Variables*. For Specific Aim 1, the phenolic-rich extracts originating from black beans were extracted using a solid:liquid extraction method, which is well suited for removing soluble fractions from an insoluble solid (Cacace et al., 2003; Shahidi & Naczk,1995; Wettasinghe & Shahidi, 1999). Applying RSM to such extractions allows for more experimental factors to be monitored with lower sample numbers compared to the univariate approach. For the purpose of this study, mass transfer rate and equilibrium were controlled by the design parameters. By using an RSM method that consisted of extracting soluble black bean phenols (which have different chemistries) and three solvents (methanol, ethanol and acetone) and adjusting for three factors at three levels,(i.e., solvent polarity, mixing time and solid:solvent ratio) to obtain black bean extracts with high levels of TP, TF and TCT (Dai et al., 2010). Reponses generated from provided the information to characterize the most effective extraction procedure and allowed an understanding of their influence (e.g., the independent and/or interactive effects of these parameters).

More specifically, the black bean samples used solvent:water ratios of 75:25, 50:50, and 25:75 for methanol, ethanol and acetone. Considering that water promotes solubility of phenolic compounds, the polarity each of the solvents were adjusted water This hypothesis was supported by Xu et al. (2007) who showed that different proportions of solvents and water affects the amount and rate of polyphenols extracted (Xu et al., 2007). Methanol was selected because it has been reported to be more efficient with a lower molecular weight polyphenols, while acetone is more efficient with higher

molecular weight such as flavonols (Metivier et al., 1980). Lastly, as ethanol has a polarity between methanol and acetone, it provided provide to provide an intermediate solvent between methanol and acetone to ensure most phenols were recovered (Metivier et al., 1980; Prior et al., 2001; Guyot et al., 2001; Labarbe et al., 1999).

The study used a solid:volume percentages of 30, 20 and 10 percent, and used a rocker to mix solvents for over a period of 180 120 and 60 minutes. These parameters were used as the literature has shown that they have been effective in isolated phenols from other natural types of natural systems (Rostanogo et al., 2004; Xu et al., 2007). The extracts were then tested to determine the optimum extraction method for TP, TF and TCT.

D.1.2 *Total Phenols.*

D.1.2.a *TP results obtained from FCCD-RSM:* This study assigned values of 1+, 0 and -1 to the independent variables (percent of solids:solvent, the ratio of solvent:water and mixing time). Each of the three independent variables were randomized and entered as absolute values into the Table 7. Data are shown as the mean ± standard deviation (n=3), whereas the range produced by each are shown in Table 8.

As evidenced by both Table 7 and 8, the greatest TP extraction efficiencies occurred with acetone; whereas the ethanol solvent mixture produced the lowest overall TP extraction. More specifically, Extract 9 of the acetone samples produced the highest TP levels (3.73 mg/g); whereas Extract 7 and 14 produced the highest TP values for the ethanol (1.99 mg/g) and the methanol systems (1.68 mg/g), respectively.

The range for the acetone extractions was much greater (Table 8) compared to methanol and ethanol; even the lowest TP levels for acetone extractions were only

Table 7: Total phenol response in mg/g using varied extraction conditions FCCD that consisted of with three center points, three solvent systems and three experimental factors for each solvent system (methanol, ethanol and acetone).

Std. Order	Methanol	Ethanol	Acetone
1	1.12 ± 0.06	1.37 ± 0.06	2.20 ± 0.08
2	1.49 ± 0.04	1.72 ± 0.01	2.70 ± 0.06
3	1.09 ± 0.02	1.46 ± 0.07	1.71 ± 0.07
4	1.19 ± 0.02	1.65 ± 0.05	2.52 ± 0.05
5	0.90 ± 0.04	0.85 ± 0.02	2.73 ± 0.15
6	1.36 ± 0.07	1.68 ± 0.03	1.99 ± 0.19
7	1.44 ± 0.03	1.99 ± 0.06	2.23 ± 0.12
8	1.32 ± 0.05	1.69 ± 0.06	2.52 ± 0.03
9	1.17 ± 0.14	1.23 ± 0.12	3.73 ± 0.13
10	0.63 ± 0.06	0.57 ± 0.01	2.23 ± 0.04
11	1.35 ± 0.07	1.78 ± 0.01	2.44 ± 0.07
12	0.88 ± 0.01	0.91 ± 0.10	3.55 ± 0.11
13	1.47 ± 0.14	1.55 ± 0.10	2.87 ± 0.03
14	1.68 ± 0.05	1.95 ± 0.05	3.71 ± 0.19
15	1.40 ± 0.05	1.61 ± 0.00	2.52 ± 0.07
16	0.78 ± 0.03	0.73 ± 0.03	1.45 ± 0.02
17	1.20 ± 0.02	1.26 ± 0.02	1.87 ± 0.08

* Data are shown as the mean ± standard deviation (n=3)

Table 8: **Ranges** of total phenols for each solvent system

Extraction Solvent	Total Phenols (mg/g)	Range (mg/g)
Methanol	0.63 – 1.68	1.05
Ethanol	0.57 – 1.99	1.42
Acetone	1.45 – 3.73	2.28

slightly lower than the highest TP levels for either methanol and ethanol extractions (Table 8). These results indicates the overall extraction procedure when using acetone has to be very specific to obtain specific TP level, while less TP values differences occurred when using different extraction factors for methanol and ethanol. Although lower levels were obtained for TP when using the methanol and ethanol, the different factors did not affect the TP values greatly making both extractions more rugged compared to acetone. It must be noted that, considering that the majority of phenols were extracted with the most non-polar solvent, the phenols had similar chemistry (more hydrophobic), which could occur by the extract containing minimal polar conjugates, such as a sugar or hydroxyl groups, and more non-polar alycones phenols (Chavan et al., 2001; Shahidi et al., 2001).

A number of other studies have demonstrated extraction process can affect TP when using different natural systems (Chavan et al., 2001; Ballard et al., 2009; Silva et al., 2007). Specifically for black beans Xu and Chang (2009), who showed that processes such as soaking, boiling and steaming significantly affect the TP content of black beans, indicating that not only the extraction parameters used, such as described herein, affect TP, but so does the unit process cooking. Most of these studies only used one extraction method. Our study utilized different matrices, more than one type of solvent and other response factors to determine TP in any natural system.

D.1.2.b *Fitting the TP model:* The statistical analyses of response values have demonstrated the best fit is the second-order polynomial equation, which provides information on the relationship between the experimental parameters and the response variables (Dean &Voss, 1999). The regression models calculates the predicted values, and then calculates the coefficient of determination (R^2). The closeness to one of the (R^2)

demonstrates high and satisfactory levels of adequacy (Le Man et al., 2010; Chauhan & Gupta, 2004). The high R^2 values for methanol (95.62%), ethanol (98.87%) and acetone (93.86%) provided assurance of low dispersion of experimental data (Table 9). Most of the variability could thus be explained and therefore supports the adequacy of this model for these extractions (Le Man et al., 2010; Chauhan & Gupta, 2004). The equations obtained were tested to determine the variability in the responses. The analysis of variance (ANOVA) of the quadratic model was adequate for all the three solvent systems showed a significant *p*-value >0.1 (Table 9) supporting the adequacy of the model for the extraction of TP for all the solvents.

D.1.2.c *Adequacy of the TP models:* In order to ensure the reliability and value of the equations, the data was examined to determine the adequacy of the data to fit the model (Myers & Montgomery, 2016). To perform this test, the experimental data was entered and statistically analyzed, so that each model could provide assurance for obtaining predictable results. The models allowed for an efficient estimation of each linear effect with a path of steepest ascent, and a significance reflecting the effect on the dependent variable to allow for *lack of fit*. All three solvents passed the "lack of fit the test" with the *p*-value of the lack of fit test for methanol (p=0.4731), ethanol (p=0.6313) and acetone (p=0.4130) all > 0.05 (insignificant) indicating that the data satisfactorily fits the model. (Table 9).

Table 9. Regression coefficients (coded) predicted by the quadratic polynomial model for TP when extracted with the cited solvent systems

Coefficient	Methanol	Ethanol	Acetone
b_o	1.403	1.650	2.695
Linear			
b_1 (SP)	-0.168**	-0.248*	-0.620**
b_2 (S:S)	-0.207**	-0.389*	0.342**
b_3 (MT)	0.093***	0.056	-0.048
Quadratic			
b_{11} (SP x SP)	-0.002	-0.011	0.213
b_{22} (S:S x S:S)	-0.269**	-0.403**	-0.371***
b_{33} (MT x MT)	-0.062	0.014	-0.123
Cross product			
b_{12} (SP x S:S)	-0.018	0.027	-0.314**
b_{13} (SP x MT)	-0.014	-0.072	-0.071
b_{23} (S:S x MT)	0.030	0.058	-0.069
R^2	95.62	98.87	93.86
p values			
Model	0.0006	0.0000	0.0018
Lack of Fit	0.4731	0.6313	0.4130

SP – Solvent Polarity, S:S – Solid: Solvent, MT – Mix Time
***** Significant at 1%, ** significant at 5%, *** significant at 10%

D.1.2.d *Regression Coefficients Equations and Pareto Charts:* The TP regression equations of the RSM extraction for each solvent described in Materials and Methods (Section C2.2.d) are provided in Table 10. The equations are based on the significance of individual regression coefficients only (p<0.01, 0.05, 0.1) (Table 9). All of these p-values were selected to capture the regression coefficients that had an effect on the model, as well as to understand the extent of that effect. Alternatively, the Pareto charts show schematically the overall contribution of each coefficient (Figure 8).The vertical line marks a point of statistical significance greater than 90 percent confidence level. All three solvents showed a negative relationship to solvent:water ratio (Figure 8). Both methanol (Figure 8a) and ethanol (Figure 8b) had a negative relationship with the solid:solvent ratio, while the solid:solvent ratio showed a positive relationship for acetone (Figure 8c). The results demonstrated the greatest influence on TP were the solid:solvent ratio and solvent:water ratio indicating the importance of solvent ratios both in purity and in regards to solids.

D.1.2.e *Final optimized TP values and processing factors:* Based on the design parameters and the model, the optimal processing factors to produce the highest overall TP were determined. All three solvents produced significant results, yet the acetone solvent was the most efficient. The results again suggest that black beans contain mostly are non-polar phenols, including procyanadins as black beans contain high levels of condensed tannins. These results may be due to the presence of these compounds. (Ek et al., 2006; Agarwal et al., 2007). (*This will be discussed later in this chapter.)*

Table 10: Regression equation of the fitted model for TP

TP Methanol = 1.404 – 0.1681*Xsp* – 0.2074*Xss* + 0.0934*Xmt* – 0.2693*Xsp* ^2
TP Ethanol = 1.6500 – 0.2481*Xsp* – 0.3898*Xss* – 0.4040*Xss* ^2
TP Acetone = 2.6960- 0.6208*Xsp* + 0.3429*Xss* – 0.3144*XspXss* – 0.3714*Xss*^2

Xsp– Solvent Polarity, ***Xss*** – Solid: Solvent, Xmt – Mix Time

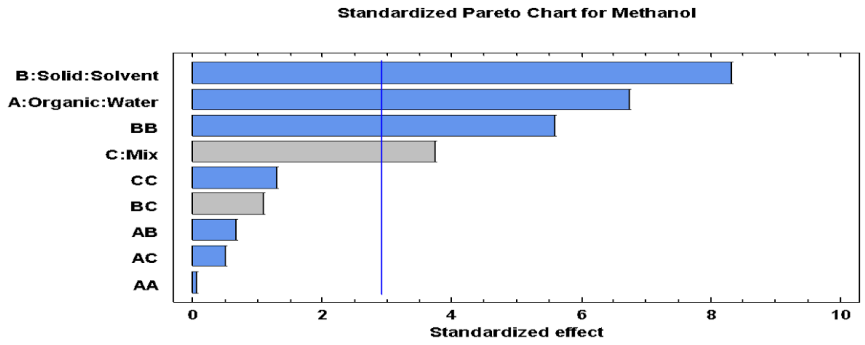

Figure 8a

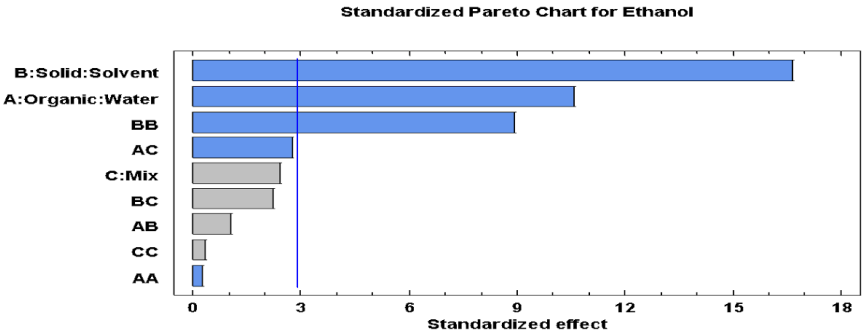

Figure 8b

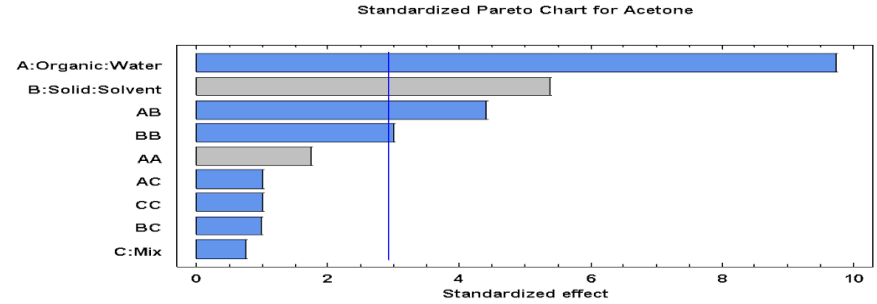

Figure 8c

Figure 8: Pareto charts showing relative effects of regression coefficient for TP accepted models in a) Methanol b) Ethanol c) Acetone. Vertical line represents $p < 0.1$.

The optimized factors generated from the acetone model are: solvent:water ratio (25:75), solid:solvent ratio (18%) and mixing time (~111 minutes) (Table 11). The results show a low proportion of solvent was necessary to attain high TP yields. Other research extracting TP from sunflower meal required a high solvent level (80% acetone) to generate the highest level of TP (Taha et al., 2011).

The main effects in plot visually demonstrated that the TP for all three solvents were negatively affected as the organic: water ratio is adjusted from a 25:75 (coded value -1) to a weaker solvent mix of 75:25 (coded value +1) (Figure 9). It demonstrated that acetone was the only solvent that was positively affected as the solid:solvent ratio increased from 10 percent (coded value -1) to 30 percent (coded value +1) (Figure 9c). Interesting, mixing time had the most effect on ethanol. Based on Figure 9a, more TP may have been obtained with longer methanol mixing time as the line continue to climb at coded +1. A minimal quadratic effect was materializing with acetone and methanol, as the curves (albeit small) were either going down for both +1 and -1 with a small optimal point (acetone) or starting to level off at +1 (methanol). If extended, the curve could have stayed level or begun to decrease.

Table 11. Optimized factors required to generate optimum TP yield (Black beans solids: Acetone solvent)

Factor	*Low*	*High*	*Optimum (coded)*	*Optimum (uncoded)*
Organic: Water	-1.0	1.0	-1.0	25:75
Solid: Solvent	-1.0	1.0	0.899897	18%
Mix	-1.0	1.0	-0.158311	≈111 minutes

Optimum value TP = 3.82463 mg/g

Figure 9a

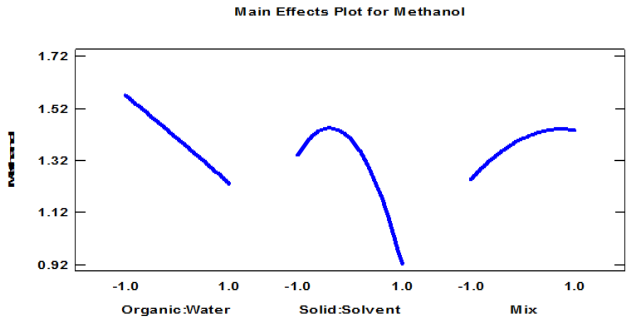

Figure 9b

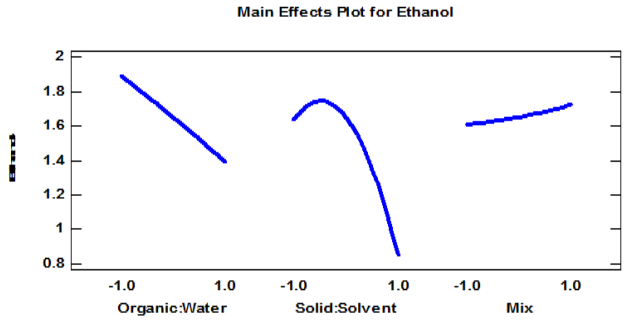

Figure 9c

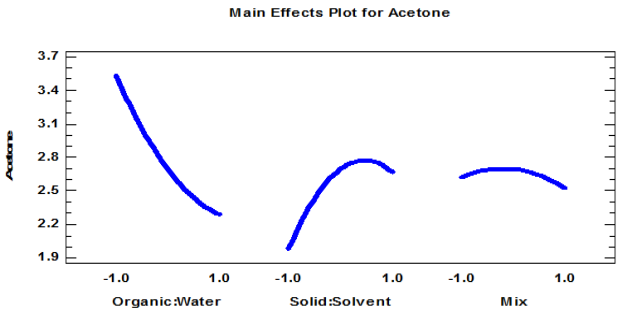

Figure 9. Main effects plot (TP).

D.1.3 Total Flavonoids (TF)

D.1.3.a *TF results obtained face centered composited design (FCCD):* To characterize TF extractions from black beans. These TF results for each extraction are shown in Table 12, whereas the range (high and low) of TF obtained from each of the 17 points are provided in Table 13. The greatest extractions efficiencies of TF were generated with the acetone solvent system; whereas the methanol solvent mixture produced the lowest overall TF extraction. The highest TF levels were extracted with acetone (3.55 mg/g, Extract 9), followed by ethanol (0.92 mg/g, Extract 14), and then methanol (0.72 mg/g, Extract 2).

The range for acetone extractions was much greater (Table 13) compared to those for either methanol and ethanol extracts. Yet, even the lowest TF levels recovered from the acetone extractions exceeded the highest TF from methanol; and were only slightly lower than the highest TF levels for ethanol extraction (Table 13). The broader range indicates that many different values can be obtained just be changing the parameters slightly resulting in very different values. Alternatively, only slight changes in the ethanol and methanol extractions resulted in values that were much closer, indicating the ruggedness of these parameters for extraction TF for these two systems. Previous results have shown aqueous acetone was able to extract high levels of TF. Metivier et al. (1980) researched the extraction of flavonoids from wine grape pomace using methanol, ethanol and water as solvents to extract TF, and determined that the methanol was 20 percent more effective than ethanol and 73 percent more effective than water (Metivier et al., 1980).

Table 12: Total flavonoids response in mg/g using varied extraction conditions FCCD that consisted of with three center points, three solvent systems and three experimental factors for each solvent system (methanol, ethanol and acetone).

Std. Order	Methanol	Ethanol	Acetone
1	0.52 ± 0.00	0.77 ± 0.01	1.79 ± 0.22
2	0.72 ± 0.05	0.81 ± 0.03	2.14 ± 0.12
3	0.39 ± 0.03	0.58 ± 0.01	1.15 ± 0.15
4	0.53 ± 0.00	0.74 ± 0.02	2.18 ± 0.04
5	0.37 ± 0.00	0.58 ± 0.00	3.00 ± 0.03
6	0.48 ± 0.06	0.67 ± 0.02	1.26 ± 0.13
7	0.62 ± 0.09	0.78 ± 0.11	1.33 ± 0.06
8	0.55 ± 0.00	0.72 ± 0.02	2.02 ± 0.03
9	0.36 ± 0.02	0.85 ± 0.03	3.55 ± 0.32
10	0.29 ± 0.01	0.51 ± 0.03	2.14 ± 0.33
11	0.57 ± 0.04	0.66 ± 0.08	1.16 ± 0.15
12	0.29 ± 0.00	0.54 ± 0.08	3.18 ± 0.24
13	0.57 ± 0.06	0.80 ± 0.02	2.52 ± 0.20
14	0.65 ± 0.03	0.92 ± 0.03	2.86 ± 0.18
15	0.59 ± 0.04	0.85 ± 0.03	1.66 ± 0.05
16	0.34 ± 0.01	0.66 ± 0.02	1.39 ± 0.04
17	0.54 ± 0.07	0.68 ± 0.03	0.81 ± 0.06

* Data are shown as the mean ± standard deviation (n=3)

Table 13. Ranges of total flavonoids (TF) for each solvent system

Extraction Solvent	Total Flavonoids (mg/g)	Range (mg/g)
Methanol	0.29 – 0.72	0.43
Ethanol	0.51 – 0.92	0.41
Acetone	0.81 – 3.55	2.74

D.1.3.b *Fitting the TF models:* Similar to fitting the TP models, multiple regression coefficients were determined for each of the three solvent systems along with the coefficient of variance and the significant of each model for each solvent (Table 14). The R^2 values for methanol (94.5%), ethanol (90.43%) and acetone (94.13%), respectively, showed that the adequacy of TF models was high for all the solvents. These values are significant as the acceptance of any model with R^2>75 (Glantz & Skinner, 1990). In addition, the results for all the TF extractions obtained from each solvent adequately described the model, the *p*-values for the models were well below $p < 0.1$ (Table 14) supporting the adequacy of the model for all solvents.

D.1.3.c *Adequacy of the TF models:* The data were examined to determine the adequacy based on it "lack of fit" to determine the variability in the responses fit (Dean & Voss, 1999). The analysis of variance (ANOVA) of the quadratic model was adequate for all the three solvent systems showed a significant *p*-value < 0.05 (Table 14). The models allowed for an efficient estimation of each linear effect with a path of steepest ascent, and a significance reflecting the effect on the dependent variable allow for *lack of fit*. A higher order model with more complex interactions between each parameter is not necessary to explain the interactions. More specifically, the *p*-value of the lack of fit test for methanol (p=0.1612), ethanol (p=0.7329) and acetone (p=0.9033) were all > 0.05 (insignificant) indicating that the data satisfactorily fits the model (Table 14).

Table 14. Regression coefficients (coded) predicted by the quadratic polynomial model for flavonoids when extracted with the cited solvent systems

Coefficient	Methanol	Ethanol	Acetone
b_o	0.587	0.790	2.214
Linear			
b_1 (SP)	-0.041**	-0.058	-0.478***
b_2 (S:S)	-0.094*	-0.024	0.755**
b_3 (MT)	0.050**	0.074***	-0.056
Quadratic			
b_{11} (SP x SP)	-0.011	0.037	0.003
b_{22} (S:S x S:S)	-0.172*	-0.158***	-0.189
b_{33} (MT x MT)	0.027	-0.010	-0.161
Cross product			
b_{12} (SP x S:S)	0.029***	-0.006	-0.333
b_{13} (SP x MT)	0.007	-0.020	-0.201
b_{23} (S:S x MT)	-0.008	0.030	-0.025
R^2	94.5	90.43	94.13
p values			
Model	0.0012	0.0077	0.0016
Lack of Fit	0.1612	0.7329	0.9033

SP – Solvent Polarity, S:S – Solid: Solvent, MT – Mix Time
***** significant at 1%, ** significant at 5%, *** significant at 10%

D.1.3.d *Regression Coefficients Equations and Pareto Charts:* The regression equations for optimizing TF with each of the three solvent systems are presented Table 15. The equations are based on the significance ($p< 0.01, 0.05, 0.1$) of individual regression coefficients. Pareto charts for each system were included as a visual demonstration of the solvent's effect on TF yields (Figure 10). For example, the solid:solvent ratio had a negative effect on TF with methanol (Figure 10a), but positively affected TF when acetone was used (Figure 10c). For mix time, a positive effect on both methanol (Figure 10a), and ethanol (Figure 10b) occurred. The quadratic coefficient (solid:solvent ratio) had a significant negative effect on both methanol and ethanol (Figure 10a-b). The solvent:water ratio had a significant negative effect on methanol (Figure 10a) and acetone (Figure 10c). Overall, methanol solvent systems showed more complex interactions, but acetone produced the optimal TF.

D.1.3.e *Final optimized TF values and processing factors:* As shown by Table 16, the optimum factors for TF level were an solvent:water ratio of 25:75 (acetone), a solid:solvent ratio of 30 percent and a mix time of approximately 143 minutes (Table 16). Similar to the TP, the polarity of the aqueous acetone is fairly low. As mentioned earlier, the optimal TP could be affected by high TCT present in black beans. (*This will be discussed later in this chapter*.) The schematic representations provided in Figures 11a-c consistently show an organic water ratio had a linear negative effect on TF extractions as the parameters increased 75:25 (1.0 coded) from 25:75 (-1.0) to indicating that a higher amount of solvent:water quantities could be used to obtain higher TF levels.

Table 15. Regression equation of the fitted model for TF

TF Methanol = 0.587 – 0.041*Xsp* – 0.094*Xss* + 0.05*Xmt* + 0.029*XspXss* – 0.172*Xss*^2
TF Ethanol = 0.790 + 0.074*Xmt* – 0.158*Xss*^2
TF Acetone = 2.214 – 0.478*Xsp* + 0.755*Xss*

Xsp– Solvent Polarity, ***Xss*** – Solid: Solvent, Xmt – Mix Time

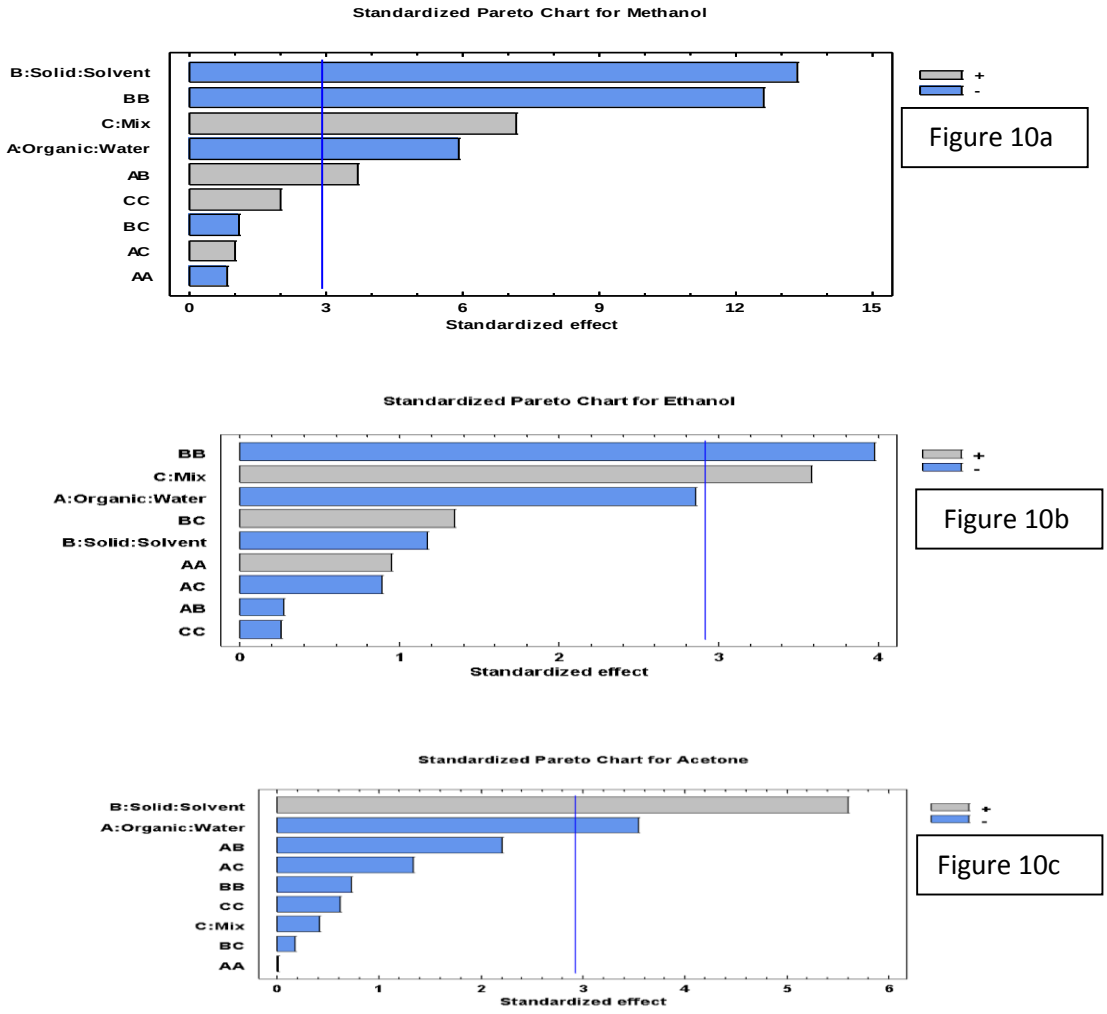

Figure 10: Pareto charts showing relative effects of regression coefficient for total flavonoids accepted models in a) Methanol b) Ethanol c) Acetone. Vertical line represents $p < 0.1$.

Table 16. Optimized factors required to generate optimum TF yield (Black bean solids: Acetone solvent)

Factor	***Low***	***High***	***Optimum (coded)***	***Optimum (uncoded)***
Organic: Water	-1.0	1.0	-1.0	25:75
Solid: Solvent	-1.0	1.0	1.0	30%
Mix	-1.0	1.0	0.377245	≈143minutes

Optimum value TF = 3.61952 mg/g

Figure 11a

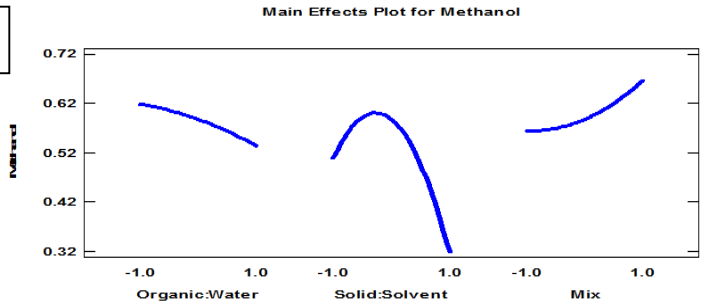

Figure 11b

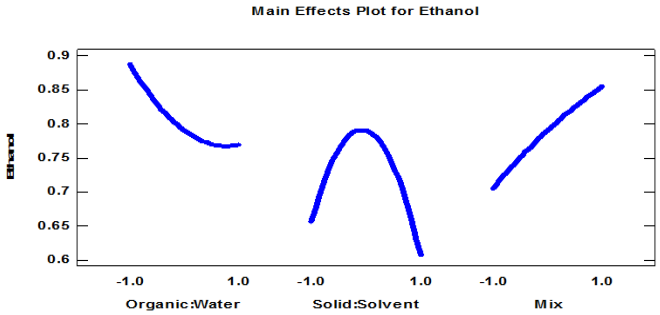

Figure 11c

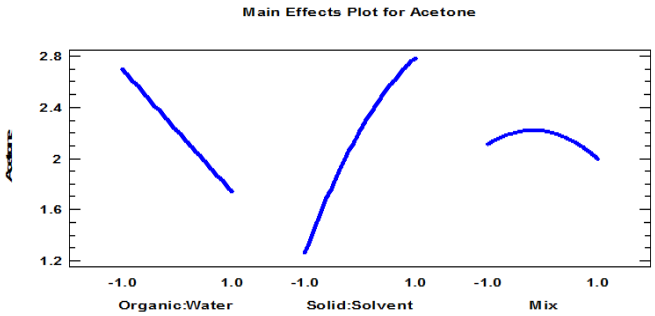

Figure 11. Main effects plot (TF).

All three schematic representations showed a significant, positive increase in TF extraction as mix time increased form 60 minutes (-1.0 coded) to 180 minutes (1.0 coded) indicating that extended extraction time could favor increased extractions of TF. In contrast, only the acetone's solid:solvent ratio demonstrated a positive TF as the solid ratio increased from 10 percent (-1.0 coded) to 30 percent (1.0 coded)(Figure 11c). The solid:solvent ratios (for methanol and ethanol showed that optimal point was located between the experiment's solid:solvent parameters (Figures 11a-b).

D.1.4 Total Condensed Tannins (TCT)

D.1.4.a *TCT results obtained face centered composited design (FCCD):* Similar to the TF and TP RSM design, extraction characterization of TCT from black beans was determined by using a 3-level faced centered composite design (FCCD) experiment with 17 runs of each solvent (with three center points included) (Table 17). A comparison of the solvent systems showed that range that the highest TCT was obtained from the acetone solvent mixture (16.15 mg/g, Extract 12). The highest TCT obtained from the other two solvents were 3.02 mg/g (Extract 14) and 2.67 mg/g (Extract 6) for ethanol and methanol respectively. It should be noted that these values were more than five times lower by comparison to the acetone extract value. In fact, the *low* TCT value (4.28 mg/g, Extract 6) obtained from acetone was not much higher the *high* value ethanol (Table 18). These results support our hypothesis that extractions parameter do affect phenols values and only one extraction, as cited in multiple manuscripts, cannot be used as means to describe the entire system. Similar to the TP and TF, the broad range of TCT obtained from acetone substantiates that extractions have to be characterized before a system can be correlated with phenol levels and a health effect.

Table 17: Total condensed tannins response in mg/g using FCCD that consisted of three center points, three solvent systems and three experimental factors for each solvent system (methanol, ethanol and acetone).

Std. Order	Methanol	Ethanol	Acetone
1	0.33 ± 0.01	2.68 ± 0.16	12.23 ± 0.42
2	0.66 ± 0.02	2.91 ± 0.26	13.12 ± 0.90
3	0.38 ± 0.03	1.88 ± 0.13	4.51 ± 0.29
4	1.89 ± 0.17	2.14 ± 0.13	11.78 ± 0.35
5	0.53 ± 0.02	0.86 ± 0.15	11.90 ± 0.30
6	2.67 ± 0.04	2.49 ± 0.18	4.28 ± 0.42
7	1.69 ± 0.18	2.60 ± 0.36	4.83 ± 0.60
8	0.63 ± 0.02	2.87 ± 0.05	11.02 ± 0.91
9	1.84 ± 0.26	0.98 ± 0.01	13.93 ± 0.49
10	0.65 ± 0.17	0.34 ± 0.03	10.19 ± 0.37
11	2.38 ± 0.49	2.44 ± 0.12	5.69 ± 0.56
12	1.22 ± 0.27	1.48 ± 0.04	16.15 ± 1.61
13	0.69 ± 0.03	2.75 ± 0.18	11.83 ± 0.53
14	0.34 ± 0.02	3.02 ± 0.18	11.02 ± 0.55
15	0.58 ± 0.01	1.16 ± 0.02	11.15 ± 0.60
16	0.32 ± 0.01	0.83 ± 0.15	12.07 ± 0.95
17	0.53 ± 0.09	2.12 ± 0.03	5.01 ± 0.32

**Data are shown as the mean ± standard deviation (n=3)*

Table 18. Ranges of total condensed tannins for each solvent system

Extraction Solvent	Total Condensed Tannins (mg/g)	Range (mg/g)
Methanol	0.32 – 2.67	2.35
Ethanol	0.34 – 3.02	2.68
Acetone	4.28 – 16.15	11.87

Additionally, comparison cannot be made between natural systems in terms of their TP unless the extraction methodology has been fully characterized for each system, which most likely will differ.

A number of studies have shown that the high phenolic compounds present in black beans consist mainly of anthocyanins and condensed tannins for legumes with a dark seed coats similar to black beans (Takahata et al., 2001; Cardador-Martinez et al., 2002; Troszynska & Ciska, 2002; Wu et al., 2004). Islam et al. (2003) investigated 790 accessions from common bean collections and reported the mean coat extract from black beans were the highest tannins with 0.29g/g followed by red beans with 0.124 g/g of seed coat. Mailoa et al. (2013) found that because tannins are highly polymerized substances, with high molecular weights over 1000, this requires a solvent capable of dissolving non-polar compounds.

D.1.4.b *Fitting the TCT models:* As cited previously, the acetone system produced the optimal total condensed tannin (TCT) yields. The experimental data were fitted to the second-order polynomial model, and the ensuing results were tested to determine the variability of responses. As shown in Table 19, ANOVA of the quadratic model was adequate for the ethanol and acetone solvent systems as their *p*-values are ($p < 0.1$). However, the fitness of the regression curve only supported these results for only ethanol (79.47%) and acetone (96.77%), as the R^2 value for methanol was < 75 percent (Table 19), it failed to satisfy the model. Thus, no more discussion on this solvent system for TCT extraction will occur in terms of the model equation and each parameters effect on TCT levels.

Table 19. Regression coefficients (coded) predicted by the quadratic polynomial model for tannins when extracted with the cited solvent systems

Coefficient	Methanol	Ethanol	Acetone
b_o	0.820	2.474	11.436
Linear			
b_1 (SP)	-0.524*	-0.266	-0.7609**
b_2 (S:S)	-0.308*	-0.705	3.990*
b_3 (MT)	-0.147**	0.115	0.0637
Quadratic			
b_{11} (SP x SP)	-0.619*	0.217	0.1139
b_{22} (S:S x S:S)	0.644*	-0.959	-3.421*
b_{33} (MT x MT)	0.318**	-0.106	0.940***
Cross product			
b_{12} (SP x S:S)	0.133**	-0.031	-0.853**
b_{13} (SP x MT)	-0.015	0.1315	0.6817**
b_{23} (S:S x MT)	0.103**	-0.0506	0.0029
R^2	65.28	79.47	96.77
p values			
Model	0.3151	0.0801	0.0002
Lack of Fit	0.0051	0.9662	0.1286

SP – Solvent Polarity, S:S – Solid: Solvent, MT – Mix Time
***** significant at 1%, ** significant at 5%, *** significant at 10%

D.1.4.c *Adequacy of the TCT models*: As shown in Table 19, the lack of fit values were > 0.05 for ethanol (p=0.9662) and acetone (p=0.1286), and none of the linear, quadratic, and cross product coefficients for ethanol were significant for ethanol. Thus, only the regression equation presented is for acetone (Table 20). As methanol (p=0.0051) also failed the lack of fit criteria, these results (along with those presented above for methanol) suggest that other models may be involved in TCT extractions when using methanol or ethanol.

D.1.4.d *Regression Coefficients Equations and Pareto Charts:* The regression equation for optimizing TCT with acetone solvent system is shown in Table 20. (Methanol and ethanol did not fit the model.) The equation for acetone is based on the significance ($p< 0.01, 0.05, 0.1$) of individual regression coefficients. The Pareto chart for acetone (Figure 12c), demonstrate the greatest effect on TCT acetone extract was the solid:solvent ratio, which had a substantial linear positive relationship. This suggests even higher TCT number may have been obtained with higher levels of solid material.

D.1.4.e *Final optimized TCT values and processing factors:* The optimum factors for TCT are an solvent:water ratio of 25:75 (acetone), a solid:solvent ratio of 14 percent, and a mix time of approximately 60 minutes (Table 21). A relatively weak acetone: water mix (25:75) may be due to the fact that black beans are high in condensed tannins, and since tannins can be divided into soluble and insoluble tannins, the soluble tannins are oligomeric proanthocyanidins (or condensed tannins) with low molecular weight (Decandia et al., 2009). Tannins are rapidly extracted with aqueous organic solvents like methanol or acetone (Decandia et al., 2009). The schematic representations provided in Figure 13 consistently show the highest TCT occurs with a low solvent:water ratio.

Table 20. Regression equation of the fitted model for TCT

TCT Acetone = 11.436 – 0.761*Xsp* + 3.99*Xss* – 0.853*XspXss* + 0.682*XspXmt* - 3.421*Xss*^2+ 0.94*Xmt*^2

Xsp– Solvent Polarity, ***Xss*** – Solid: Solvent, Xmt – Mix Time

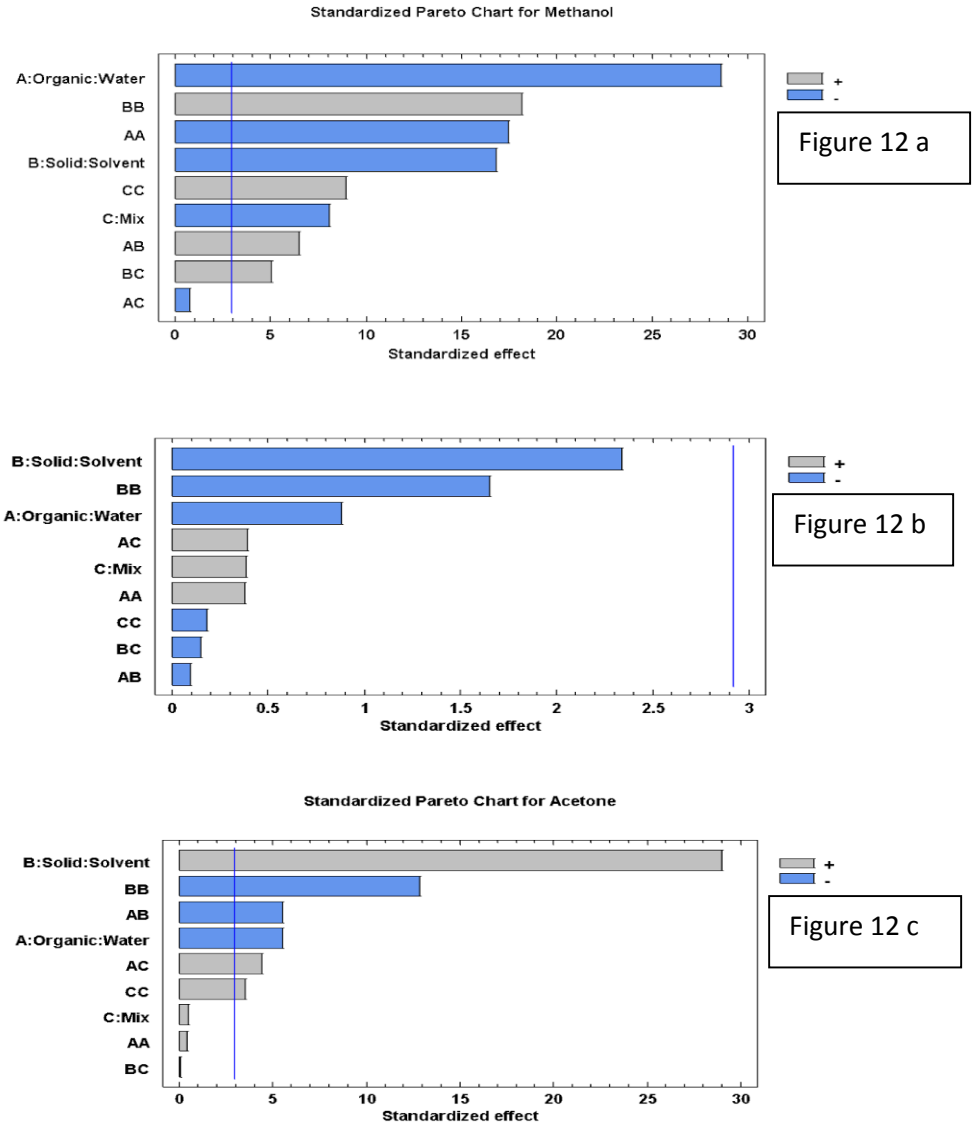

Figure 12: Pareto charts showing relative effects of regression coefficient for total condensed tannins accepted models in a) Methanol b) Ethanol c) Acetone. Vertical line represents $p < 0.1$.

Table 21. *Optimized factors required to generate optimum TCT yield (Black beans solids: Acetone solvent)*

Factor	***Low***	***High***	***Optimum (coded)***	***Optimum (uncoded)***
Organic: Water	-1.0	1.0	-1.0	25:75
Solid: Solvent	-1.0	1.0	0.707637	14%
Mix	-1.0	1.0	-1.0	60 minutes

Optimum value TCT = 15.5816 mg/g

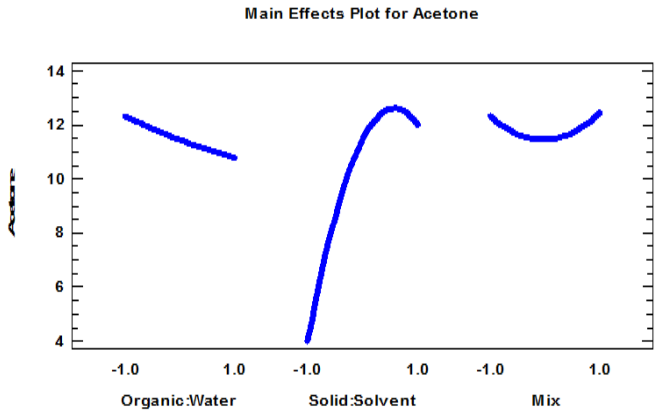

Figure 13. Main effects plot for TCT (acetone).

The schematic representations showed a significant, positive increase in TCT extraction as solid:solvent ratio increased form 10 percent (-1.0 coded) to a point near 30 percent (1.0 coded) suggesting, perhaps, a higher concentration of solid generates higher tannin content.(Figure 13). Finally, the schematic representations for acetone shows either a lower mix time or higher mix time may extract more TCT, yet 120 minutes was not the optimal mixing time (as represented by the u shaped curve). At this time, we have not explanation for this phenomenon. It is possible higher weight TCT polymers were extracted more efficiently at one time period while lower weight polymers were being extracted more efficiently at another.

D.2 ***Specific Aim 2***

D.2.1 *Adequacy of RSM to α-amylase and α-glucosidase data*. A review of the literature did not provide any references to the optimization / characterization of extraction procedures to generate extracts able to effectively inhibit α-amylase and α-glucosidase inhibition in food systems. Therefore, the objective of Specific Aim 2 was to determine RSM (FCCD) procedures for obtaining extracts from black bean to determine the extracts' ability to optimally inhibit α-amylase and α-glucosidase, and then generate predictive model equations. Unfortunately, the statistical analyses of the RSM data did not provide such a model as inhibition of α-amylase and α-glucosidase data failed to comply with the parameters stated in this study's Materials and Methods section (Section C). In spite of the lack of a predictive model equation, a comparison between the recovered extracts and their inhibitory properties demonstrated a correlation between the percent inhibition (normalized to percent per mg of extract) and the TP, TF and TCT levels for the methanol and ethanol extracts. It demonstrated a strong correlation existed

between TP, TF or TCT content and α-amylase and α-glucosidase inhibition with the acetone extractions.

D.2.2 *Overall highest inhibitions of α-amylase and α-glucosidase*. All three solvent extracts demonstrated the ability to inhibit α-amylase and α-glucosidase enzymes (Table 22). Yet, the inhibitory activities were diverse according to the solvent type used in the extraction, and the different extraction parameters. Acetone produced the highest inhibition of α-amylase (36.20 % inhibition /mg extraction) and α-glucosidase (34.10 % inhibition /mg extract) (Table 22) followed by methanol (10.84 % inhibition /mg extract) and then ethanol (10.29 % inhibition /mg/extract), which was only slightly less than methanol. However, for α-glucosidase, ethanol inhibition (16.68 % inhibition/mg) was higher than methanol inhibition (6.16 % inhibition /mg) (Table 22). Yet, for both Aim 1 and Aim 2, the acetone solvent system was the most effective system for extracting polyphenols and for producing the highest enzyme inhibition for α-amylase with a range of more than 26 percent between the optimal high (acetone) and low (ethanol). Similarly, α-glucosidase had a range of more than 28 percent between the optimal high (acetone) and low (methanol) (Table 22). In addition, the study demonstrated that the α-amylase inhibitory activity was always higher than the α-glucosidase inhibition for all the solvents used (Table 22). Previous research into α-glucosidase using baker's yeast demonstrated α-glucosidase inhibition did not correlate to a high phenolic content but high antioxidant activity extracts had a high correlation with α-amylase (porcine pancreas) inhibition (McCue et al., 2005). The study differed in that the researchers used only distilled water for extraction. However, as there was no correlation between phenol levels and α-amylase or α-glucosidase inhibitory activities, it is possible other non-phenolic

Table 22. Highest inhibitory activities of α-amylase and α-glucosidase for each solvent

Extraction Solvent	α-Amylase[a]	α-Glucosidase[a]
Methanol	10.84 ± 0.71 (3)[b]	6.16 ± 0.78 (6)[b]
Ethanol	10.29 ± 0.38 (6)[b]	16.68 ± 3.40 (9)[b]
Acetone	36.20 ± 1.99 (12)[b]	34.10 ± 1.54 (5)[b]

[a] % inhibition/mg of extract expressed as a mean ± SD of 3 replications.
[b] extraction number.

compounds may have been involved (McCue et al., 2005). A second study used 95 percent ethanol as a solvent to research the ability of Senna surattensis leaves to inhibit α-amylase and α-glucosidase (Thilagam et al., 2013). The study showed that the different inhibition between the two enzymes was most likely due to the subcultural differences of the enzymes (Chiba, 1997).

D.2.3.a *Inhibition of α-amylase and α-glucosidase for the methanol extraction:* The methanol extractions produced the lowest α-glucosidase inhibition of all extractions; whereas ethanol extracts were comparable were for the most part exerted the lowest inhibition of the for α-amylase (Table 23). (The tables are shown in the same RSM procedure order as cited in Materials and Methods section (Table 7) with TP, TF and TCT expressed now as µg /mg of extract instead of mg/g of bean as used in Specific Aim 1.) Extract 3 demonstrated the highest inhibitory effect of α-amylase (10.84 % inhibitory / mg extract), and Extract 6 demonstrated the highest inhibitory effect of α-glucosidase (6.16 % inhibitory / mg extract) (Table 23). It should be noted, Extract 14 resulted in the highest TP content (8.99 µg /mg of extract), the highest TF content (3.48 µg /mg), and the highest TCT (2.43 µg /mg) (Table 23), and yet, even though Extract 14 yielded the highest polyphenols, it did not have a direct correlation to increased α-amylase inhibition (2.46 % inhibitory / mg extract) or α-glucosidase inhibition (3.26 % inhibitory / mg extract). This indicates the higher enzyme inhibition is not directly related to the increased TP, TF or TCT. Further studies are required to determine additional factors affecting α-amylase and α-glucosidase inhibition since minimal correlation occurred when plotting each replication of α-amylase (Figure 14) and α- glucosidase inhibition (Figure 15) against its associated TP, TF and TCT concentration pre mg of extract, i.e.,

Table 23. Alpha-amylase and α-glucosidase inhibition of black beans extracts using FCCD for the methanol solvent system

NO.	TP[a]	TF[a]	TCT[a]	Amylase[b]	Glucosidase[b]	Solv.: Ratio	Solid: Vol.	Time (min)
1	**4.07 ± 1.89**	**1.89 ± 0.87**	**1.40 ± 0.65**	**6.66 ± 2.44**	**0.19 ± 0.14**	**50-50**	**30%**	**120**
2	**4.75 ± 2.71**	**2.31 ± 1.33**	**1.48 ± 0.85**	**6.2 ± 0.63**	**1.06 ± 0.30**	**50-50**	**20%**	**180**
3	**4.68 ± 0.91**	**1.70 ± 0.36**	**1.33 ± 0.26**	**10.84±0.71**	**4.91 ± 0.52**	**25-75**	**30%**	**60**
4	**6.96 ± 0.20**	**3.06 ± 0.08**	**2.20 ± 0.19**	**5.36 ± 1.22**	**3.86 ± 0.63**	**50-50**	**20%**	**60**
5	**3.97 ± 3.34**	**1.62 ± 1.36**	**0.72 ± 0.61**	**6.61 ± 0.55**	**0.81 ± 0.21**	**75:25**	**20%**	**120**
6	**4.79 ± 0.81**	**1.69 ± 0.35**	**1.48 ± 0.28**	**2.7 ± 0.72**	**6.16 ± 0.78**	**25-75**	**20%**	**120**
7	**3.97 ± 0.21**	**1.66 ± 0.18**	**1.44 ± 0.06**	**2.92 ± 1.37**	**5.01 ± 1.35**	**25-75**	**10%**	**180**
8	**3.73 ± 0.97**	**1.55 ± 0.42**	**1.38 ± 0.36**	**1.62 ± 0.01**	**1.03 ± 0.58**	**50-50**	**20%**	**120**
9	**1.81 ± 1.36**	**0.57 ± 0.42**	**0.39 ± 0.30**	**2.50 ± 0.14**	**0.53 ± 0.18**	**75:25**	**10%**	**180**
10	**2.23 ± 1.23**	**1.03 ± 0.57**	**0.41 ± 0.24**	**1.14 ± 0.34**	**1.08 ± 0.00**	**75:25**	**30%**	**60**
11	**3.93 ± 0.37**	**1.62 ± 0.12**	**1.10 ± 0.26**	**1.55 ± 0.26**	**4.85 ± 1.31**	**25-75**	**10%**	**60**
12	**1.43 ± 1.05**	**0.48 ± 0.35**	**0.27 ± 0.22**	**1.89 ± 0.33**	**0.98 ± 0.17**	**75:25**	**10%**	**60**
13	**8.83 ± 0.77**	**3.43 ± 0.34**	**2.34 ± 0.11**	**3.46 ± 0.31**	**4.12 ± 0.89**	**50-50**	**20%**	**120**
14	**8.99 ± 0.54**	**3.48 ± 0.27**	**2.43 ± 0.17**	**2.46 ± 0.27**	**3.26 ± 1.53**	**50-50**	**10%**	**120**
15	**7.73 ± 0.24**	**3.25 ± 0.18**	**2.12 ± 0.07**	**1.93 ± 0.33**	**1.40 ± 0.02**	**50-50**	**20%**	**120**
16	**4.80 ± 3.92**	**2.08 ± 1.70**	**0.90 ± 0.74**	**1.56 ± 0.29**	**1.38 ± 0.16**	**75:25**	**30%**	**180**
17	**4.73 ± 1.11**	**2.11 ± 0.55**	**1.23 ± 0.29**	**9.13 ± 0.25**	**5.64 ± 0.40**	**25-75**	**30%**	**180**

[a] Data are shown as a mean (μg polyphenol /mg of extract) ± SD of at least 5 replications.
[b] Data are shown as a mean (% inhibition/mg of extract) ± SD of 3 replications relative to acarbose (positive control).

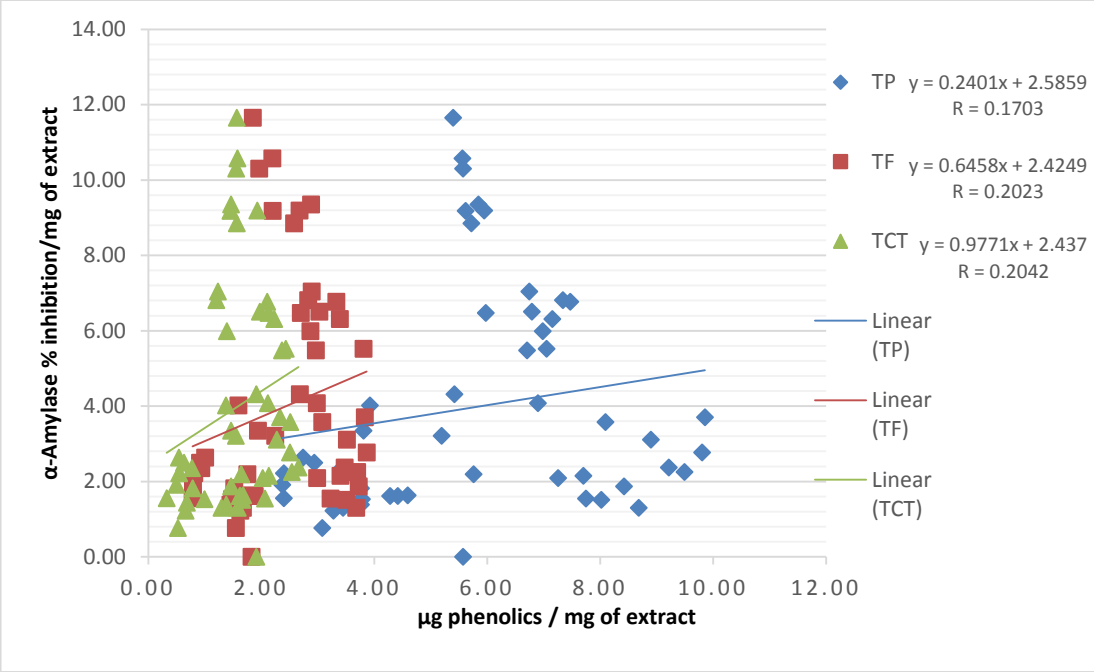

Figure 14: Effect of TP, TF and TCT concentrations on α-amylase inhibitory activities for the methanol solvent. The scatter plot contains data of each replication (trendline equations are for TP, TF and TCT, respectively).

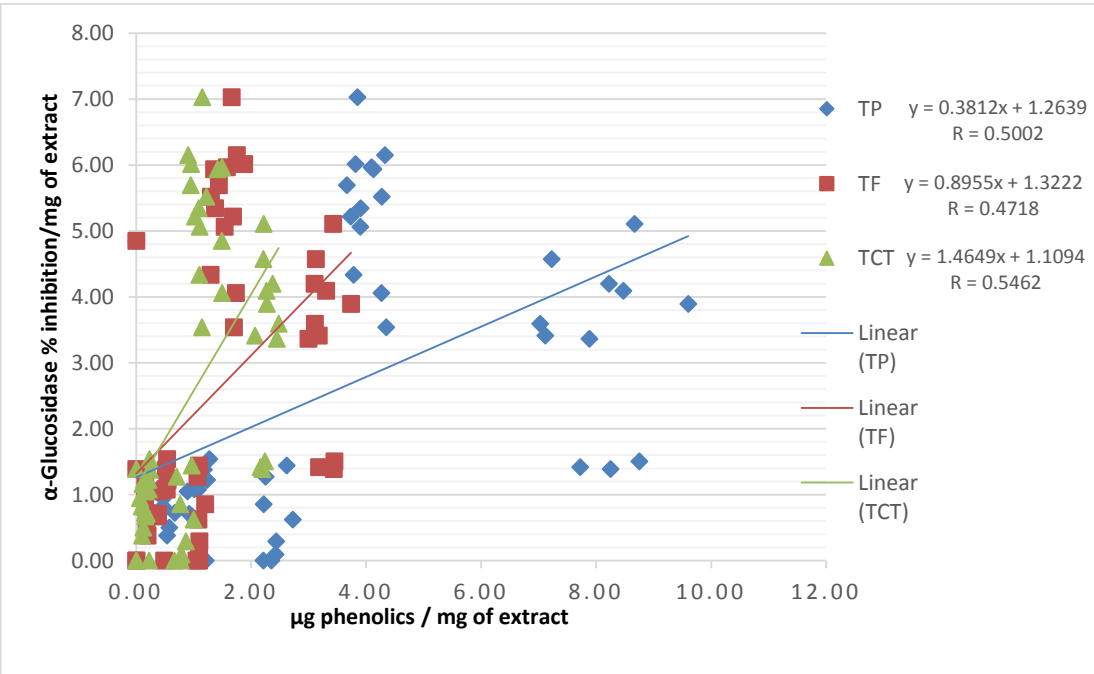

Figure 15: Effect of TP, TF, and TCT concentrations on α-glucosidase inhibitory activities for the methanol solvent. The scatter plot contains data of each replication (trendline equations are for TP, TF and TCT, respectively).

especially for α- glucosidase and TCT, as evidenced by the R values. For α-amylase, the R values were as follows: (R =0.17) for TP, (R=0.20) for TF and (R =0.20) for TCT (Figure 15). Nevertheless, in the case of α-glucosidase, there was moderate correlation as the R values were (R =0.50) for TP, (R =0.47) for TF and (R =0.54) for TCT (Figure 15). While a trend occurred along the lines for inhibition of α-amylase versus the various individual points, two groups are shown in Figure 15 for a-glucosidase as evidenced by on group clustered at the bottom of the chart and the others at the top. This could be due to different types (single or multiple) of phenols acting together to inhibit this enzyme or to completely different group of phenols. Moreover, other items, such as sugars, minerals, proteins, vitamins and complex extracts are extracted with methanol and may be contributing to this effect.

These results differ from an earlier study in which the inhibition of pancreatic α-amylase had a positive correlation with the TP extracts from berries, green tea and red cabbage (McDougall et al., 2005). It must be noted, that in this study, the researchers initially used only one extraction and took steps to release and isolate the bound phenols. The pancreatic α-amylase inhibition was attributed to the tannins present in the extracts, and the research proposed that the polyphenols might be exerting a synergistic effect (McDougall et al., 2005). Still other studies show weak correlations between TP and inhibitory properties (McCue et al., 2005; Ranilla et al., 2010). McCue et al. (2005) demonstrated that a high phenolic content does not always correlate to α-glucosidase inhibition, and that non-phenolic compounds in bean extracts are involved in the inhibitory properties, as stated previously. Ranilla et al. (2010) showed a correlation between α-amylase inhibition and TP levels in cooked beans indicating, perhaps the

correlation is associated with TP and residual protein-type α-amylase inhibitors, but again these researchers used limited extraction procedures.

D.2.3.b *Inhibition of α-amylase and α-glucosidase for the ethanol extraction: A*s cited above, ethanol extracts were similar to methanol extracts in terms of inhibiting α-amylase, but produced higher inhibitory capabilities for α-glucosidase compared to methanol. The highest inhibition for α-amylase occurred with Extract 6 (10.29 % inhibition /mg extract), and the highest inhibition for α-glucosidase occurred with Extract 9 (16.68 % inhibition /mg extract) (Table 24). Unlike methanol, the optimum ethanol extracts were distributed across varying parameters. All extracts were able to inhibit α-amylase and α-glucosidase. However, adjusting each extraction parameter resulted in different inhibitory values, once again showing extraction parameters used, in addition to the solvent system, affected the results most. This is likely due to the levels and/or types of compounds that were extracted (Table 24).

Correlation of TP, TF and TCT levels in response to α-amylase and α-glucosidase inhibitory activities for the ethanol extracted samples are shown on Figures 16 and 17. The scatter plot (Figure 16) show low correlation of TP and TF with α-amylase inhibition, as their R values were 0.20, 0.23, respectively (less than the required >0.5). There was a moderate correlation between the TCT and α-amylase inhibition, as the R value was 0.41 This confirms the study cited earlier, that attributed α-amylase inhibition to tannins in multiple food systems (McDougall et al., 2005). However, the plot chart generated by this study for the α-glucosidase inhibitory correlation (Figure 17) demonstrated a moderate correlation with TF as its R value was the only one that exceeded the >0.05 threshold by (R=0.66).

Table 24. Alpha-amylase and α-glucosidase inhibition of black beans extracts using FCCD for the ethanol solvent system

No.	TP[a]	TF[a]	TCT[a]	Amylase[b]	Glucosidase[b]	Solv.: Ratio	Solid: Vol.	Time (min)
1	**7.90 ± 0.30**	**4.20 ± 0.08**	**15.06 ± 1.00**	**5.01 ± 0.76**	**5.41 ± 0.63**	**50-50**	**30%**	**120**
2	**9.30 ± 0.29**	**4.35 ± 0.23**	**16.21 ± 2.50**	**4.91 ± 0.69**	**7.35 ± 0.52**	**50-50**	**20%**	**180**
3	**5.85 ± 0.30**	**2.34 ± 0.08**	**10.53 ± 0.46**	**8.11 ± 0.42**	**2.19 ± 0.75**	**25-75**	**30%**	**60**
4	**9.30 ± 0.40**	**4.19 ± 0.17**	**14.38 ± 0.63**	**4.55 ± 0.33**	**7.54 ± 0.46**	**50-50**	**20%**	**60**
5	**5.99 ± 0.27**	**4.09 ± 0.16**	**8.41 ± 0.83**	**1.90 ± 1.00**	**11.65 ± 1.15**	**75:25**	**20%**	**120**
6	**5.96 ± 0.26**	**2.38 ± 0.12**	**10.04 ± 0.42**	**10.29 ±0.38**	**4.04 ± 0.63**	**25-75**	**20%**	**120**
7	**6.17 ± 0.63**	**2.40 ± 0.40**	**8.40 ± 0.84**	**4.77 ± 0.52**	**2.49 ± 1.12**	**25-75**	**10%**	**180**
8	**7.55 ± 2.05**	**3.23 ± 0.87**	**10.52 ± 2.84**	**3.90 ± 0.03**	**7.93 ± 0.78**	**50-50**	**20%**	**120**
9	**5.64 ± 1.84**	**3.90 ± 1.23**	**5.65 ± 1.77**	**2.06 ± 0.91**	**16.68 ± 3.40**	**75:25**	**10%**	**180**
10	**4.69 ± 1.28**	**4.16 ± 1.17**	**3.29 ± 0.92**	**2.33 ± 0.36**	**11.53 ± 2.69**	**75:25**	**30%**	**60**
11	**5.23 ± 1.17**	**1.91 ± 0.52**	**6.83 ± 1.57**	**4.22 ± 0.06**	**1.86 ± 0.97**	**25-75**	**10%**	**60**
12	**4.24 ± 1.20**	**2.53 ± 0.89**	**3.89 ± 1.24**	**3.03 ± 0.78**	**13.87 ± 4.26**	**75:25**	**10%**	**60**
13	**7.14 ± 2.29**	**3.72 ± 1.09**	**12.04 ± 3.53**	**4.15 ± 0.51**	**7.20 ± 0.76**	**50-50**	**20%**	**120**
14	**7.91 ± 2.90**	**3.73 ± 1.41**	**12.35 ± 4.68**	**3.91 ± 0.79**	**12.26 ± 1.10**	**50-50**	**10%**	**120**
15	**9.18 ± 0.21**	**4.86 ± 0.19**	**14.17 ± 0.44**	**4.77 ± 0.90**	**7.39 ± 0.91**	**50-50**	**20%**	**120**
16	**7.38 ± 0.32**	**6.68 ± 0.38**	**7.18 ± 0.41**	**1.11 ± 0.80**	**11.29 ± 0.98**	**75:25**	**30%**	**180**
17	**4.69 ± 0.27**	**2.55 ± 0.17**	**8.59 ± 0.87**	**8.35 ± 0.23**	**3.98 ± 0.15**	**25-75**	**30%**	**180**

[a] Data are shown as a mean (µg polyphenol /mg of extract) ± SD of at least 5 replications.
[b] Data are shown as a mean (% inhibition/mg of extract) ± SD of 3 replications relative to acarbose (positive control).

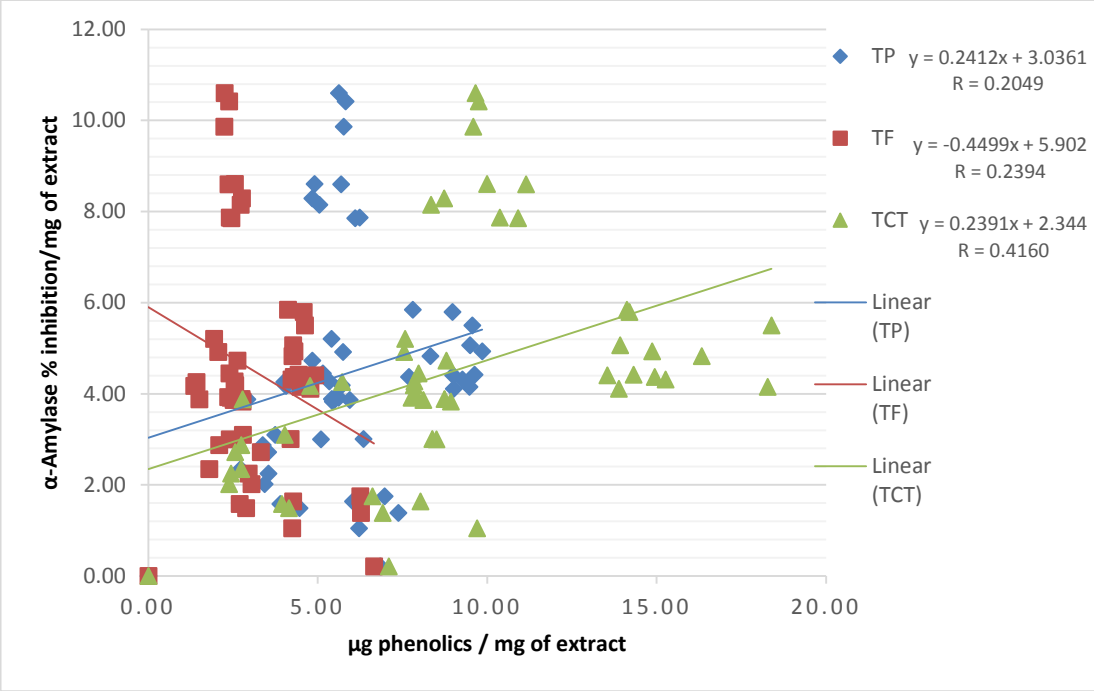

Figure 16: Effect of TP, TF and TCT concentrations on α-amylase inhibitory activities for the ethanol solvent. The scatter plot contains data of each replication (trendline equations are for TP, TF and TCT, respectively).

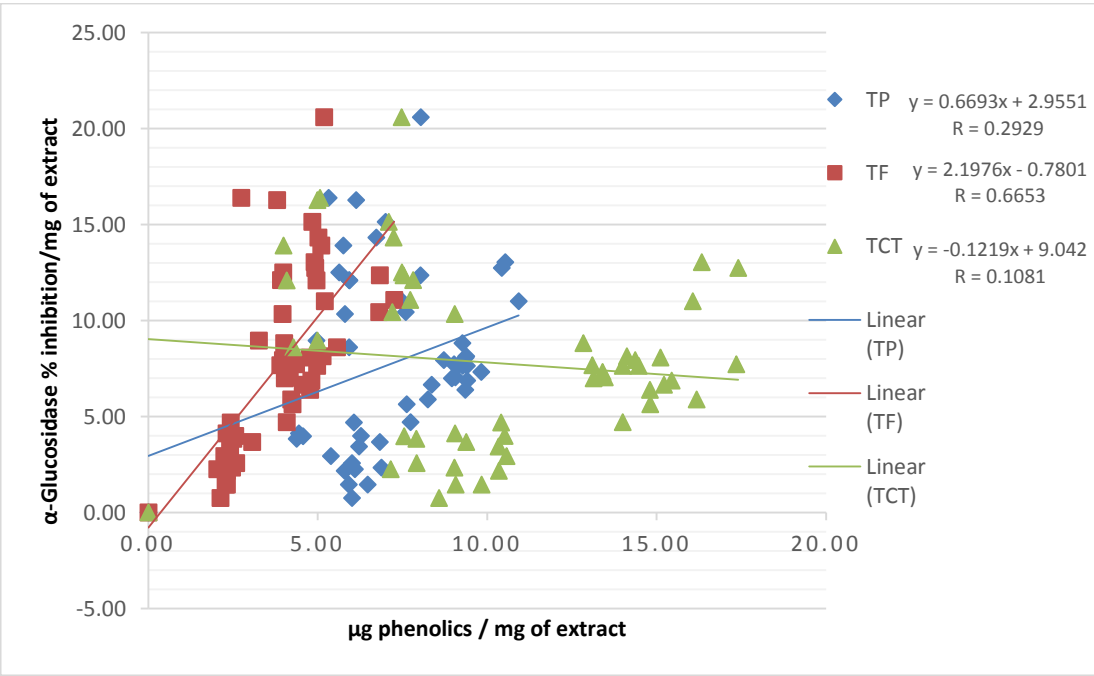

Figure 17: Effect of TP, TF and TCT concentrations on α-glucosidase inhibitory activities for the ethanol solvent. The scatter plot contains data of each replication (trendline equations are for TP, TF and TCT, respectively).

There was weak correlation between α-glucosidase inhibitory and TP and TCT indicated by their R values: (R=0.29) for TP and (R=0.10) for TCT (Figure 17).

D.2.3.c *Inhibition of α-amylase and α-glucosidase for the acetone extraction:*

The greatest α-amylase and α-glucosidase inhibition occurred for the acetone extracts generating inhibitory levels much higher than for either the methanol or the ethanol extracts (Table 25). The extraction that produced the highest TP were Extract 10 (38.84 µg/mg), Extract 5 for TF (42.01 µg/mg) and Extract 16 for TCT (13.84 µg/mg), even though Extract 10 contained the highest TP, TF and TCT levels i.e., 38.84 µg/mg, 37.22 µg/mg and 11.83 µg/mg of extract, respectively (Table 25).

The highest inhibition for α-amylase was exerted by Extract 9 at 36.65 percent inhibition/mg of extract (Table 25); whereas Extract 5 produced this the highest inhibition for α-glucosidase (34.10 % inhibition/mg extract) (Table 25). Total phenol, TF and TCT all showed a strong correlation to the highest α-amylase inhibition with R values of: R=83, R=83, and R=79, respectively (Figure 18). Inhibition of α-glucosidase inhibition was highly correlated with TP, TF and TCT with values of: R=97, R=96, and R=91, respectively (Figure 19). The high R values supports the results from a study of twenty-two flavonoids which determined they exhibited strong inhibitory against yeast α-glucosidase and α-amylase (Kim et al., 2000). The scatter plots demonstrate strong correlations of the phenolics (TP, TF and TCT) with α-amylase (Figure 18) and α-glucosidase (Figure 19) inhibition. As cited before, several studies highlight the effect of isolated flavonoids on the inhibition of α-amylase and α-glucosidase (Kim et al., 2000; Tadera et al., 2005; Li et al., 2009).

Table 25. Alpha-amylase and α-glucosidase inhibition of black beans extracts using FCCD for the acetone solvent system

No	TP[a]	TF[a]	TCT[a]	Amylase[b]	Glucosidase[b]	Solv.: Ratio	Soli: Vol.	Time (min)
1	**14.37 ± 0.65**	**11.77 ± 1.36**	**5.34 ± 0.23**	**4.91 ± 0.90**	**10.96 ± 0.97**	**50-50**	**30%**	**120**
2	**17.21 ± 1.78**	**13.66 ± 1.54**	**5.57 ± 0.66**	**4.44 ± 0.13**	**11.92 ± 0.64**	**50-50**	**20%**	**180**
3	**8.03 ± 1.14**	**5.38 ± 0.97**	**1.41 ± 0.21**	**7.59 ± 0.16**	**6.81 ± 0.09**	**25-75**	**30%**	**60**
4	**16.68 ± 1.70**	**14.41 ± 1.47**	**5.20 ± 0.54**	**4.95 ± 1.24**	**13.27 ± 0.89**	**50-50**	**20%**	**60**
5	**38.22 ± 5.35**	**42.01 ± 5.50**	**11.09±1.47**	**26.80 ± 0.71**	**34.10 ± 1.54**	**75:25**	**20%**	**120**
6	**8.48 ± 0.73**	**5.37 ± 0.50**	**1.21 ± 0.11**	**11.23 ± 0.30**	**6.42 ± 0.81**	**25-75**	**20%**	**120**
7	**7.82 ± 0.36**	**4.66 ± 0.18**	**1.13 ± 0.13**	**6.67 ± 0.70**	**5.59 ± 1.05**	**25-75**	**10%**	**180**
8	**8.08 ± 7.51**	**6.47 ± 6.01**	**2.35 ± 2.20**	**4.15 ± 0.29**	**0.99 ± 0.16**	**50-50**	**20%**	**120**
9	**33.43 ± 2.41**	**31.87 ± 3.31**	**8.33 ± 0.61**	**35.65 ± 1.96**	**31.16 ± 0.96**	**75:25**	**10%**	**180**
10	**38.84 ± 1.39**	**37.22 ± 5.37**	**11.83±0.59**	**28.92 ± 1.58**	**25.07 ± 1.78**	**75:25**	**30%**	**60**
11	**5.01 ± 4.63**	**2.38 ± 2.23**	**0.78 ± 0.72**	**8.51 ± 2.15**	**1.12 ± 0.13**	**25-75**	**10%**	**60**
12	**31.91 ± 3.82**	**28.54 ± 3.70**	**9.67 ± 1.37**	**36.20 ± 1.99**	**21.76 ± 5.75**	**75:25**	**10%**	**60**
13	**9.88 ± 8.13**	**8.66 ± 7.17**	**2.71 ± 2.24**	**3.99 ± 0.87**	**2.13 ± 0.07**	**50-50**	**20%**	**120**
14	**10.12 ± 9.37**	**7.80 ± 7.21**	**2.01 ± 1.85**	**2.82 ± 2.62**	**1.27 ± 0.16**	**50-50**	**10%**	**120**
15	**8.92 ± 6.29**	**5.90 ± 4.14**	**2.63 ± 1.85**	**4.19 ± 0.18**	**2.93 ± 0.07**	**50-50**	**20%**	**120**
16	**24.89 ± 2.81**	**24.04 ± 2.70**	**13.84±1.81**	**26.69 ± 1.50**	**26.19 ± 0.33**	**75:25**	**30%**	**180**
17	**4.77 ± 3.66**	**2.07 ± 1.60**	**0.85 ± 0.65**	**8.51 ± 0.15**	**1.50 ± 0.17**	**25-75**	**30%**	**180**

[a] Data are shown as a mean (µg polyphenol /mg of extract) ± SD of at least 5 replications.
[b] Data are shown as a mean (% inhibition/mg of extract) ± SD of 3 replications relative to acarbose (positive control)

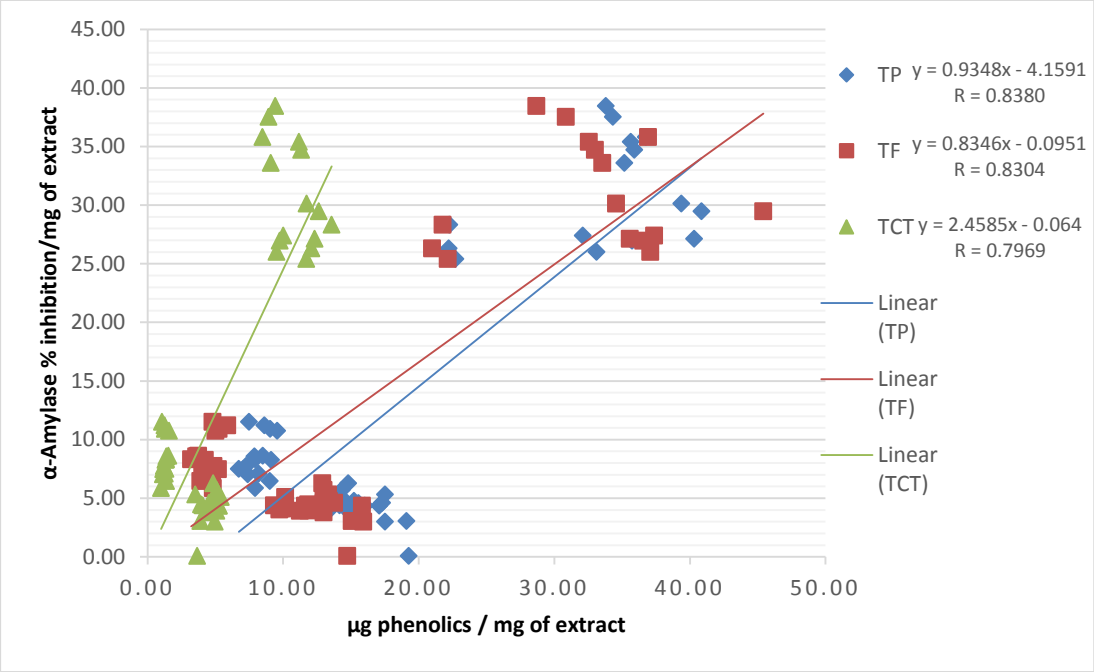

Figure 18: Effect of TP, TF and TCT concentrations on α-amylase inhibitory activities for the acetone solvent. The scatter plot contains data of each replication (trendline equations are for TP, TF and TCT, respectively).

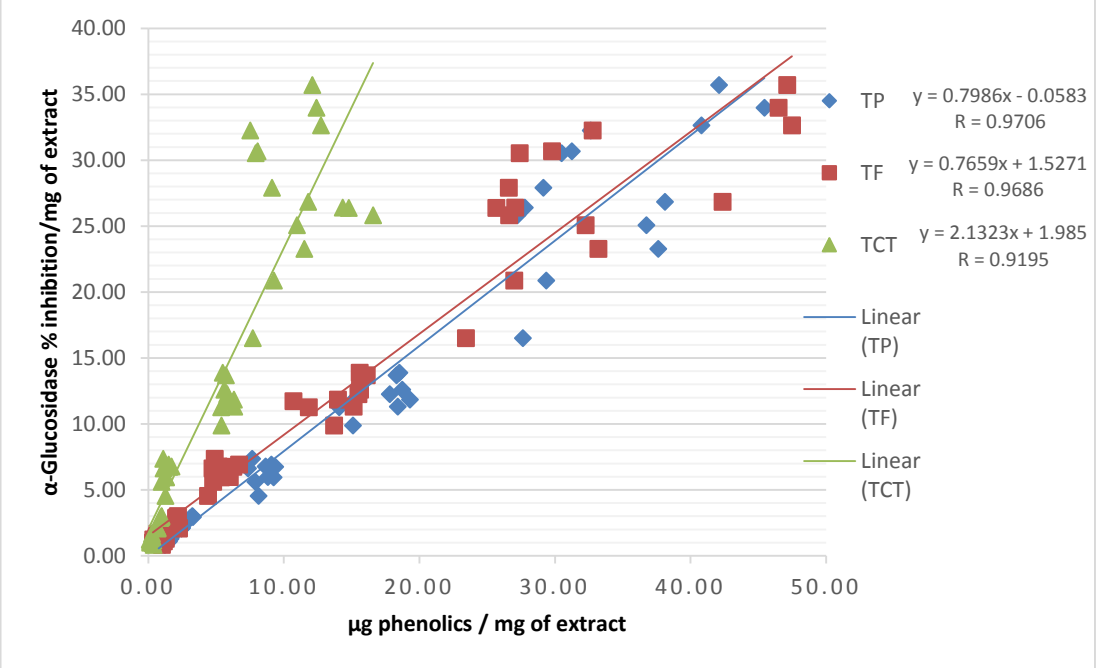

Figure 19: Effect of TP, TF and TCT concentrations on α-glucosidase inhibitory activities for the acetone solvent. The scatter plot contains data of each replication (trendline equations are for TP, TF and TCT, respectively).

A study of several dry bean cultivars (four yellow cultivars, a red coat, two white cultivars, a black bean, a mottled brown bean, and a yellow-brown bean) reported all bean extracts in the study had a capacity to inhibit α-glucosidase, and a significant inhibitor of α-amylase (Ranilla et al., 2010).

E. CONCLUSIONS

In conclusion, findings of this project indicate that the response surface methodology (RSM) was successful for obtaining TP, TF and TCT from black beans. The optimized factors for TP predicted by RSM consists of an acetone: water ratio of 25:75, a solid: solvent ratio of 18 percent and mix time of approximately 111 minutes. In addition, for TF the optimal factors were 25:75 acetone: water ratio, a solid: solvent ratio of 30 percent, and a mix time of approximately 143 minutes. Moreover, the optimum levels of TCT were achieved with a 25:75 acetone: water, a solid: solvent ratio of 14 percent, and a mix time of approximately 60 minutes.

Acetone was the most effective solvent solution for extracting TP, TF and TCT from black beans. Also in most cases, a second-order polynomial could be used to optimize extractions, more complex interactions may be better explained with a higher order model.

The study demonstrated black bean polyphenols were capable of inhibiting α-amylase at an optimal value 36.2 percent and α-glucosidase by 34.1 percent. In addition, the non-phenolic compounds in bean extracts may be involved in the inhibitory properties of key carbohydrate-hydrolysis enzymes. Furthermore, in many cases, direct correlations occurred for inhibition of the enzymes and TP amounts with the acetone extracts

producing the highest correlation in terms of total amounts. Additionally non-polar polyphenols in black beans are more susceptible to inhibit α-amylase and α-glucosidase. Finally, the extraction parameters used must be considered (and characterized) when studying the phenolic levels and their possible health-promoting effect.

F. FUTURE STUDIES

There is a need for future studies to examine black bean polyphenolic extracts with methanol solvents and ethanol solvents to determine if their ability to inhibit carbohydrate- hydrolyzing enzymes (α-amylase and α-glucosidase) has a synergistic effect with other black bean properties. This would explain why optimal inhibition did not always occur in the Extract with the greatest TP, TF and TCT, and the lack of correlation.

Future studies could research the effect of polyphenols in vivo to determine the level of glucose absorption blocked as the result of α-amylase and α-glucosidase inhibition. This would confirm the hypothesis that inhibiting α-amylase and α-glucosidase in the gut will help mitigate the effects of glucose absorption. By tracking the blood glucose levels in the sample population, the success of the strategy can be verified.

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