4-2016

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

Mohammed Alrugaibah
University of Nebraska-Lincoln, m.alrugaibah@gmail.com

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

by

Mohammed A. Alrugaibah

A Thesis

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Food Science and Technology

Under the Supervision of Professor Vicki Schlegel

Lincoln, Nebraska

May, 2016

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

Mohammed Alrugaibah, M.S.

University of Nebraska, 2016

Advisor: Vicki Schlegel

Pinto beans contain high levels of diverse phenols, known mainly for their potent antioxidative properties. However, reports have shown that phenols can inhibit the carbohydrate–hydrolysis enzymes, α-amylase and α-glucosidase, thereby retarding glucose absorption. However, a severe gap in knowledge exists on the ability of pinto beans to inhibit these enzymes. Therefore, the objective of this project was to determine the ability of phenolic rich extracts obtained from pinto beans (BaJa) to inhibit α-amylase and α-glucosidase. The hypothesis was that pinto beans would be able to inhibit these enzymes due to the presence of high levels of chemically diverse phenols. Response surface methodology (RSM) was initially used to characterize extraction parameters that produced extracts with high total phenols (TP), total flavonoids (TF) and total condensed tannins (TCT) levels. This approach provided a starting point to identify the phenolic rich extracts and then to compare their inhibitory effects relative to one another. The project was completed by adjusting the polarity of methanol, ethanol, acetone and altering for three mixing times and solid:solvent ratios. Seventeen extracts produced by each solvent system was examined for TP, TF, TCT and their ability to inhibit α-amylase and α-glucosidase.

The optimum factors as predicted by the analysis of RSM of the quadratic model for TP yields were 75:25 acetone:water, 10 percent solid:solvent and 87 minutes of mixing. For optimal TF extraction 75:25 acetone:water, 10 percent solid:solvent and 119 minutes of mixing were required. Maximum TCT values were achieved with 62:38 acetone:water, 20 percent solid:solvent and 180 minutes of mixing. Acetone extracts were also the most effective for inhibiting α-amylase and α-glucosidase (57.83, 17.59 percent /mg extract, respectively). Alpha-amylase or α-glucosidase inhibition did not correlate with TP, TF or TCT for the methanol extracts, but the correlation increased with the highest occurring with the acetone extracts. In summary, the significance of this project was that extracts originating from pinto beans are capable of inhibiting key carbohydrate–hydrolysis enzymes, but this property depends on the extract, most likely due to different phenol levels / types, and the presence of other nonephenolic components. Nonetheless, this food system may have potent health benefits for diabetics.

DEDICATION

I dedicate this work to my family and many friends. I cannot thank enough my supportive and loving wife as well as my five months old daughter for the inspiration and motivation. A special feeling of gratitude to my loving parents, whose words of encouragement and a push for tenacity ringing in my ears. My brothers and sisters who have supported me throughout the process and never left my side are very special. I also dedicate this work and give special thanks to Qassim University in Saudi Arabia for the overwhelming scholarship.

ACKNOWLEDGEMENTS

First and for most, I thank Allah (GOD), the almighty for easing this path for me. Prophet Mohammed [SAWS] said, "Whosoever does not thank people, has not thanked Allah." This project would not have been possible without the encouragement and support of many people. I would like to sincerely thank my advisor, Dr. Schlegel for her patience, understanding, the endless and numerous help, guidance and most of all the dedication she has given me throughout my whole master's journey.

In addition, I would like to thank my committee members, Dr. Jeyam Subbiah and Dr. Soonkyu Chung for their enlightening comments and commitment to serve on my committee. Very special thanks go to Dr. Carlos Urrea from Panhandle Research and Extension Center at the University of Nebraska-Lincoln for the generous gift of pinto beans for this study. I am also eternally grateful for the help and training by Richard Zbasnik. Indeed, I express my appreciation to all my lab mates, classmates, professors and whomever I learned from for their love, advice and encouragement during my graduate studies. Finally, numerous thanks to the University of Nebraska-Lincoln that provided me with the necessary help to complete this work.

Table of Contents

List of Figures

List of Tables

A. LITERATURE REVIEW

A.1 Background of Dry Edible Beans - Pinto Beans:

Dry edible beans, *Phaseolus vulgaris L.,* (or the common bean) are highly consumed in Central and South America, as well as in other parts of the world (Messina, 1999). More than 40,000 varieties of dry beans exist, and each type has its own subclasses (McGinnis & Suszkiw, 2006). Internationally, India is the largest producer, followed by Myanmar, Brazil, United States and Mexico, respectively (FAOSTAT, 2014). In 2013, the United States produced roughly 1.3 million tons of dry edible beans totaling $977 million and harvested approximately 1.3 million acres (Zahniser & Wells, 2014). By the end of the same year (2013), the U.S. exports of dry beans were 452,000 tons, and the imports were 135,000 tons (FAOSTAT, 2014). Dry beans are grown for their green pods, green leaves and dry seeds, which position this legume as an economically viable crop throughout the world (Jones, 1999).

Nationally, North Dakota is the largest producer of dry beans with 30 percent of the total market share followed by Michigan (18 percent), Minnesota (13 percent) and Nebraska coming in fourth at 10 percent (Figure 1) (USDA, 2016) with different types of market classes grown in the US shown in Figure 2. Nevertheless, dry bean consumption in North America is comparably low despite its rich bean production history, which officially dates back to 1900s, and their diversity and high nutritional composition (Mitchell et al., 2009). More specifically, the average U.S. intake of dry beans is below the recommended value for the vegetable group (DHHS & USDA, 2015). Nonetheless, dry bean consumption has been steadily increasing in the U.S. primarily due to an increased population of Hispanic-Americans (Luthria & Pastor-Corrales, 2006). In the U.S., approximately 7.5 lb of dry beans are consumed annually per capita (USDBC, 2015).

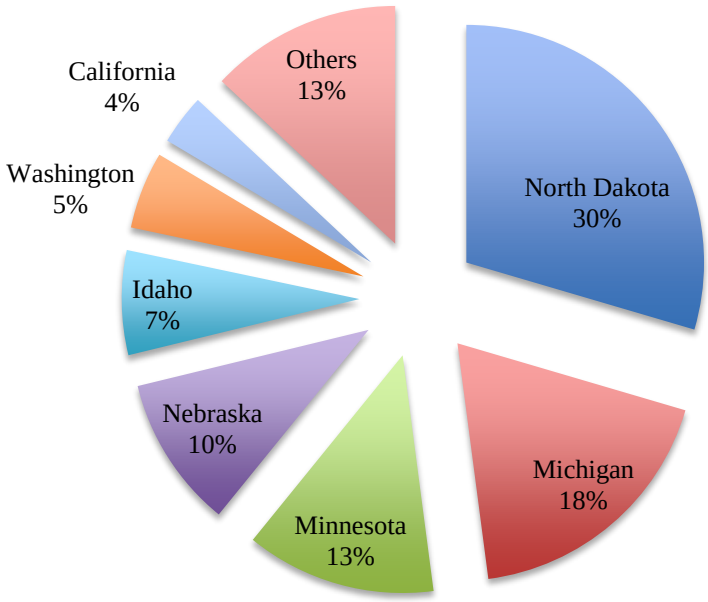


Figure 1. Pie chart shows the leading dry bean producing states (USDA, 2016).

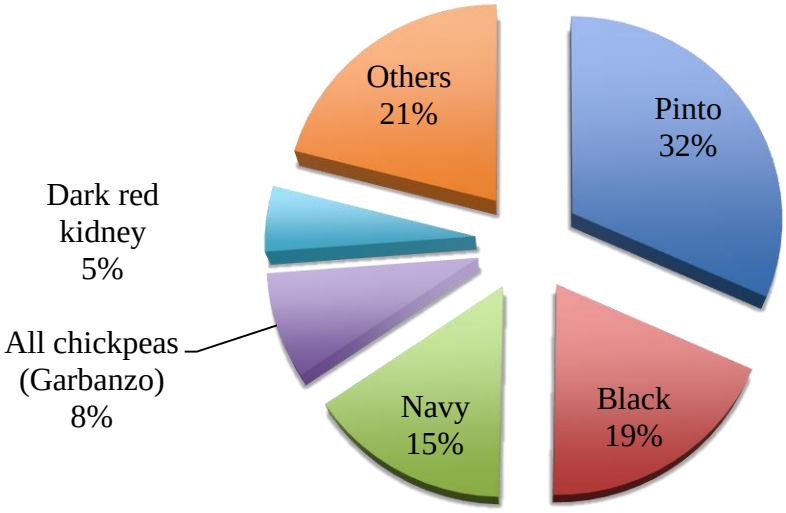


Figure 2. Pie chart shows major types of dry bean production in the U.S. (USDA, 2016).

Among the dry beans market classes, pinto beans are the highest consumed dry bean per capita (3.36 lb) followed by navy (1.7 lb), kidney (0.6 lb) and finally the great northern bean (0.4 lb) (Messina, 1999). Dry beans have also gained in popularity due to their long shelf life, economic affordability and ease of storing and cooking (Jones, 1999). Moreover, dry beans have been characterized as a nearly perfect food because of their high protein content, fiber, complex carbohydrates, vitamin B and other health-benefiting phytochemicals (Madhujith & Shahidi, 2005; Timoracká et al., 2011; Lin et al., 2008; Winham et al., 2008). For example, dry beans contain a diverse group of micronutrients, such as polyphenols that for some market classes (pinto beans, black beans and red beans) are higher than many fruits and vegetables (Lin et al., 2008). Polyphenols have been linked to preventing or remediating cellular conditions, such as oxidation and inflammation, that if left unchecked can lead to other diseases, such as heart disease, cancer, diabetes, arthritis, etc. (Madhujith & Shahidi, 2005; Hughes et al., 1997; Mensack et al., 2012; Bennink, 2002). However, a severe gap in knowledge exists that directly links dry beans to these health-promoting properties.

Pinto beans were selected for this project because they are consumed by many people, as stated previously, in both developed and undeveloped countries (Lin at al., 2008). This project is further tied to pinto beans because Nebraska is the second largest grower of this bean in the U.S. (USDA, 2016) and the highest produced bean in the U.S. (Figure 2). In 2015, Nebraska produced approximately 105,000 tons of pinto beans (USDA, 2016). Nonetheless, significant challenges are facing the long-term production of dry bean in our state, including higher prices of competing corps and the influx of dry beans from international sources. This is particular affecting the Nebraska Panhandle where most of the Nebraska dry beans are grown, as this region

is currently experiencing flat and declining populations. Firmly establishing pinto beans as human health-benefiting food is needed to increase pinto bean market demand and prices, while protecting the overall health of our citizens against western-based diseases.

A.2 Dry Bean Composition:

Dry beans have high nutritional value as stated above (Saari et al., 2006; Kalogeropoulos et al., 2010; Timoracká et al., 2011). More specifically, most of dry beans market classes share the same composition in their major components (Table 1), but differ in their micronutrients (USDA, 2015). For example, one-half cup of cooked dry beans provides approximately the following daily values (DV): protein (15 percent), carbohydrate (8 percent), fiber (30 percent), calcium (4 percent), magnesium (12.5 percent) and folate (34 percent) (USDA, 2006). Additionally, the energy content of dry beans is low, which ranges between 103 and 151 Kcal/100 g beans for different market classes (Kalogeropoulos et al., 2010). It is important to mention that many factors affect the micronutrients composition of dry beans, including the market class, drought, soil fertility, storage conditions, and thermal processing (Luthria & Pastor-Corrales, 2006; Xu & Chang, 2009; Dannehl & Josuttis, 2014; Shrestha & St Clair, 2014).

Dry beans in general have low fat levels (1–2 percent) with the exception of chickpeas (15 percent) (Messina, 1999). Therefore, dry beans are good alternative to red meat in the diet due to their lower content of fat (Anderson et al., 1999) in combination with their high protein levels, as will be discussed latter. The major types of fatty acids in dry beans are essential polyunsaturated fatty acids, such as linoleic acid (LA) (n-6, 18:2) and α-linolenic fatty acid (ALA) (n-3, 18:3) (USDA, 2015) being the most abundant. In pinto beans, ALA content is 328 mg/100 g boiled pinto

Table 1. Nutritional values of raw pinto beans (USDA, 2015*)

Nutrient	Unit	Value Per 100 g (Raw)
Main Components		
Water	g	11.3
Energy	kcal	347
Protein	g	21.4
Total lipid (fat)	g	1.2
Carbohydrate	g	62.5
Fiber, total dietary	g	15.5
Sugars, total	g	2.1
Minerals		
Calcium, Ca	mg	113
Iron, Fe	mg	5.07
Magnesium, Mg	mg	176
Phosphorus, P	mg	411
Potassium, K	mg	1393
Sodium, Na	mg	12
Zinc, Zn	mg	2.2
Vitamins		
Vitamin C, total ascorbic acid	mg	6.3
Thiamin	mg	0.71
Riboflavin	mg	0.21
Niacin	mg	1.17
Vitamin B-6	mg	0.47
Folate, DFE	µg	525
Vitamin E (alpha-tocopherol)	mg	0.21
Vitamin K (phylloquinone)	µg	5.6
Lipids		
Fatty acids, total saturated	g	0.23
Fatty acids, total monounsaturated	g	0.22
Fatty acids, total polyunsaturated	g	0.40

***** National Nutrient Database for Standard Reference (Release No. 28).

bean (Kalogeropoulos et al., 2010). Moreover, omega 3 fatty acids in dry beans range between 2–40 percent of the total fatty acids for different market classes, while omega 6 fatty acids range between 20–50 percent. Lastly, trans fatty acids are less than 1 percent (Kalogeropoulos et al., 2010). Omega 3 and 6 fatty acids have positive effects on lipid peroxidation and platelet activities. In particular, lipid peroxidation can cause negative effects, by generating highly toxic free radicals, which in turn can damage main cellular components (DNA, proteins and lipid membranes) (Maritim et al., 2003). Eicosapentaenoic acid (EPA) can be elongated by α-linolenic in humans; although, EPA can be transformed into docosahexaenoic acid (DHA), but the conversion efficiency is low (5 percent). Both EPA acid and DHA are polyunsaturated fatty acids and have been shown multiple health promoting properties (Messina, 1999).

Based on a study of different dry bean market classes and their cultivars, their phytosterols (plant sterols) content was between 13.5 mg/100 g in cooked black-eyed beans and 48.9 mg/100 g in cooked chickpeas, while pinto beans contained 21.5 mg/100 g (Table 2) (Kalogeropoulos et al., 2010). According to Kalogeropoulos et al. (2010), the most predominant phytosterol was β-sitosterol for all the pinto bean cultivars examined (12.5 mg/100 g cooked) Yet, a severe gab of knowledge exists on phytosterols levels in many common beans, *Phaseolus vulgaris L.*, due mainly to the low fat content. Nevertheless, phytosterols have received extensive attention for their effective role in protecting against low-density lipoprotein (LDL) by inhibiting intestinal cholesterol absorption; thereby, reducing cholesterol in the plasma (Peterson, 1951; Moghadasian & Frohlich, 1999; Law, 2000). Dry beans also contain tocopherols and squalene in their lipid fractions (Table 2). Squalene is a biochemical intermediate in the synthesis of plant and animal sterols (Kelly, 1999).

Table 2. Tocopherols, squalene and phytosterols content in cooked pinto beans[a]

Class	Subclass	Content (mg/100 g)[b]
Tocopherols	α-	0.01
	(β + γ)-	0.08
	δ-	0.32
	Total	0.41
Squalene		0.23 ± 0.02
Phytosterols	β-Sitosterol	12.5 ± 0.9
	Campesterol	1.75 ± 0.11
	Stigmasterol	4.82 ± 0.43
	Δ^5-Avenasterol	2.44 ± 0.17
	Total	21.5 ± 1.9

[a] Pinto beans were soaked in water overnight, then boiled for 50 minutes.
[b] Results are shown as average ± standard deviation of three replication (Adapted from Kalogeropoulos et al., 2010).

Squalene content in dry beans is very low, ranging between 0.45 and 0.94 mg/100 g and 0.23 mg/100 g in pinto beans (Kalogeropoulos et al., 2010). Dry beans contain tocopherols as low as 0.26 mg/100 g in cooked giant beans to 1.78 mg/100 g in cooked chickpeas. Alternatively, total tocopherol in cooked pinto beans is approximately 0.40 mg/100 g (Kalogeropoulos et al., 2010). The previous study examined tocopherols after a boiling process (30–100 minutes) and showed tocopherols types and levels among the various beans and other tested legumes. The most predominate tocopherol in pinto beans is delta (δ- tocopherol), while alpha (α-) is the least abundant in most dry beans (Boschin & Arnoldi, 2011; Kalogeropoulos et al., 2010). Vitamin E, which encompasses all three tocopherols isomers and three tocotrienols isomers, serves as a fat-soluble antioxidant that protects polysaturated fatty acids from lipid peroxidation (Horwitt, 1986; Di Mascio et al., 1991; Jiang at al., 2001).

Dry beans contain comparably high levels of proteins, which represent about 20–30 percent of its total energy. Raw pinto beans are composed of ≈ 20–23 g/100 g protein for different cultivars (Table 1) and 9 g/100 g in cooked beans (USDA, 2015). Almost all dry beans cultivars are poor in the sulfur-based amino acids, such as methionine and cysteine, in comparison to the other essential amino acids (Kannan et al., 2001; Koehler et al., 1987). However, lysine is the most predominant amino acid in all dry beans varieties; whereas pinto bean has the greatest protein quality among the dry beans (relative nutritive value >90) (Koehler et al., 1987). As noted by the Protein Digestibility Corrected Amino Acid Score (PDCAAS), which was developed by the Food and Agriculture Organization (FAO) and the World health Organization (WHO) in 1991, as a preferred method for the assessments of the protein quality in the human diet (Schaafsma, 2000), the PDCAAS of most dry beans are relatively high

(Sarwar & McDonough, 1989). In 2013, FAO proposed a new method for measuring protein quality to replace the PDCAAS, called the Digestible Indispensable Amino Acid Score (DIAAS), which has not been completed as of yet on dry beans.

Many dry beans verities have higher levels of dietary fibers than most food types (USDA, 2015). Total dietary fiber in dry bean classes range between 5.3–10.5 g/100 g cooked dry bean (USDA, 2015). In fact, cooked dry beans (≈100 g) provide 21 up to 42 percent of the DV of fiber based on an intake of 2,000 calories for adults and children four years or older (USDA, 2015; FDA, 2014). Total dietary fiber in cooked pinto beans is approximately 10 g/100 g, providing 36 percent of the DV of fiber (USDA, 2015). Nevertheless, Kutos et al. (2003) reported that processing of cooking and pretreatment of pinto beans significantly affected total dietary fiber (TDF), soluble dietary fiber (SDF), insoluble dietary fiber (IDF) and resistant starch (RS) levels. They determined that IDF decreased substantially as did TDF levels for all thermally processed samples. Moreover, the authors showed that processing (soaking and cooking) of pinto beans significantly decreased RS relative to raw pinto beans. In contrast, another study showed that thermal processing of two common beans (black and flor de mayo) increased the resistance starch content (Osorio-Díaz et al., 2003).

Dry beans are recognized in the Eighth Edition of the Dietary Guidelines for Americans (2015-2020). The key recommendations for healthy eating patterns by the dietary guidelines placed legumes (beans and peas) in two categories, i.e., the vegetable group and the protein group. Because beans are classified as both a vegetable and a protein source; they can be consumed as either to meet the recommended intake. The recommended amount of vegetables (i.e., beans) for a 2,000-calorie diet is 2.5 cup-equivalents of vegetables per day and 5.5 ounce-

equivalents of protein per day (DHHS & USDA, 2015). Interestingly, the new Dietary Guidelines calls for a shift in the consumption of protein foods, i.e., to vary from dry beans and peas with red and white meat.

A.3 Phenolic Compounds:

Approximately 100,000 known secondary metabolites have been characterized in plants (Mazid et al., 2011). Secondary metabolites act as defense mechanisms to protect the host organism against environmental stresses, such as biotic stress, i.e., bacteria, fungi, insects, mammals, birds, etc., and/or abiotic stress, i.e., temperature change, drought, salinity, U.V. stress, etc. (Lattanzio et al., 2008; Gómez-Caravaca et al., 2013). It should be noted, however, that drought for plants is considered to be the most harmful abiotic stress (Chaves et al., 2003). In this section, a brief discussion is presented on polyphenols, a category of secondary metabolites ubiquitous to all plants (Appel & Schultz, 1992), their classes, and the most common distributed phenolic components present in dry edible beans, with an emphasis on pinto beans.

Phenols are characterized in various ways according to their structure considering that more than 7,000 chemically diverse phenols have been identified. These compounds range from free mono-phenols to highly-polymerized compounds (Gómez-Caravaca et al., 2013). Biologically, phenolic compounds evolve from two metabolic pathways: the shikimate-phenylpropanoid pathway, where most phenolic compounds are synthesized, and the acetic-acid pathway, where the main molecules consist of a simple phenol (Chen at al., 2009; Giada, 2013; Gómez-Caravaca et al., 2013). Furthermore, the combination of these two pathways leads to the formation of flavonoids, which are the largest group of phenolic compounds in nature (Giada, 2013). Phenolic compounds are characterized by the presence of at least one phenol

(or aromatic ring) bonded to one or more hydroxyl groups (Strack, 1997; Gómez-Caravaca et al., 2013). The most common polyphenols occur when derivatized to other molecules, such as sugars (i.e., glycosyl residue), amino acids, organic acids or lipids. They also may exist in a free form (unconjugated), which are called aglycones (Clifford & Brown, 2006; Giada, 2013). Polyphenols have been categorized into four main classes (Figure 3): flavonoids, phenolic acids, stilbenes and lignans (Butterfield et al., 2002; Han et al., 2007; Gómez-Caravaca et al., 2013).

Polyphenols are distributed in different locations in the plant system, including the cell nuclei, the cellular and subcellular tissues, cell membrane (i.e., insoluble phenolics) and/or the plant-cell vacuoles (i.e., soluble phenolics) (Hutzler et al., 1998; Pandey & Rizvi, 2009). Considering that these compounds react to environmental stresses, the levels of phenols in a food system can differ according to the degree of ripeness, farming practices, environmental factors and/or processing and storage conditions (Pandey & Rizvi, 2009; Dannehl & Josuttis, 2014; Shrestha & St Clair, 2014).

A.3.1. ***Flavonoids*:** Flavonoids are the most studied group of polyphenols (Lattanzio et al., 2008; Clifford et al., 2006). In "*The Handbook of Natural Flavonoids*," by Harborne & Baxter (1999), the authors listed 6,467 flavonoid structures. However, identification of other flavonoids has been increasing rapidly. In 2006, Andersen et al. (2006) reported more than 7,000 structures of different flavonoids classes in their book, "Flavonoids: Chemistry, Biochemistry and Applications." From a chemical perspective, flavonoids are characterized by a C6−C3−C6 structure, which consist of two phenyl rings and a heterocycle ring (Manach et al., 2004; Clifford et al., 2006).

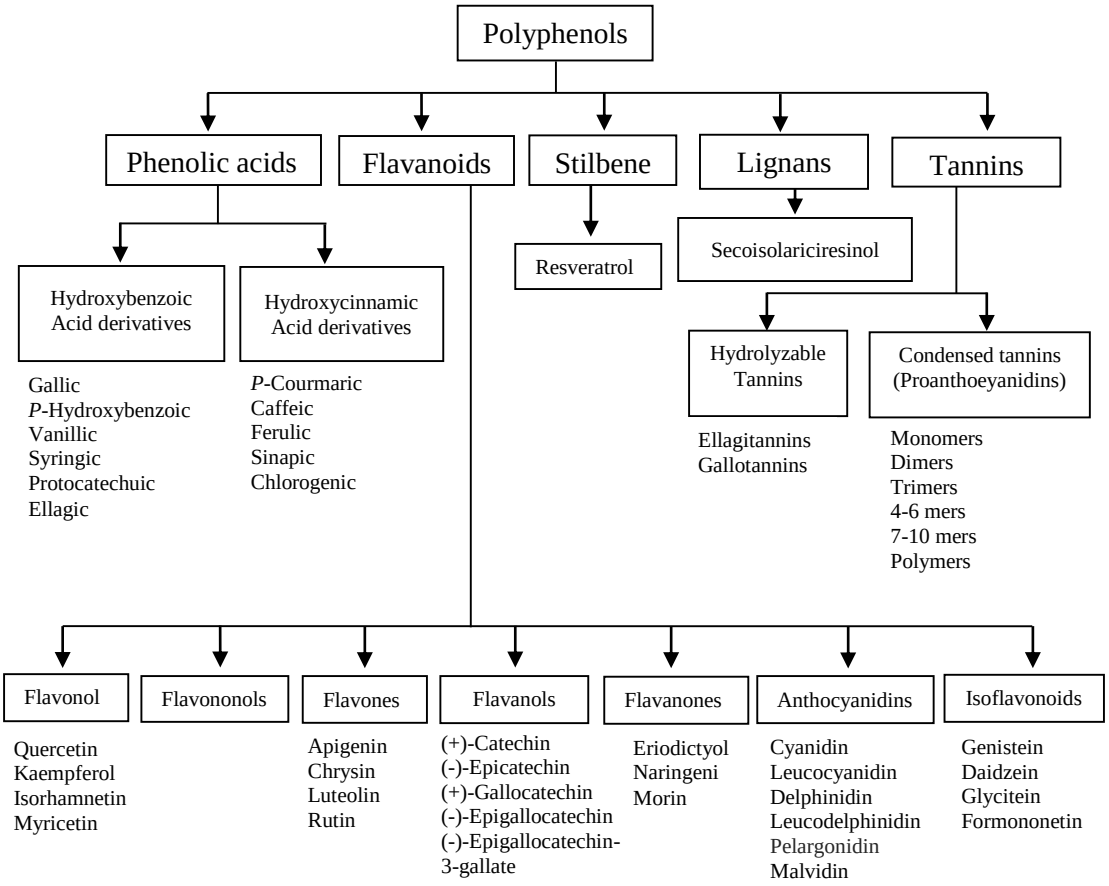
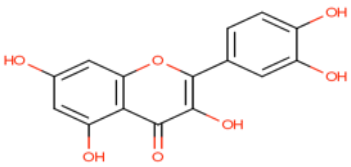


Figure 3. Classification of polyphenols (Shahidi & Ambigaipalan, 2015).

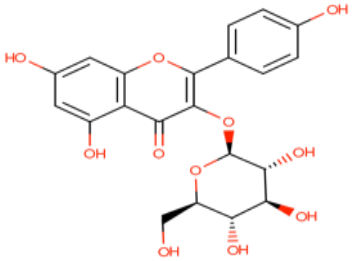
Flavonoids are further divided into six subclasses: flavonols, flavanones, flavanols, flavones, isoflavones and anthocyanins (Figure 3), which is based on the location of various functional groups but mainly the hydroxyl groups (Pandey et al., 2009; Tsao, 2010). Many of the flavonoids add color to foods (i.e., anthocaynins), but some are colorless (i.e., flavanones), or now are thought to lack pigments in the visible region (Giada, 2013). The average daily intake of flavonoids in the United States ranges from 100 mg to 2 g (Clifford, 2004). Common flavonoids in pinto beans are shown in Figure 4.

A.3.2. *Phenolic Acids:* Phenolic acids are also present in all plant based systems, including dry edible beans. They are divided into two main groups (Figure 3): the hydroxybenzoic and hydrocinnamic acids, each of which can be diravitized with other compounds (Giada, 2013; Pandey & Rizvi, 2009). Phenolic acids are classified by a benzene ring linked to a carboxylic acid and at least one or more hydroxyl groups (Giada, 2013). The cinnamic acids have a $C_6{-}C_3$ structure (Figure 5) and are more common in plants (i.e., dry beans) than the benzoic acids, which have a $C_6{-}C_1$ structure (Figure 6) (Rice-Evans et al., 1996; Lin et al., 2008; Shahidi & Ambigaipalan, 2015).

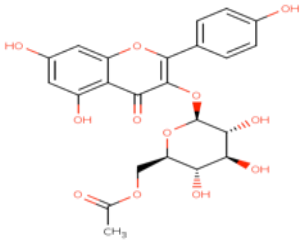
Phenolic acids in plants take primarily four forms: free (aglycones), insoluble-bound, esterified and/or glycosides (Krygier et al., 1982; Naczk & Shahidi, 1989). However, phenolic acids are mostly present in the bound form (Shahidi & Ambigaipalan, 2015), such as lignins, which are cross-linked phenolic polymers. Depending on the diet consumed, it has been estimated that the dietary intake of phenolic acids is approximately 25 mg to 1 g /day (Shahidi & Ambigaipalan, 2015; Clifford, 2004).



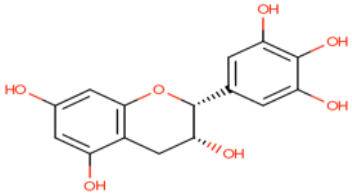
Quercetin



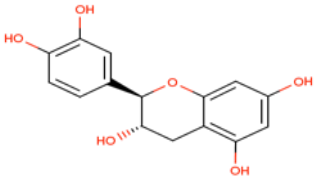
Kaempferol 3-O-glucoside



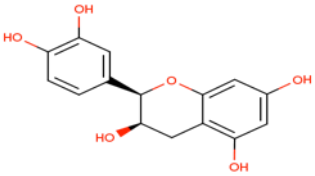
Kaempferol-3-O-acetylglucoside



(+)-Catechin

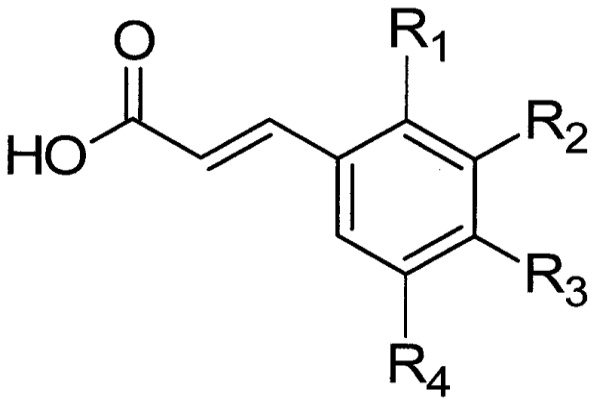


(-)-Epicatechin



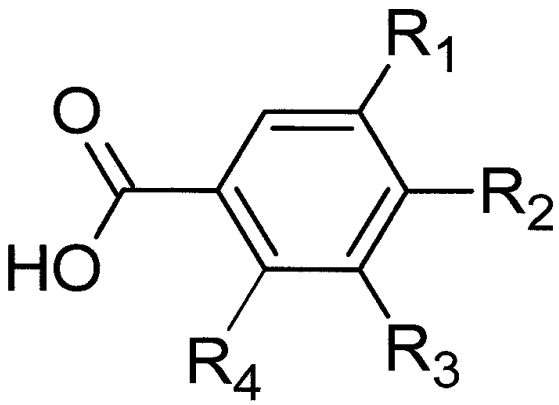
(-)-Epigallocatechin

Figure 4. Major subclasses of flavonoids found in pinto beans (Phenol-Explorer database, 2016; Xu & Chang, 2009; Espinosa-Alonso et al., 2006; de Pascual-Teresa et al., 2000).



Ceramic acid ($R_1 = R_2 = R_3 = R_4 = H$)
o-Coumaric acid ($R_1 = OH$; $R_2, R_3, R_4 = H$)
m-Coumaric acid ($R_2 = OH$; $R_1, R_3, R_4 = H$)
p-Coumaric acid ($R_3 = OH$; $R_1, R_2, R_4 = H$)
Caffeic acid ($R_2 = R_3 = OH$; $R_1, R_4 = H$)
Ferulic acid ($R_2 = OCH_3$; $R_3 = OH$; $R_1, R_4 = H$)
Sinapic acid ($R_2 = R_4 = OCH_3$; $R_3 = OH$; $R_1 = H$)

Figure 5. The chemical structure and formulas of the main cinnamic acids (Adapted from Giada, 2013).

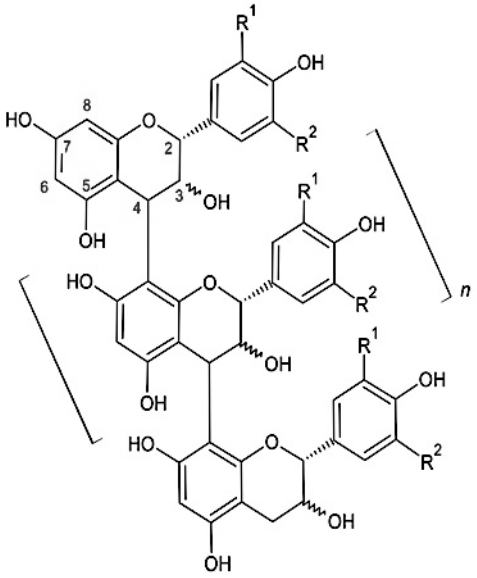


Salicylic acid ($R_4 = OH$, $R_1, R_2, R_3 = H$);
Gentisic acid ($R_1, R_3 = OH$; $R_2, R_4 = H$);
p-Hydroxybenzoic acid ($R_2 = OH$, $R_1, R_3, R_4 = H$);
Protocatechuic acid ($R_1, R_2 = OH$; $R_3, R_4 = H$);
Vanillic acid ($R_1 = OCH_3$, $R_2 = OH$; $R_3, R_4 = H$);
Gallic acid ($R_1, R_2, R_3 = OH$; $R_4 = H$);
Syringic acid ($R_1, R_3 = OCH_3$; $R_2 = OH$; $R_4 = H$)

Figure 6. The chemical structure and formulas of the main benzoic acids (Adapted from Giada, 2013).

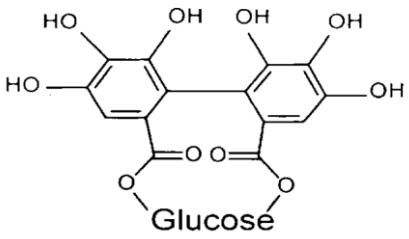
A.3.3. *Tannins*: Tannins are also considered polyphenols, but they are cross linked flavonoids only or with flavonoids and phenolic acids that can form large polymers. The molecular weight of tannins ranges were initially proposed to be between 500 and 3,000 Da (Naczk et al., 1994), but these levels have now changed. It is now postulated that tannins' molecular weight (i.e., proanthocyanidins) can be as high as to 20,000 Da (Cannas, 2010; Cheynier, 2012). Tannins are known for their ability to bind and precipitate proteins (Vermerris & Nicholson, 2008). Nevertheless, tannins may form complexes with starch, cellulose and/or minerals (Cannas, 2010).

Tannins are categorized into two main groups (Figure 3): hydrolysable tannins and condensed tannins, which are often called *proanthocyanidins* (Chung et al., 1998; Giada, 2013). The hydrolysable tannins (Figure 7) are derivatives of gallic acid or hexahydroxydiphenic acid, which form gallotannins and ellagitannins, respectively (Okuda et al., 1995; Cheynier, 2012). In contrast, the proanthocyanidins, or condensed tannins (Figure 7), are oligomers or polymers of flavan-3-ols and mostly linked by carbon-carbon bonds (type-B) or doubly linked (type-A) (USDA, 2004). Proanthocyanidins are the second-most abundant phenolic compound after lignans (Gu et al., 2004). Proanthocyanidins take the form of procyanidin (monomers of catechin or epicatechin), prodelphinidin (monomers of gallocatechin or epigallocatechin) and/or propelargonidin (monomer of afzelechin) (Gu et al., 2004; USDA, 2004). Nevertheless, the most abundant type of proanthocyanidins is procyanidins, which may exist as polymers composed of units as high as 50 (Crozier et al., 2012). Tannins are naturally present in foods, such as tea, chocolate, fruits (i.e., berries, raspberries, grape seeds, etc.), legumes (i.e., dry beans and sorghum), nuts (i.e., hazelnuts, pecans, pistachios, almonds, etc.) and spices (i.e., cinnamon and curry) (Gu et al., 2004; de Pascual-Teresa et al., 2000; USDA, 2004).



Propelargonidin	$R^1 = H$
	$R^2 = H$
Procyanidin	$R^1 = OH$
	$R^2 = H$
Prodelphinidin	$R^1 = OH$
	$R^2 = OH$

A. Proanthocyanidins



B. Ellagitannins

Figure 7. Chemical structure of proanthocyanidins (condensed tannins) and hydrolysable tannins (ellagitannins) (Adapted from Van Huynh & Bevington, 2014; Wilson & Hagerman, 1990).

A.3.4. *Specific Phenolic Compounds in Pinto Beans:* In terms of human health, phenols have been linked to the prevention of multiple chronic diseases as stated above (Madhujith & Shahidi, 2005; Hughes et al., 1997; Mensack et al., 2012; Bennink, 2002). It is important to note again that the phenolics compounds are present in many dry beans, such as pinto beans (Table 3) at levels greater than in most fruits and vegetables (de Pascual-Teresa et al., 2000; Xu et al., 2007; Xu & Chang, 2009; Bhagwat, et al., 2014).

Pinto beans have been shown to contain specific phenolic acids, such as *p*-coumaric acid, ferulic acid, sinapic acid (Table 4) (Luthria & Pastor-Corrales, 2006; Ross et al., 2009), chlorogenic acid (Xu & Chang, 2009; Mojica et al., 2015), protocatechuic acid, *p*-Hydroxybenzoic acid, gentisic acid (Ross et al., 2009), vanillic acid and syringic acid (Mojica et al., 2015). Pinto beans also contain flavonols (Table 5), such as kaempferol 3-*O*-glucoside and kaempferol 3-*O*-acetylglucoside (Xu & Chang., 2009; Lin et al., 2008), quercetin (Espinosa-Alonso et al., 2006), and quercetin 3-*O*-glucoside (Mojica et al., 2015). Specific flavanols (flavan-3-ols) are present in pinto beans (Table 5), such as (+)-catechin, (-)-epicatechin and (-)-epigallocatechin (de Pascual-Teresa et al., 2000), but again the amounts vary depending on the cultivar, farming practices, storage conditions, processing and cooking. Importantly, extraction methods greatly influence how much phenols are cited in the literature of a given legume as will be confirmed by this study, and has been by other unpublished studies in our lab. Several phenolics have also been detected in pinto beans, i.e., the isoflavones, (daidzein and genistein) as well as the lignans, such as secoisolariciresinol (Mazur et al., 1998).

Additionally, proanthocyanidins (condensed tannins) (Table 6) are present in pinto beans mainly in two forms: monomers or polymers of procyanidin (PC) and

Table 3. Total phenols, TF and TCT contents of different market classes of dry edible beans

Bean Class	TP-Total Phenol	TF-Total Flavonoid	TCT-Condensed Tannin
Pinto	3.76 ± 0.06	2.99 ± 0.12	3.23 ± 0.11
Small Red	5.76 ± 0.38	4.24 ± 0.10	5.16 ± 0.11
Black	3.37 ± 0.15	2.51 ± 0.12	4.09 ± 0.10
Red kidney	4.05 ± 0.05	3.39 ± 0.09	2.87 ± 0.09
Navy	0.57 ± 0.05	0.92 ± 0.02	0.47 ± 0.01

Values reported for TP, TF and TCT are in mg/g. Results are shown as a mean +/- standard deviation (n=3) on dry weight basis. Different market classes of common beans (*Phaseolus vulgaris L*) were used. The bean flour (0.5 g each) were extracted with mixture of acetone/water/acetic acid (70:29.5:0.5, v/v/v). The mixture were extracted for 3 hours under horizontal shake for 300 rpm followed by another 12 hours of overnight incubation in dark. The residues were re-extracted with 5 ml of respective extraction solvents and the extracts were combined and stored at 4 °C. Total phenol, flavonoid, and condensed tannins were determined using a colorimetric method (Xu et al., 2007).

Table 4. Phenolic acids content of different market classes of dry edible beans

Bean Class (Cultivar)	Phenolic acids concentration (mg/100 g) (n=3)				Total phenolic acids content (mg/100 g)
	Caffeic acid	*p*-Coumaric acid	Ferulic acid	Sinapic acid	
Pinto (Maverick)	ND*	4.5	16.0	9.0	29.5
Great Northern (Norstar)	ND	4.0	17.0	9.4	30.4
Navy (Vista)	ND	12.4	26.6	9.2	48.2
Black (T-39)	1.1	9.42	20.62	7.2	37.25
Dark Red Kidney (Red Hawk)	ND	1.8	15.3	3.8	20.9
Red Mex (UI 239)	ND	5.8	17.4	5.4	28.6

*Not Detected (ND). Ground beans were extracted with MeOH containing 0.2% TBH (2, 3-tertbutyl- 4-hydroxy anisole) and 10% acetic acid (85:15). The mixture was then sonicated for 30 minutes and the volume of the extract was adjusted to 10 mL with distill water. Individual phenolic acids were quantitated by HPLC diode array detector (Luthria & Pastor-Corrales, 2006).

Table 5. Content of flavonoid subclasses in pinto beans

Subclass	Flavonoid	Content (mg/100g)	Reference
Flavonols	Kaempferol 3-*O*-glucoside	14.7	Xu & Chang, 2009
	Kaempferol-3-*O*-acetylglucoside	3.00	
	Quercetin	0.22	Espinosa-Alonso et al., 2006
Flavanols (flavan-3-ols)	(+)-Catechin	5.07	de Pascual-Teresa et al., 2000
	(-)-Epicatechin	0.14	
	(-)-Epigallocatechin	0.05	

Data are shown as an average of 2-3 replications of a raw weight.

Table 6. Content of proanthocyanidins (condensed tannins) in pinto beans

Pinto bean	Proanthocyanidin	mg / 100 g
Cooked, boiled, without salt	Monomers	1.75 ± 0.06
	Dimers	4.40 ± 0.36
	Trimers	3.91 ± 0.32
	4-6mers	10.52 ± 5.46
	7-10mers	4.32 ± 5.48
	Polymers	1.41 ± 1.66
Raw	Monomers	10.72 ± 4.06
	Dimers	19.22 ± 12.69
	Trimers	16.18 ± 12.00
	4-6mers	125.90 ± 9.21
	7-10mers	135.62 ± 10.43
	Polymers	459.63 ± 34.15

Data are shown as means of 4-7 replications ± standard deviation (de Pascual-Teresa et al., 2000; Gu et al., 2004; USDA, 2004).

propelargonidin (PP) with C-C linkages (Gu et al., 2004; USDA, 2004). In addition, the phenolic aldehyde, vanillin, is present in different pinto bean cultivars (Mojica et al., 2015). Notably, the literature has not reported the presence of anthocyanins in pinto beans to our knowledge (Espinosa-Alonso et al., 2006; Lin et al., 2008; Xu & Chang, 2009; Mojica et al., 2015).

A.4 Diabetes Mellitus:

Type 1 diabetes – previously called "juvenile" diabetes – is usually diagnosed in children and young adults. It occurs when the body limits or stops the production and regulation of insulin (CDC, 2014). On the other hand, Type 2 diabetes presents when the body does not utilize or respond to insulin appropriately (is resistant to insulin), followed by a failure of beta cells in the pancreas to produce sufficient amounts of insulin (ADA, 2016; CDC, 2014). Type 2 diabetes is the most common, occurring in 90-95 percent of the cases, whereas type 1 account for 5 percent of total diabetics (CDC, 2014).

In 2001, WHO released statistics on trends in worldwide diabetes. It was projected that total diabetics worldwide will reach 370 million by 2030 (WHO, 2001), but in 2014 sadly, people with diabetes exceeded 380 million (WHO, 2014). In 2012, approximately 9.3 percent of the U.S. population (29.1 million) presented with diabetes with 21 million diagnosed and 8.1 million remaining undiagnosed (CDC, 2014). Moreover, an estimated 86 million U.S. adults (1 out of 3) have prediabetes, and it is estimated that 90 percent of these individual do not know they have the condition (CDC, 2014). As an outcome, the total medical costs and loss of wages for people diagnosed with diabetes was approximately $245 million in 2012 (CDC, 2014).

Diabetes was the seventh leading cause of death in 2010 in the U.S. (CDC, 2014), and the eighth leading cause of death in the world (WHO, 2014). Diabetes can also lead to other serious complications, such as hypoglycemia, hypertension, dyslipidemia, cardiovascular disease, heart attack, stroke, kidney failure and blindness (CDC, 2014; WHO, 2014). Therefore, diabetes mellitus is considered the major epidemic of the 21st century. The etiology of diabetes is still ambiguous, and treatment protocols remain limited (Maritim et al., 2003), although Type 2 diabetes can be managed with healthy eating habits, exercise and weight control (Nelson et al., 2002; Sigal et al., 2006; Klein et al., 2004).

A.5 <u>Carbohydrate digestive-based enzymes (α-amylase and α-glucosidase)</u>:

Alpha-amylase (EC 3.2.1.1) hydrolyzes the long chains of starch (amylose and amylopectin) into lighter weight molecules of α–limited-dextrins, maltose, and maltotrios (Hanhineva et al., 2010). Alpha-amylase is endo-acting on the α–1,4 glycosidic linkages of starch (Figure 8a) (Buisson et al., 1987; Stephen et al., 2014). In terms of its source, α-amylase is present in mammals (pancreas & saliva), plants (malt from barely or wheat) and microorganisms (i.e. *Bacillus* & *Aspergillus*) (Damodaran, et al., 2007; Buisson et al., 1987). The pH stability of α-amylase ranges between 6 and 10, and the thermal stability depends on the source of the enzyme (Damodaran, et al., 2007). (Figure 8b shows the protein tertiary structures of α-amylase and α-glucosidase). Alternatively, α-glucosidase (EC 3.2.1.20), located in the epithelium of the small intestines, hydrolyzes disaccharides into monosaccharides (Matsui et al., 2001; Striegel et al., 2015; Thilagam et al., 2013) by targeting the terminal α–1,4 glycosidic linkage residues (Hanhineva et al., 2010).

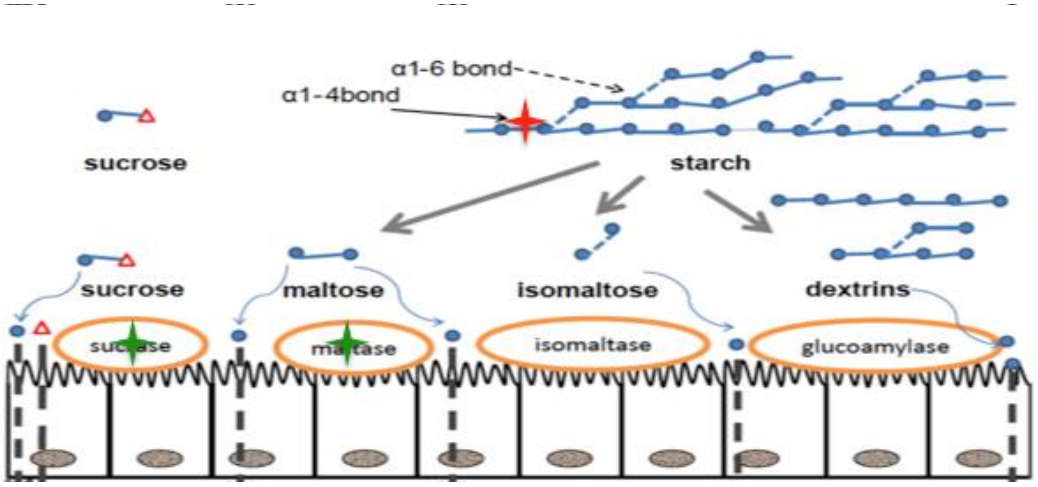
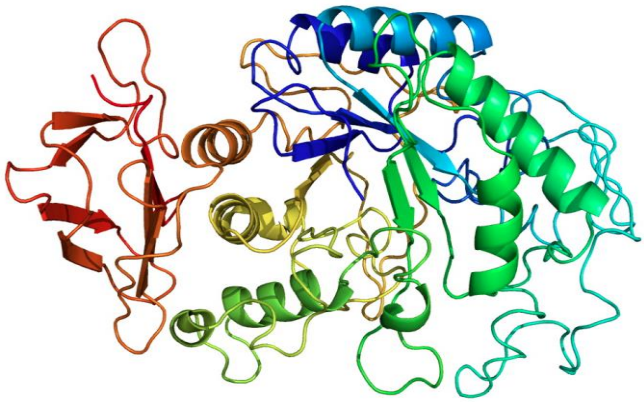


Figure 8a: Starch degradation and site of action of α-amylase (red star) and α-glucosidase (green star) (adapted from Tappy, 2012).

i.



ii.

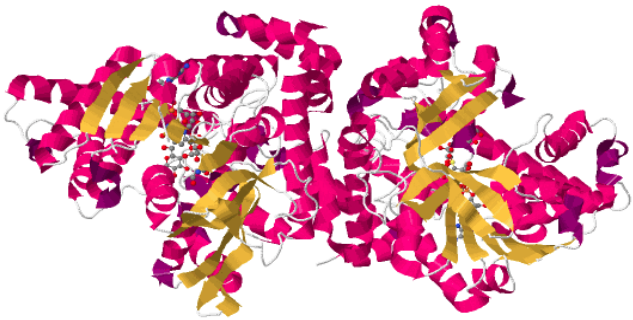


Figure 8b. Crystal structures of human pancreatic α-amylase (i) and α-glucosidase (ii) (Adapted from RCSB PDB, 2015).

Indeed, the inhibition of α-amylase and α-glucosidase may reduce or delay the absorption of glucose in the digestive track and hence, its level in the blood stream. Such delayed rates can aid in the management or regulation of type 2 diabetes (Hogan et al., 2010; Vinson et al., 2009; Udani et al., 2009; Sheliya et al., 2015). The most commonly used drugs for inhibiting α-glucosidase, and consequently for treating type 2 diabetes, are acarbose, miglitol and voglibose. Acarbose also inhibits α-amylase. However, these drugs may cause several side effects (Luna & Feinglos, 2001; Dabhi et al., 2013).

Recent reports have shown that polyphenols can affect glucose utilization (Broadhurst et al., 2000; Bhandari et al., 2008), even though the health benefits of polyphenols have been attributed mainly to their antioxidative properties, i.e., scavenging free radicals, chelating redox metals, inhibiting oxidative enzymes and/or activating anti-oxidative enzymes (Madhujith et al., 2005; Oomah et al., 2010; Kim et al., 2000; McDougall et al., 2005). Nevertheless, numerous studies have been conducted on phenol-rich extracts present in multiple plant systems that inhibit α-glucosidase and α-amylase, which in turn retard glucose absorption. For example, inhibition of α-glucosidase and α-amylase was detected in, but not limited to, the flowering plant systems: purple sweet potatoes, cabbage, strawberries, blueberries, blackcurrant, the Nepalese herb (Pakhanbhed), allspice, cinnamon, teucrium, witch hazel and black and green tea (Broadhurst et al., 2000; Matsui et al., 2001; McDougall et al., 2005; Bhandari et al., 2008; Dastjerdi et al., 2015). However, Oki et al. (1999) investigated the effect of α-glucosidase inhibitors, in vitro, according to its origin, and concluded that the origin of the enzyme (i.e., baker's yeast, rat small intestine, rabbit small intestine and/or pig small intestine) plays a significant role in the inhibitory properties of α-glucosidase. Also, different substrates (PNPG, maltose, sucrose) were

examined using rat α-glucosidase with various inhibitors resulting in different degrees of inhibition for each substrate (Oki et al., 1999; Tadera et al., 2006).

While definitive research on the effect of polyphenols in inhibiting α-glucosidase and α-amylase is still in progress, numerous studies have attributed the inhibition effect to different phenolic components or groups. For instance, Matsui et al. (2001) examined the inhibitory activity of α-glucosidase and α-amylase on 16 plant extracts and were the first to determine that inhibiting α-glucosidase was due to the anthocyanins. These results were supported by a study conducted on soft fruits by McDougall et al. (2005). They indicated that the inhibition of α-amylase was more effective with strawberry and raspberry extracts compared to blueberry, blackcurrant and red cabbage. This was mainly due to the tannins (ellagitannins) content in strawberry and raspberry, although they suggested that the phenolics might have a synergistic influence. In contrast, blueberry and blackcurrant extracts were more effective in inhibiting α-glucosidase, which was attributed to their elevated anthocaynins content, especially the unbound anthocyanin fractions (McDougall et al., 2005). Furthermore, Boath et al. (2012) concluded that tannin-like components (ellagitannins and proanthocyanidins) in different types of berries were able to inhibit α-amylase, but not α-glucosidase. The researchers also suggested that anthocaynins, along with other unidentified phenolics in blackcurrants were able to inhibit α-glucosidase more effectively (Boath et al. 2012). Research by Striegel et al. (2015) also concluded that phenolic compounds high in molecular weight, such as proanthocyanidins in black tea, were hypothesized to inhibit both α-glucosidase and α-amylase. Nonetheless, they determined that the inhibition of 50 percent of the enzyme activity (IC50) of α-glucosidase was higher than α-amylase test samples (Striegel et al., 2015). Likewise, Sreerama et al. (2012) supported anthocaynins as the

major contributor in inhibiting α-glucosidase in some beans (*Vigna species*) (mung, moth and adzuki, red and black). The highest inhibition was induced by the adzuki black beans, which contained the highest amounts of anthocaynins. However, because the adzuki red bean, which contained higher levels of anthocyanins, had lower α-glucosidase inhibition compared to both mung or moth beans. As such, the authors stated that other phenolic components may be contributing to the inhibitory activity of the enzyme (Sreerama et al., 2012).

Furthermore, Mojica et al. (2015) conducted an in vitro study to examine the inhibitory activities of α-amylase and α-glucosidase of 15 market classes of dry beans. Black beans had the highest content of anthocaynins, specifically the cultivar of *otomi*, which exhibited the highest inhibition of α-glucosidase. Alternatively, the highest inhibition of α-amylase was exhibited by the pinto bean (*saltillo*), which had a high antioxidant activity against reactive oxygen species (ROS), particularly nitric oxide (Mojica et al., 2015). However, no correlation was specified for most of the tested beans that inhibited α-amylase and α-glucosidase, nor was any specific phenolic or phenolic components identified as the responsible effectors. Also, only one extraction method was used, (1:50 w/v, 100 percent methanol and mixing time 24 hours at 20 °C). A completely different extraction method could have provided different results as will be shown in this study.

Other studies have highlighted the efficacy of flavonoids against α-amylase and α-glucosidase. For example, Kim et al. (2000) investigated the inhibitory effect of twenty-one flavonoids on α-glucosidase in vitro and determined that at a dose of 0.5 mg/ml, luteolin inhibited yeast α-glucosidase at a rate 36 percent greater than acarbose. Also, Tadera al. (2005) evaluated the inhibitory activity of several specific phenolics from six flavonoid groups against α-glucosidase and α-amylase.

The highest inhibitions against yeast α-glucosidase were as follows (high to low): anthocyanin (cyanidin IC50= 4 μM); isoflavone (genistein IC50= 7 μM and daidzein IC50= 14 μM); flavonol (myricetin IC50= 5 μM, quercetin IC50= 7 μM and kaempferol IC50= 12 μM); flavone (luteolin IC50= 21 μM); flavanone (naringenin IC50= 75 μM); and flavan-3-ol (Epigallocatechin-3-gallate IC50= 2 μM and Epigallocatechin IC50= 75 μM). For the α-amylase inhibition, the most effective flavonoids were luteolin, myricetin and quercetin, with IC50 values less than 0.50 μM (Tadera al., 2005). Moreover, Yao et al. (2011) isolated two flavones, vitexin (apigenin 8-C-glucoside) and isovitexin (Apigenin 6-C-glucoside) from adzuki beans extracts (*Vigna angularis*), which exhibited high inhibition against α-glucosidase. Furthermore, Sheliya et al. (2015) studied the inhibitory effect of α-glucosidase in vitro and in vivo. They discovered that specific pernylated flavonoids from (*euohorbia hirta L*.) herb (i.e., hirtacoumaroflavonoside and hirtaflavonoside-B) were more effective in inhibiting α-glucosidase compared to non-pernylated flavonoids (i.e., quercetrin, dimethoxy quercetrin) (Sheliya et al., 2015).

Lastly, it is again important to note that the results and conclusions of the above studies were based on non-uniform factors such as extraction procedure (i.e., solvent type, solvent:water ratio, incubation time, solid:volume ratio, etc.), instrumentation, samples concentration and type of control or subtract; this makes it very challenging to compare and contrast between the studies (Mojica et al., 2015).

A.6 Response-Surface Methodology (RSM)

Response-surface methodology (RSM) is an experimental design established by Box and Wilson in 1951 (Hill & Hunter, 1966; Bezerra et al., 2008). Response-surface methodology consists of multivariate methods that generate and establish a model based on the relationship between a response (y) and several controlled

independent variables (X_1, X_2,…X_n) (Bezerra et al., 2008; Hanrahan & Lu, 2006; Khuri & Mukhopadhyay, 2010). Response-surface methodology is a practical method for developing, improving and optimizing / characterizing procedures to obtain specific analytes or study various treatment groups (Myers & Montgomery, 2002). The RSM is widely used in analytical chemistry (Hill & Hunter, 1966; Bezerra et al., 2008). However, several other fields utilize RSM, such as food and industrial processes, environmental, pharmaceutical and biological analysis, but with different experimental sub-designs for applications (Hanrahan & Lu, 2006).

The most common RSM designs are three-level-factorial, Box-Behnken, central composite (CCD) and Doehlert designs (Bezerra et al., 2008; Hanrahan & Lu, 2006; Khuri & Mukhopadhyay, 2010). Choosing among these designs depends on several aspects, i.e., the objective of the experiment or research, the most important factor, and a number other variables that may affect the experimental design or recovering an analytes (Bezerra et al., 2008; Hanrahan & Lu, 2006). Two models can be generated from RSM, a first-order and second-order model with latter being the most widely used. The second-order model is flexible, various parameters are relatively easy to estimate and is reliable for problem solving (Myers & Montgomery, 2002). Many studies on dietary systems have applied RSM of different designs to extract phenols from their matrices, including wheat (Liyana-Pathirana & Shahidi, 2005), fruits of euterpe oleracea (Pompeu et al., 2009) and gluten-free sorghum bread (Schober et al., 2005). The RSM approach, as opposed to the classical, univariate methods, provides essential, clear and accurate information for researchers using a limited number of samples (Bezerra et al., 2008; Hanrahan & Lu, 2006). Lastly, it requires less sample analysis than changing a single variable at a time.

B. OBEJECTIVES and SPECIFIC AIMS

The objective of this project was to determine the ability of phenolic rich extract obtained from pinto beans (*BaJa*) to cause effects in glucose regulation by inhibiting α-amylase and α-glucosidase, the two carbohydrate–hydrolysis enzymes of starch and/of disaccharides. *The hypothesis of this project* was that phenols present in pinto beans (*BaJa*) are able to inhibit these enzymes due to the presence of chemically diverse phenolic groups. In order to perform a comprehensive evaluation on the pinto bean (*BaJa*), however, RSM was initially used to characterize the parameters that produced the highest amounts of TP, TF and TCT. As these extracts are chemical diverse, this provided a starting point with which to identify the phenolic rich extract for the effect and to compare the inhibitory effects of the different extractions to one another. The objective and hypothesis of this research were fulfilled through the following specific aims:

***Specific Aim 1*:** To determine the optimal extraction procedures for methanol, ethanol, and acetone using an RSM in terms of solvent polarity, solid:solvent ratio and mixing time for obtaining pinto beans (BaJa) extracts with high levels of TP, TF and TCT. Also, to determine predictive equations from the same RSM data (Specific Aim 1) for TP, TF and TCT.

***Specific Aim 2*:** To compare the extracts ability to inhibit the carbohydrate–hydrolysis enzymes (α-glucosidase and α-amylase) with the TP, TF and TCT data obtained from Specific Aim 1 using regression analysis.

C. MATERIALS and METHODS

C.1 Bean Samples, Chemicals and Reagents:

Pinto beans (BaJa class grown in 2013) were provided by UNL Extension Center at Scottsbluff, NE as a kind gift from Dr. Carlos Urrea. The beans were stored in a freezer at approximately −20 ^{o}C for the duration of the research project. Extraction solvents (lab grade), including methanol, ethanol, acetone and other chemicals, such as dimethysulfoxide (DMSO) and hydrochloric acid, were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Fisher Scientific Co. also supplied sodium carbonate, sodium nitrite, sodium chloride, sodium phosphate monobasic monohydrate and sodium potassium tartrate. Other reagents were purchased from various vendors, including Folin-Ciocalteu (MP Biomedical LLC.; Solon, OH), aluminum chloride and vanillin (ACROS Organics Inc.; Fair Lawn, NJ), sodium hydroxide (BDH; West Chester, PA), starch (J.T. Baker; Center Valley, PA). Standards and subtracts, including gallic acid, catechin, 3,5-dintorsalicylic acid (DNS), 4-nitrophenyl β-d-glucopyranoside (PNPG), procine amylase enzyme and rat intestinal powder were obtained from Sigma-Aldrich (St. Louis, MO). Acarbose was purchased from LKT Laboratories Inc. (St Paul, MN). All chemicals and reagents were stored according to the label's instructions by the manufactures.

C.2 Specific Aim 1:

C.2.1. *Response Surface Methodology (RSM):* An RSM experimental design was used to obtain TP, TF and TCT rich extracts. This procedure consisted of using three different solvents (methanol, ethanol and acetone) while also adjusting for solvent polarity ratio, solid:solvent ratio and mixing time (Table 7). Therefore, a three-factor face centered cube design (FCCD) consisting of 17 samples (three center points) were used (Table 8), with three replications for each extraction parameter

Table 7. Independent variables and their coded (actual) values used for RSM

Independent Variable	Units	Coded Levels		
		-1	0	+ 1
Solvent*: Water ratio	% (v/v)	25:75	50:50	75:25
Solid: Volume ratio	% (w/v)	10%	20%	30%
Mixing Time	min	60	120	180

*Methanol, ethanol and acetone.

Table 8. Three-factor, three-level face-centered cube design (FCCD) used for RSM

Standard Order	Run Order	Solvent:Water (V/V)	Solid:Volume (W/V)	Mixing Time (min)
1	2	(+1) 75:25	(0) 20%	(0) 120
2	7	(0) 50:50	(0) 20%	(+1) 180
3	14	(+1) 75:25	(-1) 10%	(-1) 60
4	1	(0) 50:50	(0) 20%	(-1) 60
5	9	(0) 50:50	(+1) 30%	(0) 120
6	16	(0) 50:50	(-1) 10%	(0) 120
7	4	(-1) 25:75	(-1) 10%	(+1) 180
8	8	(0) 50:50	(0) 20%	(0) 120
9	13	(-1) 25:75	(+1) 30%	(+1) 180
10	15	(+1) 75:25	(+1) 30%	(-1) 60
11	6	(-1) 25:75	(-1) 10%	(-1) 60
12	11	(-1) 25:75	(+1) 30%	(-1) 60
13	3	(0) 50:50	(0) 20%	(0) 120
14	12	(-1) 25:75	(0) 20%	(0) 120
15	17	(0) 50:50	(0) 20%	(0) 120
16	5	(+1) 75:25	(+1) 30%	(+1) 180
17	10	(+1) 75:25	(-1) 10%	(+1) 180

preformed. The order of the 17 different combinations was analyzed randomly to minimize any bias between the variables (Table 8). These solvent systems and the other parameters were selected because multiple studies have used these conditions as single factor extractions to obtain phenolics from various natural systems (Liyana-Pathirana & Shahidi, 2005; Silva et al., 2007; Karacabey & Mazza, 2010; Mojica et al., 2015).

C.2.2. *Sample Preparation / Extraction:* Pinto beans (*BaJa*) were homogenized in quantities needed for a given experiment into a fine ground powder using a coffee grinder (Salton, MD# GC-5, China). The samples were then passed through a 9 mesh-sieve (correspond to opening size of 2.00 mm) to ensure a similar particle size. Afterwards, the samples were suspended into each of the solvent:water and solid:volume ratios cited on Tables 7 and 8. Next, the samples were placed on a platform shaker at room temperature applying the mixing time durations indicated on Table 8. After the designated time had elapsed, the samples were instantly centrifuged at 25 ^{o}C until a clear supernatant was obtained. If the samples were not clear after centrifugation, which was typical for the more non-polar solvents containing higher water ratio, the samples were filtered through a Whatman #1 filter paper to remove any small particles. The supernatants then were analyzed immediately in triplicate for TP, TF and TCT. The remaining supernatants were stored in the freezer (≈ -20 ^{o}C) after adding a layer of nitrogen for future analyses. All extractions preparation, procedure and order were completed consistently for the entire research as described herein and shown on Tables 7 and 8.

C.2.3. *Phenolic Analyses* (*Total Phenols):* The Folin-Ciocalteu method was used to determine TP quantities in the collected supernatants as described by Singleton & Rossi (1965). Briefly, a sample aliquot of a 100 µL was mixed with 100

µL of Folin-Ciocalteu reagent and 4.5 mL of nanopure water. After mixing on a vortexer, 0.3 mL of 2 percent (w/v) sodium carbonate was added. The samples then were incubated at room temperature for 2 hours. A *Bechman DU8000 Spectrophotometer* was set at 760 nm and the samples intensity (without any dilution) were measured. A standard calibration curve using 6 different concentrations of gallic acid was plotted (the independent variable) to calculate the phenol concentrations (the dependent variable) in the samples. The TP were expressed as a mean mg *galic acid* / g pinto bean (BaJa) powder ± standard deviation (SD).

C.2.4. *Phenolic Analyses (Total Flavonoids):* To measure TF, 125 µL of the prepared sample supernatant as described above (refer to C.2.1 and C.2.2) was combined with 37 µL of 5 percent (w/v) sodium nitrite plus 1.0250 mL of nanopure water according to Adom et al. (2002). After 4-6 minutes, 75 µL of 10 percent (w/v) aluminum chloride was added to the samples followed by mixing and incubation for 5 minute at room temperature. Next, 0.25 mL of 1.0 N sodium hydroxide was added to each tube followed by mixing. Then, the mixture then was measured at a wavelength of 510 nm. A standard calibration curve using external standard ((+)-*catechin*) was plotted to calculate TF in the samples. The TF were expressed as a mean mg *(+)-catechin* / g pinto bean (BaJa) powder ± SD.

C.2.5. *Phenolic Analyses (Total Tannins):* Total condensed tannins were determined according to Bhat et al. (2007). Briefly, 250 µL of prepared bean supernatant were combined with 1.00 mL of 4 percent vanillin. Afterwards, 0.500 mL of concentrated hydrochloric acid was added into the samples followed by mixing. The samples then were incubated in the dark at room temperature for 20 minutes. Lastly, the absorbance was monitored spectrophotometrically at 500 nm. A standard calibration curve using external standard (*(+)-catechin*) was plotted to calculate TCT

in the samples. The TCT were expressed as a mean mg *(+)-catechin* / g pinto bean (BaJa) powder ± SD.

C.2.6. *Data Analysis:* All methods and procedures were completed in triplicate and the experimental results were expressed as a mean ± SD. The statistical analyses were performed using StatsGraphics® Centerium (Version XVI; Warrenton, VA). The RSM experimental data were analyzed by multiple regression analysis using the least squares test (LSD). Two different tests, i.e., the sequential sum of the squares and model summary statistics, were applied to the experimental data to determine the adequacy of various models. The model, the regression coefficients involved in the model and their effect were analyzed by Pareto ANOVA and were considered significant at $p < 0.10$. The fitness of the regression curve was further evaluated by determining the correlation coefficient for the model R^2 (>75) (Le et al., 2010; Chauhan & Gupta, 2004); whereas the ability of the model to fit the experimental data was assessed by the lack of fit test ($p>0.05$). Regression equations were formulated based on whether the data obtained from each solvent system fulfilled the criteria stated in this section.

Pareto charts of the response (TP, TF and TCT) in relation to the three independent variables were constructed by the StatsGraphics® software. The data from the RSM design were fitted to the second-order polynomial model shown below and the significantly regression coefficients were determined.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ii} X_i X_j$$

Where Y is the response, β_0, β_i, β_{ii}, are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i, and X_j are the independent variables.

C.3 Specific Aim 2:

C.3.1. *Sample Preparation:* For completing the carbohydrate enzymatic assays (Specific Aim 2), phenolic extraction from the beans was preformed exactly the same as the extraction procedure for Specific Aim 1 (refer to sections C.2.1. and C.2.2.). The fresh supernatants were initially transferred to pre-weighed test tubes, and then a line was drawn at the top of the supernatant level on each tube. Next, the samples were placed into a vacuum evaporation oven (Fisher Scientific™ Isotemp™ Model 280A) adjusted to ≈25 ^{o}C and a pressure of -25 psi until a gummy-like residue formed. The samples then were periodically purged with nitrogen to complete the drying process. The test tubes then were re-weighted to determine the amount of residue (the concentration) in each test tube. These residues were then re-suspended to the mark using one percent DMSO. Lastly, the samples were subjected to the following enzymatic assessments based on their ability to inhibit the following enzymes immediately after being re-suspended.

C.3.2. *Carbohydrate–Hydrolysis Enzymatic Assay (Alpha-amylase):* This assay was preformed according to Subramanian et al. (2008) and Ali et al. (2006). Briefly, a sample blank (not containing the enzyme) was prepared by adding 150 μl of the re-suspended samples (refer to section C.3.1) to 150 μl of 20 mM phosphate buffer (sodium phosphate monobasic monohydrate with 0.006 M sodium chloride, pH 6.9). The experimental re-suspended samples (150 μl) were analyzed for their ability to inhibit α-amylase by combining 150 μl of 0.5 mg/ml amylase solution (procine amylase enzyme diluted in chilled 20 mM phosphate buffer kept in ice bath) to each sample. The control sample included 150 μl of 0.5 mg/ml amylase solution and 150 μl of one percent DMSO. The blank sample contained 150 μl of 20 mM phosphate solution and 150 μl of one percent DMSO. Moreover, the positive control included

150 µl of 1 mM acarbose (dissolved in one percent DMSO) and 150 µl of α-amylase solution. Then, each sample prepared as described above, was gently mixed and pre-incubated at room temperature for 10 minutes. To initiate the reaction, 150 µl of 0.5 percent starch solution (potato starch combined with 20 mM phosphate buffer; boiled for 15 minutes) was added into each sample followed by mixing gently. The reaction was then allowed to incubate at room temperature for 30 minutes. Next, 300 µl of DNS color reactant (sodium potassium tartrate + 3,5-dintorsalicylic acid + 2 M sodium hydroxide) was added into the samples, followed again by a gentle mixing. The reaction was stopped, by placing the samples (covered with aluminum foil) in a boiling water bath for 10 minutes. Lastly, all samples were allowed to cool down to room temperature. The samples were then diluted with 2 mL nanopure water. Finally, the absorbance was determined at 540 nm. The percentage of amylase inhibitory activity of each sample was calculated by using the following equations:

$$\text{Abs. of Sample B} = \boldsymbol{Abs.of\ sample}\ (\mathbf{540}) - \boldsymbol{Abs.of\ sample\ Blank}\ (\mathbf{540})$$

$$\%\ \text{Inhibition} = \frac{\boldsymbol{Abs.of\ Control}\ (\mathbf{540}) - \boldsymbol{Abs.of\ Sample\ B}\ (\mathbf{540})}{\boldsymbol{Abs.of\ Control}\ (\mathbf{540})} \times \mathbf{100}$$

C.3.3. *Carbohydrate–Hydrolysis Enzymatic Assays (Alpha-glucosidase):* This assay was conducted by using the 4-nitrophenyl β-D-glucopyranoside (PNPG) method according to Matsui et al. (1996), Kim et al. (2004) and Önal et al. (2005). The rat intestinal solution, which contain the α-glucosidase, was prepared by dissolving 0.100 g of rat intestinal powder into 3 ml of chilled 0.9 percent sodium chloride solution followed by intermittent sonication and then centrifuged. The control blank included 200 µl of one percent DMSO, while the positive control contained 100 µl of 1 mM acarbose (dissolved in one percent DMSO) and 100 µl of the rat intestinal solution. For the experimental samples, 100 µl of the re-suspended samples (refer to section C.3.1) were combined with 100 µl rat intestinal solution. The

experimental sample blank contained 100 µl of the re-suspended samples and 100 µl one percent DMSO. Each sample was then incubated at 37 °C for 10 minutes. To initiate the reaction, 300 µl of 1 mM PNPG (PNPG combined with 50 mM phosphate buffer) was added into each sample. Subsequently, the samples were incubated again at 37 °C for an additional 20 minutes. The reaction was stopped by placing the samples (covered with aluminum foil) in a boiling water bath for 10 minutes. Next, all samples were allowed to cool to room temperature. The samples were then diluted with 1.5 mL of nanopure water and were centrifuged until any solid participate pelleted resulting in a clear supernatant. Lastly, the absorbance was measured spectrophotometrically at 400 nm. The percentage inhibitory activity of each sample was calculated by using the following equations:

$$\text{Abs. of Sample B} = \boldsymbol{Abs.of\ sample}\ (\mathbf{400}) - \boldsymbol{Abs.of\ sample\ Blank}\ (\mathbf{400})$$

$$\%\ \text{Inhibition} = \frac{\boldsymbol{Abs.of\ Control}\ (\mathbf{400}) - \boldsymbol{Abs.of\ Sample\ B}\ (\mathbf{400})}{\boldsymbol{Abs.of\ Control}\ (\mathbf{400})} \times \mathbf{100}$$

C.3.4. *Data Analysis:* All methods and procedures were completed in triplicate and the results were normalized to percent inhibition/mg of residues, which is expressed as a mean ± SD as two different extraction preparation and drying methods were completed. Also, TP, TF and TCT were calculated in each of the residue based on the original extract amounts obtained from the RSM values (Aim 1), but normalized and expressed as a mean µg / mg of extract ± SD. The standard statistical analyses including the scatter plots were performed by Microsoft Excel (version 2016; Redmond, WA). The generated standard analysis was used to determine if there was a relationship between the α-amylase and α-glucosidase inhibition and TP, TF and TCT contents.

D. RESULTS and DISCUSSION

D.1 ***Specific Aim 1:***

D.1.1.a *RSM Characterization: Selection of Independent Variables:*

Wet-chemistry extraction (solvent extraction), as opposed to other extraction techniques that use instrumentation, such as ultrasound-assisted extraction, microwave-assisted extraction and pressurized liquid extraction, (Galili & Hovav, 2014), is widely applicable, economically affordable and efficient. Still, solvent extraction drawbacks of analytes from any natural system require multiple extraction factors. The most influential factors are solvent type, solvent:water ratio, solvent:solid ratio, extraction steps, additives to the solvent (such as HCl or NaOH), extraction period and extraction temperature (Galili & Hovav, 2014).

In the case of polyphenols, these compounds are typically extracted using 100 percent water or aqueous organic solvents, such as methanol, ethanol, acetone and/or ethyl acetate (Xu & Chang, 2007). Selecting one of these organic solvents is particularly critical for an extraction's efficiency of any targeted analyte (Xu & Chang, 2007; Zhao et al., 2006). However, polyphenols are even more susceptible to these parameters considering their chemical diversity that can differ based on many factors as cited previously (i.e., bean market classes or environmental conditions during growth) (Dannehl & Josuttis, 2014; Shrestha & St Clair, 2014). Moreover, several other factors can interfere with the choice of solvent, such as the polarity (water content) in the solvent and the solute matrix (Al-Farsi & Lee; 2008; Galili & Hovav, 2014). Numerous studies have used aqueous organic solvents to recover polyphenols from dry seeds with solvent:water ratios ranging from 50 percent to 100 percent (Madhujith & Shahidi, 2005; Espinosa-Alonso et al., 2006; Lin et al., 2008; Oomah et al., 2010). Indeed, Rostagno et al. (2004) showed that a minimum of 25

percent water with an upper range of 75 percent of organic solvents promote the solubility of phenolic compounds from soybeans. Therefore, in this research, three solvent:water ratios percent (v/v) were selected (25:75, 50:50 and 75:25) for each of the three solvents (methanol, ethanol and acetone) so as to capture multiple solvent polarities. Although few studies have successfully used acidified organic solvents to extract polyphenols from different legumes (Xu & Chang, 2007; Sreerama et al., 2012); previous studies conducted in our lab showed that acidified solvents did not increase the extraction efficiency of TP and TF from pinto beans (*LaPaz*) or blue green algae (*Spirulina*) (*LaPaz*) (Aldawsari, 2014; Salamatullah, 2014) under constant mixing at ambient room temperature. However, higher solvent:solid ratios increases the extraction yield regardless of the solvent type used (Al-Farsi & Lee, 2008; Pinelo et al., 2003; Pompeu et al., 2009; Galili & Hovav, 2014). Therefore, we investigated three solid:volume ratios percent (w/v) (10, 20 and 30 percent).

The extraction mixing time of polyphenols from multiple cultivars of common beans as reported in the literature ranged from one hour to 24 hours (Kalogeropoulos et al., 2010; Madhujith & Shahidi, 2005; Espinosa-Alonso et al., 2006; Lin et al., 2008). Based upon these studies, the extraction period impact differed based on the natural system or other parameters used. For example, mixing time had minimum or no effect on TP content in ground date seeds (Al-Farsi & Lee, 2008), wheat (Liyana-Pathirana & Shahidi, 2005) and pistachio (Rajaei et al., 2010). Also, longer extraction periods resulted in the degradation of total anthocyanins from fruits of *Euterpe oleracea* (Pompeu et al., 2009). Moreover, using a shaker for the extraction of phenols of several medicinal plants resulted in higher levels of TP contents and higher antioxidant activity compared to the extracts obtained by using a refluxing technique (Sultana et al., 2009). The authors speculated that the thermal treatment from the

reflux method may have accelerated the oxidation of some polyphenols and reduced their content (Sultana et al., 2009). Thus, a short mixing times were used for this study and included 60, 120 and 180 minutes using a mixing platform.

As stated previously, many studies have used methanol, ethanol or acetone with different water ratios to recover polyphenols from fruits, vegetables and cereals (Shahidi & Ambigaipalan, 2015; Mattila, et al., 2000; Labarbe et al., 1999; Sun & Ho, 2005; Zadernowski et al., 2005), but few studies have used RSM for optimizing extraction procedures of phenolics in food systems with a few exceptions (Liyana-Pathirana & Shahidi, 2005; Pompeu et al., 2009; Karacabey & Mazza, 2010), all of which will be integrated in the discussion without data as presented in the next sections.

D.1.2. *Total Phenols*:

D.1.2.a *Total phenol results obtained from FCCD-RSM:* Total phenolic levels for each solvent system (methanol, ethanol and acetone) were determined using FCCD, as described in the Materials and Methods (Section C). By adjusting for three factors, (solvent:water ratio, solid:volume ratio and mixing time) using three levels, 17 extractions were completed (Table 8). The TP results obtained for each solvent are shown in Table 9, and are expressed as a mean ± SD of three replicates. The range (lowest to highest) of TP results was also determined (Table 10).

As shown by these Tables, the acetone system exhibited the greatest TP levels (2.31 mg/g). The lowest response occurred with methanol (0.32 mg/g) resulting in a difference of ≈ 2 mg/g difference when compared to the acetone extracted TP levels. Methanol and ethanol yielded similar TP levels, suggesting that these solvents do not improve the TP recovery with respect to the other independent variables.

Table 9. Total phenols (in mg/g) of pinto beans (*BaJa*) extracts under different extraction conditions and solvent systems using face centered cubic design

Std. Order	Methanol	Ethanol	Acetone
1	0.32 ± 0.03	0.52 ± 0.00	1.64 ± 0.02
2	0.53 ± 0.02	0.80 ± 0.01	1.56 ± 0.01
3	0.61 ± 0.07	0.69 ± 0.00	2.07 ± 0.02
4	0.58 ± 0.02	0.77 ± 0.01	1.84 ± 0.01
5	0.50 ± 0.01	0.77 ± 0.01	1.37 ± 0.02
6	0.88 ± 0.06	1.11 ± 0.00	2.19 ± 0.01
7	0.87 ± 0.02	1.01 ± 0.01	1.40 ± 0.01
8	0.63 ± 0.03	0.90 ± 0.01	1.51 ± 0.03
9	0.74 ± 0.04	0.78 ± 0.03	1.17 ± 0.03
10	0.63 ± 0.02	0.40 ± 0.00	1.31 ± 0.01
11	0.77 ± 0.13	1.20 ± 0.01	1.60 ± 0.03
12	0.63 ± 0.05	0.76 ± 0.01	1.13 ± 0.01
13	0.65 ± 0.07	0.78 ± 0.01	1.72 ± 0.01
14	0.79 ± 0.06	0.82 ± 0.01	1.35 ± 0.02
15	0.56 ± 0.04	0.76 ± 0.01	1.62 ± 0.02
16	0.36 ± 0.03	0.36 ± 0.01	1.51 ± 0.01
17	0.40 ± 0.09	0.69 ± 0.00	2.31 ± 0.02

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

Table 10. Range of total phenols for each solvent system (lowest to highest)

Extraction Solvent	Total Phenols (mg/g)	Range (mg/g)
Methanol	0.32 – 0.88	0.56
Ethanol	0.36 –1.20	0.84
Acetone	1.13 – 2.31	1.18

The ability of acetone to extract optima TP yields indicates that the majority of the phenols present in pinto beans (*BaJa*) are non-polar or lack of polar conjugates. Alternatively, the range of TP (high versus low levels) was much less for the methanol extraction indicating that different procedures could be used to produce the most similar results. In other words, the methanol-based extractions were more robust even though less TP were obtained compared to ethanol or acetone (Table 10). In this context, "The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage" (ICH, 2005; Burns et al., 2009).

Ballard et al. (2009) studied the extraction efficiencies of ethanol and methanol on TP yields from peanut skins also using RSM. The highest TP yields occurred with aqueous ethanol while adjusting for solvent:water composition, temperature and mixing time. Interestingly, many studies have completed phenolic based extractions with ethanol (Durling et al., 2007; Inglett et al., 2010; Chew et al., 2011), but only a few have been reported using acetone (Eberhardt et al., 2000; Nakatani et al., 2000). No explanations were provided why these solvents or solvent:water ratio were selected. Clearly, as proven in this study, the acetone under certain mixing and solid:solvent ratio provided the highest TP yield from pinto beans.

D.1.2.b *Fitting a Second-Order TP model*: Regression coefficients were determined using the least-squares method (LSM) from the TP results (response) obtained for each solvent system to predict the quadratic polynomial equations. The R^2 value was ≈91 percent for methanol, ≈96 percent for ethanol and ≈95 percent for acetone, indicating that most of the variability could be explained for these solvents-based TP extractions (Table 11). A high R^2 indicates that the variability between the

Table 11. Regression coefficients of the predicted quadratic model for TP when extracted with the solvent systems cited for pinto beans (*BaJa*)

Coefficient	Methanol	Ethanol	Acetone
b_o	0.612	0.821	1.616
Linear			
b_1 (SP)	-0.174*	-0.190*	0.217**
b_2 (S:S)	-0.093**	-0.161*	-0.307*
b_3 (MT)	-0.004	-0.018	0.036
Quadratic			
b_{11} (SP x SP)	-0.052	-0.158^	-0.120
b_{22} (S:S x S:S)	0.082^	0.113	0.164
b_{33} (MT x MT)	-0.052	-0.038	-0.097
Cross product			
b_{12} (SP x S:S)	-0.002	0.006	-0.107
b_{13} (SP x MT)	0.053^	0.016	0.076
b_{23} (S:S x MT)	0.028	0.021	0.024
R^2	91.32	96.47	95.90
p values			
Model	0.0056	0.0003	0.0005
Lack of Fit	0.2276	0.7241	0.6295

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

observed values and the fitted values are small. The *p*-values for the models also were well below $p < 0.10$ (Table 11, final section), again supporting the adequacy of the second-order model for all three solvents.

D.1.2.c *Adequacy of the TP models (lack of fit)*: It is important to examine the "lack of fit" of the data to assure reliable and valuable equations. Any violation or failure of the data to fit the models will lead to inaccurate or misleading outcomes (Myers & Montgomery, 2002). Therefore, this criterion was evaluated by comparing the difference between the residuals of the current model (Maran et al., 2013). If the model's residuals correspond to that of the experimental with a *p*-value > 0.05 (insignificant), the data satisfactorily fit the model. For the TP data, all three solvents for the RSM extraction of pinto beans passed this test (Table 11).

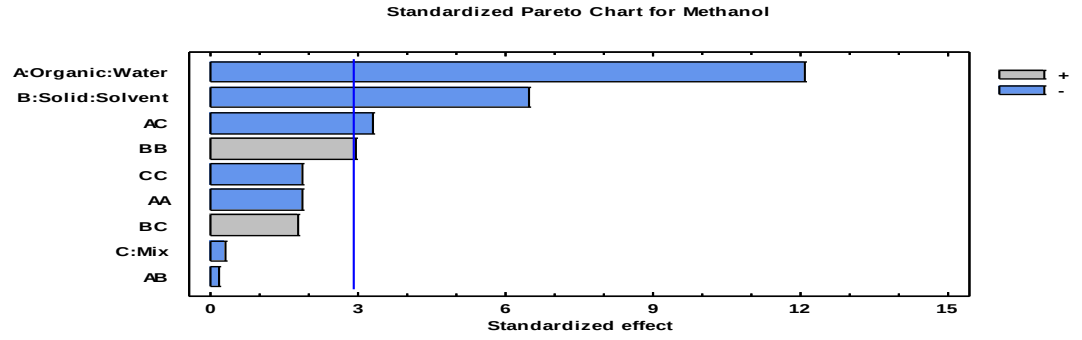
D.1.2.d *Regression coefficients equations and Pareto charts*: Regression coefficients and Pareto charts were generated to determine the factors that had the greatest effect and how they affected the response (Figure 9a-c), i.e., particularly if cross products were involved. (The TP regression equations for all solvent systems of the RSM extraction of pinto beans are provided in Table 12). These generated equations are based only on the significance of individual regression coefficients ($p < 0.1$) (Table 11). Any bar beyond the vertical line on the Pareto chart that represents a coefficient is statistically significant at the 90 percent confidence level. "The length of each bar is proportional to the value of a t-statistic calculated for the corresponding effect" (StatsGraphics, 2014). The blue bar (−) is negatively proportional with the effect of its coefficient, whereas the gray bar (+) is positively proportional.

All investigated solvents resulted in unique effects on TP extraction from pinto beans (*BaJa*) as shown by a visual comparison of the Pareto charts (Figures 9a-c). The TP extractions with methanol and ethanol alike were mainly effected by two

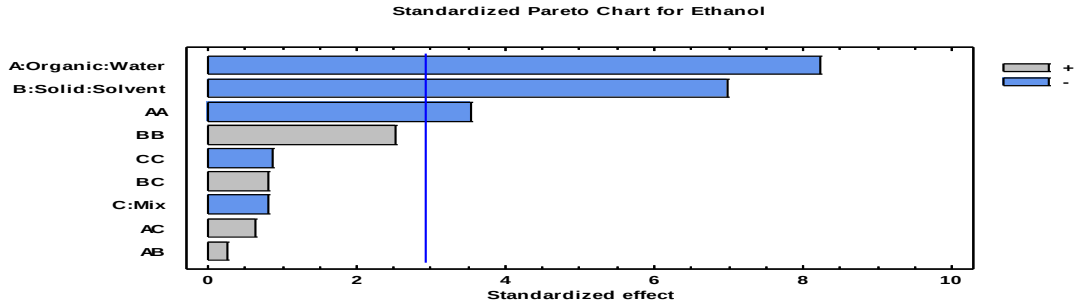
Table 12: Regression equations fit to the model (passed the lack of fit test) for TP

TP methanol = 0.612 – 0.174*_Xsp_ – 0.093*_Xss_ + 0.053*_XspXmt_ + 0.082*_XssXss_
TP ethanol = 0.821 – 0.190*_Xsp_ – 0.161*_Xss_ – 0.158*_XspXsp_
TP acetone = 1.616 + 0.217*_Xsp_ – 0.307*_Xss_

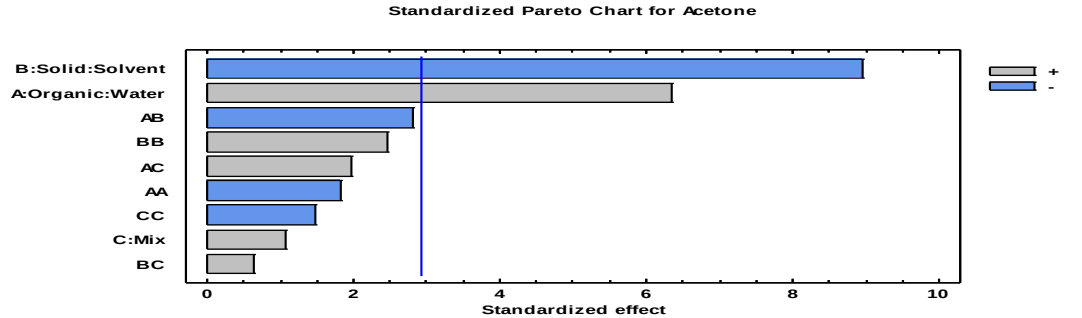
Xsp: solvent polarity, Xss: solid:solvent, Xmt: mixing time.



(a)



(b)



(C)

Figure 9: Pareto charts show relative effects of regression coefficients for accepted TP models for methanol (a), ethanol (b) and acetone (c) for pinto bean (*BaJa*). The vertical line represents $p < 0.1$.

factors, the solvent:water ratio and solid:solvent ratio, which had an inversely proportional effect on TP yields for both linear coefficients (Figures 9a and 9b). Similarly, the solvent:water ratio and solid:solvent ratio were also statistically significant ($p < 0.1$) for the acetone solvent, but the higher solvent:water ratio, the higher TP yields and the higher solid:solvent ratio, the lower TP yields (Figure 9c). The mixing time parameter was not statistically significant at the 90 percent confidence level for all solvent systems (Figures 9a-c).

D.1.2.e *Final optimized TP values and processing factors*: The optimal processing factors were determined based on the generated acetone model, which is expected to produce the highest TP yields (Table 13 and Figure 10c). As cited previously, the overall result indicated that the phenols present in pinto beans (*BaJa*) are more non-polar as the acetone system yielded the highest TP value. *These high values could be due to the condensed tannins (non-hydrolysable tannins), as their content is high in pinto beans, which will be discussed later in this chapter*.

The optimum factors generated from the acetone model are: solvent:water ratio (75:25), solid:volume ratio (10 percent) and mixing time (≈87 minutes). As indicated in Table 13 and shown in Figure 10c, a high proportion of solvent relative to water was needed for the optimum TP values. Also, the main effect of the solvent:water ratio for methanol and ethanol (Figures 10a and 10b) has a similar pattern, which indicates that the optimum TP yields were reached with a lower solvent:water ratio, but again the opposite effect occurred for the acetone system. In this case, the optimal solvent:water ratio for the acetone was 75:25 acetone:water. Al-Farsi & Lee (2008) investigated the optimization of phenolics' extraction from ground date seeds and determined the highest TP yields occurred with 50:50 acetone:water, whereas the lowest yields were obtained with 100 percent acetone.

Table 13: Optimized factors required to generate optimum TP yield for the acetone system for pinto beans (*BaJa*)

Optimum value = 2.31254 mg/g of beans

Factor	*Low*	*High*	*Optimum (coded)*	*Optimum (uncoded)*
Solvent:Water	-1.0	1.0	1	75:25
Solid:Solvent	-1.0	1.0	-1.0	10%
Mixing Time	-1.0	1.0	0.450772	≈87 min

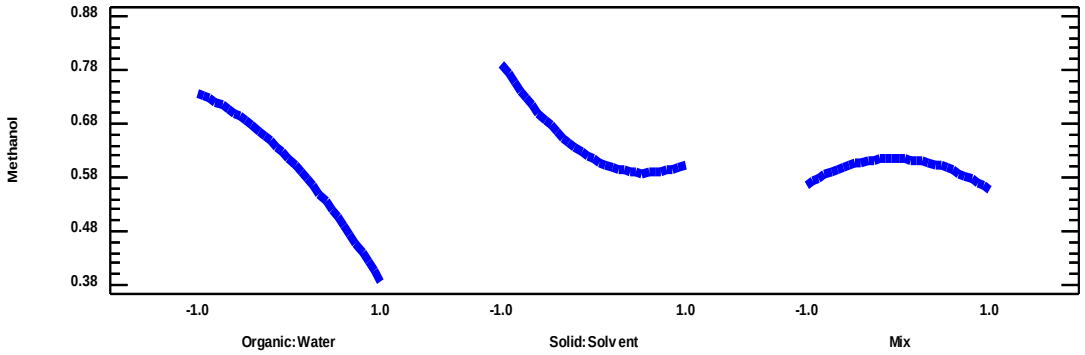


Figure 10a: Main effects plot for methanol (TP).

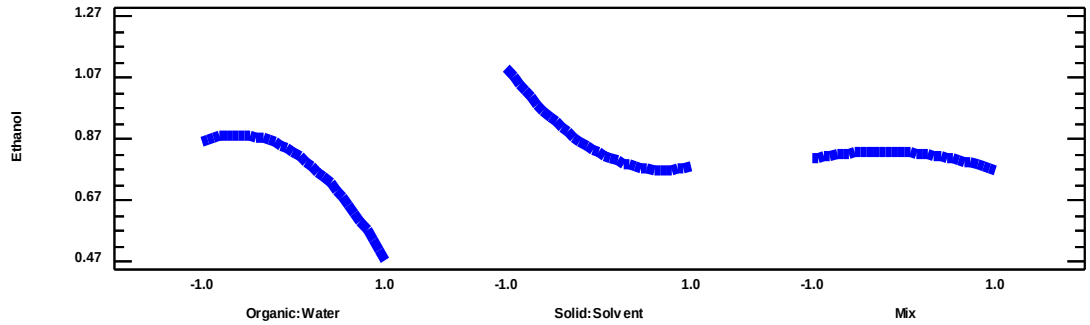


Figure 10b. Main effects plot for ethanol (TP).

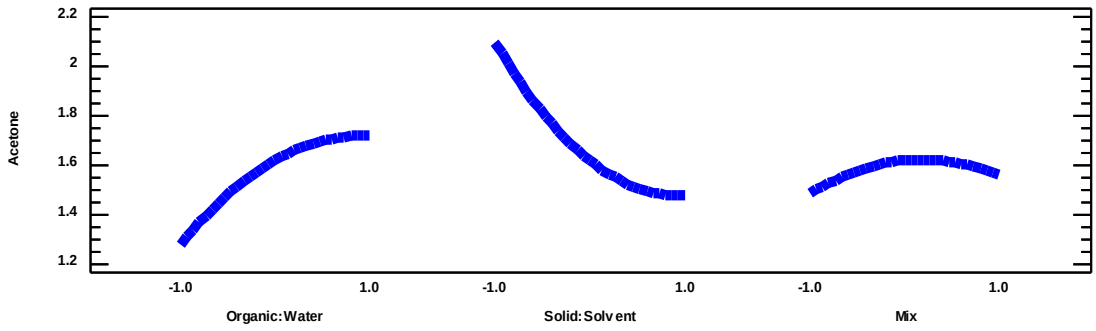


Figure 10c. Main effects plot for acetone (TP).

The researchers of the previous study investigated seven solvent systems (100 percent water, ethanol, methanol, acetone, 50 percent ethanol, 50 percent methanol and 50 percent acetone) along with other independently tested parameters. Their outcome was relatively similar with our results, suggesting that an increase of acetone beyond 75:25 could result in a reduction in TP content.

The curve in Figure 10c (solvent:water) further indicates a maximum at the 75:25 solvent polarity. Total phenols yield may increase with even lower solid:solvent ratios more than that achieved with 10 percent solid:solvent ratio, as evidenced by the upward curve for the coded -1 for solid:solvent curve. Similarly, Pinelo et al., (2004) applied a three solvent:solid ratios (5:1, 7.5:1 and 10:1) to the extract polyphenols from pin sawdust and almond hulls. The authors concluded that the lower solid:solvent ratio produced the highest TP yields, which is consistent with our data. These results most likely occur because the solvent to surface contact is higher for lower solid:solvent ratios resulting in higher TP yields.

Despite the optimum mixing time of 87 minutes for the acetone, the time parameter had no significant effect (*p*-value=0.39). Liyana-Pathirana & Shahidi, (2005) also reported that mixing time did not significantly affect polyphenols extractions present in ground date seeds. To briefly elaborate, these researchers investigated three mixing periods (45, 60 and 75 minutes). Longer extraction times did not correlate with higher TP values when the ground date seeds were subjected to either 100 percent water or 50 percent acetone (Al-Farsi & Lee, 2008). Moreover, water extraction of the polyphenols from pistachio hulls showed no significant difference between 60 and 40 minutes in TP levels (Rajaei et al., 2010). Indeed, shorter mixing durations not only save time but also prevent the possibility of compromising the phenols, such as oxidation.

D.1.3. *Total Flavonoids:*

D.1.3.a *Total flavonoids results obtained from FCCD-RSM:* Total flavonoid levels for each solvent system (methanol, ethanol, and acetone) were also determined using FCCD. The same three factors, (solvent:water ratio, solid:volume ratio and mixing time) used for TP process were applied to TF extraction (Table 8).
The results obtained for each solvent are shown in Table 14, expressed as a mean ± SD of three replicates. The ranges (lowest-to-highest response) of TF results are listed in Table 15. The highest TF yields were once again recovered by the acetone system (3.79 mg/g) followed by ethanol (0.94 mg/g) and then methanol (0.19 mg/g), where the last was 3 times less than the acetone.

The behavior of methanol and ethanol was similar, except for mixing time effect, and yielded relatively comparable TF levels. Similar to the TP studies, these results shows that methanol and ethanol do not improve the TF extraction regardless of the other independent variables. As shown in Table 15, TF ranges for methanol and ethanol were not as large as compared to acetone. Again, these results confirm that the former processes are more rugged than the acetone extraction. "Ruggedness is normally expressed as the lack of influence of operational and environmental factors of the analytical method." (ICH, 2005; Burns et al., 2009). In fact, the lowest value obtained from the acetone was 0.78 mg/g, which was the highest TF value for methanol (Table 15). In addition, there was a 3 mg/g difference just within acetone extractions. These data clearly demonstrate that extraction processes for polyphenols should be characterized or their procedures clearly defined if one is to compare phenolic content with other reports. Nonetheless, the ability of acetone to extract the optimum overall TF content reveals that the majority of the TF present in pinto beans (*BaJa*) were also non-polar conjugates or alglycones.

Table 14. Total flavonoids (in mg/g) of pinto beans (*BaJa*) extracts under different extraction conditions and solvent systems using face centered cubic design

Std. Order	Methanol	Ethanol	Acetone
1	0.19 ± 0.01	0.57 ± 0.02	2.60 ± 0.10
2	0.38 ± 0.02	0.86 ± 0.03	1.64 ± 0.06
3	0.45 ± 0.01	0.87 ± 0.01	3.22 ± 0.09
4	0.52 ± 0.02	0.69 ± 0.02	1.79 ± 0.11
5	0.42 ± 0.02	0.70 ± 0.04	2.09 ± 0.10
6	0.76 ± 0.01	0.94 ± 0.00	2.49 ± 0.06
7	0.78 ± 0.02	0.75 ± 0.03	1.08 ± 0.00
8	0.57 ± 0.02	0.83 ± 0.03	2.00 ± 0.07
9	0.35 ± 0.01	0.49 ± 0.03	0.94 ± 0.02
10	0.22 ± 0.01	0.42 ± 0.02	2.52 ± 0.18
11	0.63 ± 0.02	0.69 ± 0.04	1.34 ± 0.02
12	0.43 ± 0.00	0.48 ± 0.04	0.78 ± 0.04
13	0.49 ± 0.01	0.67 ± 0.03	2.29 ± 0.04
14	0.52 ± 0.01	0.49 ± 0.03	1.05 ± 0.05
15	0.45 ± 0.00	0.66 ± 0.01	2.23 ± 0.09
16	0.24 ± 0.00	0.33 ± 0.01	2.91 ± 0.07
17	0.27 ± 0.01	0.77 ± 0.02	3.79 ± 0.05

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

Table 15. Range of total flavonoids for each solvent system (lowest to highest)

Extraction Solvent	Total Phenols (mg/g)	Range (mg/g)
Methanol	0.19 – 0.78	0.59
Ethanol	0.33 – 0.94	0.61
Acetone	0.78 – 3.79	3.01

D.1.3.b *Fitting a Second-Order TF model*: Regression coefficients were again determined using the LSM from the TF results to calculate model's equations for each solvent system. The R^2 values for methanol, ethanol and acetone were 90.8, 90.1 and 93.9 percent, respectively, (Table 16). Therefore, most of the variability could be explained for these solvent-based TF extractions. The models generated by all three solvents were also significant in that their *p*-values were much lower than $p < 0.1$ (Table 16).

D.1.3.c *Adequacy of the TF models (lack of fit)*: The model's adequacy was then evaluated for lack of fit. As cited previously, the lack of fit criteria for all solvents was *p*-value > 0.05 (insignificant), which indicates that the data satisfactorily fit the model. The lack of fit *p*-value was 0.4113 for methanol, 0.6526 for ethanol, and 0.1743 for acetone (Table 16).

D.1.3.d *Regression coefficients equations and Pareto charts*: The TF regression equations for the three solvent systems generated by the RSM extraction studies are shown in Table 17. These equations, as cited earlier, are based only on the significance of individual regression coefficients ($p < 0.01$, 0.05 and 0.1). The Pareto charts of the regression coefficients for each solvent system are shown in Figures 11a-c. As shown by the equations and charts, all the investigated solvents indicated different behavior effects on TF yields. The RSM extraction of TF with methanol was mainly affected by two parameters, the solvent polarity and solid:solvent ratio (linear coefficient), and the quadratic solvent polarity. All were inversely proportional with TF yields (Figure 11a). For ethanol, one linear coefficient (solid:solvent) and one quadratic coefficient (solvent polarity) was statistically significant at $p < 0.05$, and $p < 0.1$, respectively. Additionally, each parameter was inversely proportional with TF yields (Figure 11b).

Table 16. Regression coefficients of the predicted quadratic model for TF when extracted with the solvent systems cited for pinto beans (*BaJa*)

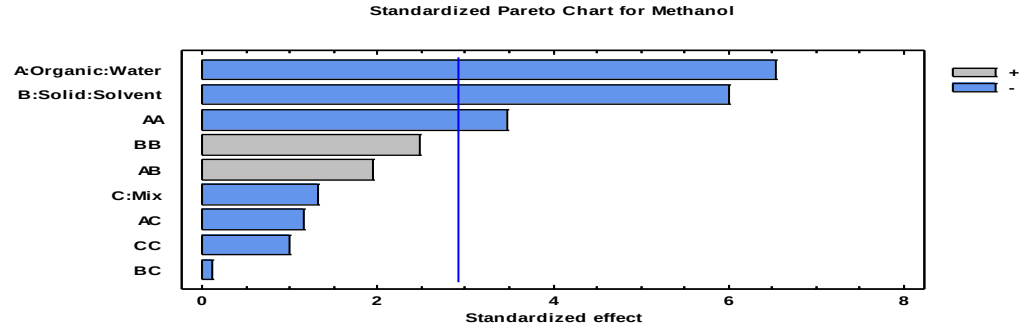
Coefficient	Methanol	Ethanol	Acetone
b_o	0.495	0.733	1.999
Linear			
b_1 (SP)	-0.131**	-0.014	0.844*
b_2 (S:S)	-0.120**	-0.178**	-0.409**
b_3 (MT)	-0.026	0.024	0.211*
Quadratic			
b_{11} (SP x SP)	-0.135^	-0.212^	-0.043
b_{22} (S:S x S:S)	0.096	0.076	0.419**
b_{33} (MT x MT)	-0.038	0.029	-0.157
Cross product			
b_{12} (SP x S:S)	0.043	-0.028	0.067
b_{13} (SP x MT)	-0.026	-0.055	-0.044
b_{23} (S:S x MT)	-0.002	-0.027	-0.146
R^2	90.80	90.10	93.86
p values			
Model	0.0068	0.0086	0.0018
Lack of Fit	0.4113	0.6526	0.1743

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

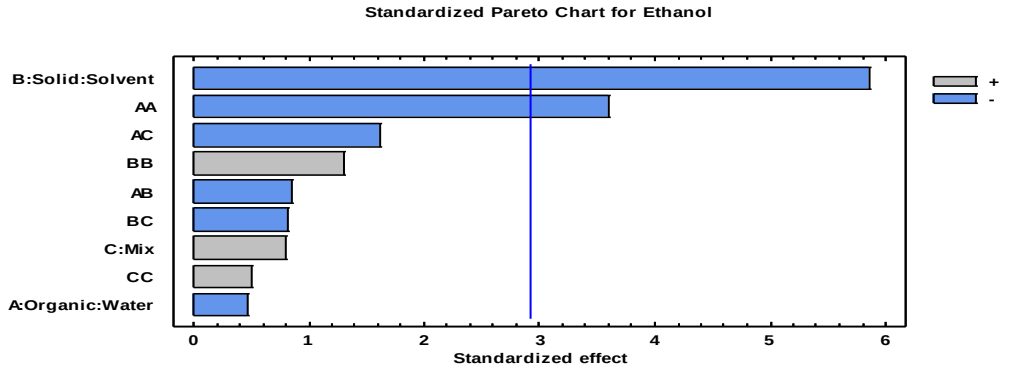
Table 17: Regression equations' fit to the model (passed the lack of fit test) for TF

TF methanol = 0.495 – 0.131**Xsp* **– 0.120****Xss* **– 0.135****XspXsp**
TF ethanol = 0.733 – 0.1781**Xss* **– 0.212****XspXsp**
TF acetone = 1.999 + 0.844**Xsp* **– 0.409****Xss* **+ 0.211****Xmt* **+ 0.419****XssXss**

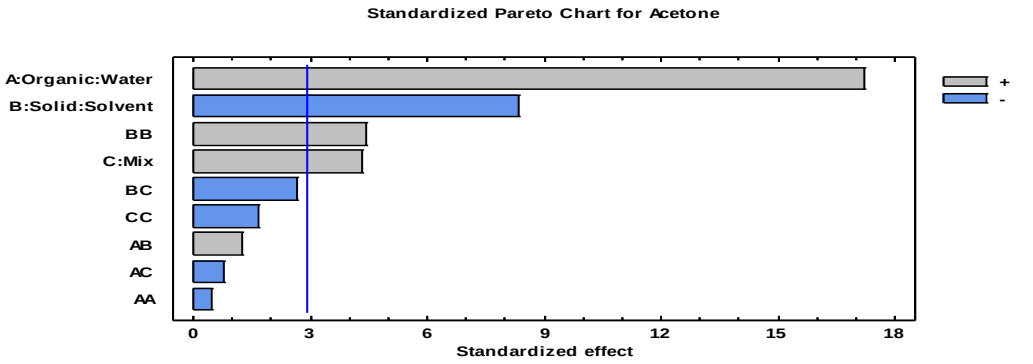
Xsp: solvent polarity, Xss: solid:solvent, Xmt: mixing time.



(a)



(b)



(c)

Figure 11: Pareto charts show relative effects of regression coefficients for accepted TF models for methanol (a), ethanol (b) and acetone (c) for pinto bean (*BaJa*). The vertical line represents $p < 0.1$.

The mixing time parameter (linear, quadratic or cross product) was not statistically significant for either methanol or ethanol (Figures 11a-b). Alternatively, for the acetone, the significant linear parameters included mixing time as well as solvent polarity, solid:solvent ratio combined with the quadratic coefficient of solid:solvent ratio. The relationship between the linear coefficients, solvent polarity and mixing time with TF yields were directly proportional, but inversely proportional to solid:solvent ratio. Interesting, TF increased proportionally with the quadratic coefficient, solid:solvent ratio.

D.1.3.e *Final optimized TF values and processing factors*: The optimal processing factors were determined based on the generated acetone model for TF. It should be noted that these results may be due to a breakdown of tannins. Some of the polymerized tannins may have been broken down to monomer flavonoids during homogenization. It would be expected that these monomers would be more non-polar, and thus be recovered by the acetone, as they would not be conjugated to a sugar or other more hydrophilic group. Thus, the assay for TF (aluminum chloride) would be able to detect these possible remnants of the tannin fraction. Clearly, this hypothesis has to be verified. The optimum factors using the acetone system for TF are similar to those for the TP, except for the mixing time, which for the uncoded values included: solvent:water ratio (75:25), solid:volume ratio (10 percent) and mixing time (≈119 minutes) (Table 18). (Figures 12a-c show the main effect plots for each solvent). A high proportion of acetone to water was needed for the solvent:water ratio (75:25) to obtain the highest TF values, similar to TP. As cited previously and shown in Figure 12c, the main effect of solvent:water ratio increased sharply from the lowest point (-1) to the highest point (+1), generating a linear trendline. Therefore, TF yields are likely to increase with higher solvent proportions (i.e., 85:15).

Table 18: Optimized factors required to generate optimum TF yield for the acetone system for pinto beans (*BaJa*)

Optimum value = 3.7168 mg/g of beans

Factor	*Low*	*High*	*Optimum (coded)*	*Optimum (uncoded)*
Solvent:Water	-1.0	1.0	1	75:25
Solid:Volume	-1.0	1.0	-1.0	10%
Mixing Time	-1.0	1.0	0.995	≈119 min

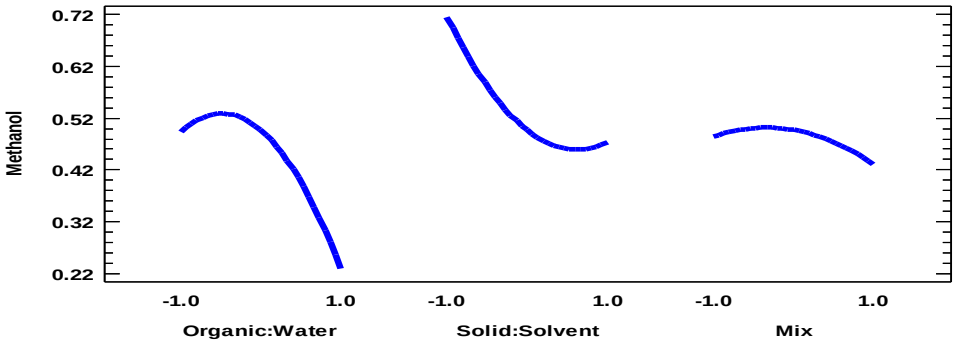


Figure 12a. Main effects plot for methanol (TF).

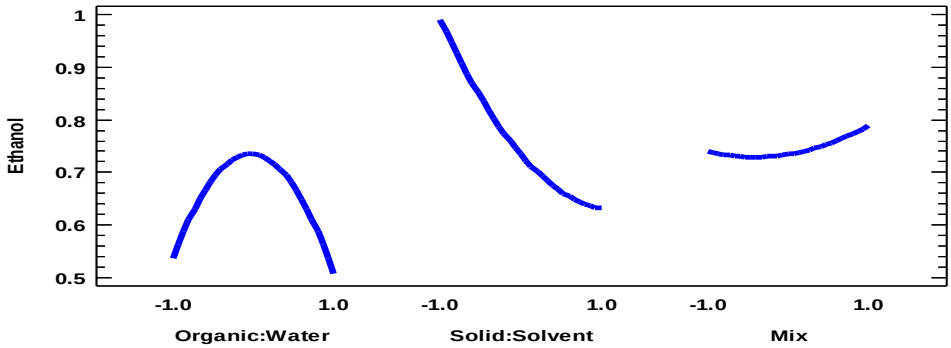


Figure 12b. Main effects plot for ethanol (TF).

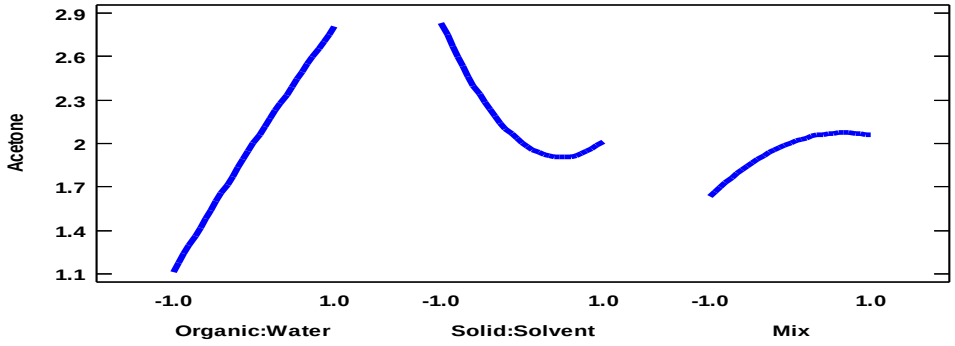


Figure 12c. Main effects plot for acetone (TF).

Zhao et al. (2006) indicated that 80 percent acetone was superior to 80 percent ethanol or 80 percent methanol for extracting TF from barley (*Hordeum vulgare L.*). However, Al-Farsi & Lee (2008) concluded that 100 percent acetone resulted in lower TF yields, whereas 50 percent resulted in the highest TF yields. Yet, it is unclear whether a higher polarity would increase TF for the pinto beans, considering the two previously cited studies. In addition, higher solvent:water ratios may cause the current upward linear line to maximize and then to decrease.

Interestingly, the main effect of solid:volume ratio was the same for all solvent systems (Figures 12a-c), which was (-1) (10 percent) as an optimum value. As cited earlier, even lower solid:volume ratio may increase TF yields. Various studies have demonstrated that lower solid:volume ratios resulted in higher polyphenols in multiple food systems (Pinelo et al., 2004; Al-Farsi & Lee 2008; Rajaei et al., 2010; Pompeu et al., 2009). The optimum mixing time predicted for the acetone was ≈119 minutes, which had a significant effect ($p < 0.10$) and was only a minute less than the longest mixing time tested (120 min) (Figures 11c and 12c). Again, as citied earlier, some studies reported that mixing time had no significant effect on polyphenols extractions (Al-Farsi & Lee, 2008; Liyana-Pathirana & Shahidi, 2005; Rajaei et al., 2010). However, Pompeu et al. (2009) showed that 240 minutes was the optimal mixing time for extracting phenolics from the fruits of (*Euterpe oleracea*). The study consisted of monitoring the extraction of phenols from 1 minute to 500 minutes. The longer mixing time required by acetone may be related to the higher TF levels compared to methanol and ethanol and as shown by the model equation (Table 17). Considering that the acetone was able to extract more TF, compared to methanol or ethanol, it have impacted by solid:solvent ratio, requiring

longer mixing times. Indeed, the main effects plots for acetone (Figure 12c) shows that mixing time and solid:solvent ratio plots are inverted relative to each other.

D.1.4. *Total Condensed Tannins:*

D.1.4.a *Total condensed tannins results obtained from FCCD-RSM:* Total condensed tannins levels for each solvent system (methanol, ethanol and acetone) were also determined using FCCD. The same three factors, (solvent:water ratio, solid:volume ratio and mixing time) employed for TP and TF procedures were applied to TCT extraction (Table 8). The results obtained for each solvent system are shown in Table 19, whereas ranges (lowest-to-highest response) of TCT are indicated in Table 20. Although, the ethanol system yielded higher amounts of TCT than methanol by only 0.16 mg/g, the acetone system was again substantially more effective than methanol and ethanol. The optimum TCT yields were recovered by the acetone system was 12.57 mg/g followed by ethanol (1.86 mg/g) and then methanol (1.70 mg/g), which was 7 times lower than the acetone. These results show that methanol and ethanol did not improve the extraction of TCT regardless of the other independent variables applied.

Similar to TP and TF data reported in the previous sections, the TCT ranges for methanol and ethanol did not vary widely in overall range compared to the acetone (Table 20), which had an 11 mg/g difference between the highest and the lowest response. Nonetheless, as tannins are mostly polymerized molecules, acetone (lower in polarity than methanol and ethanol) was expected to be the most effective solvent to extract optimum TCT levels. Yet, it must be emphasized that the combination of factors has to be considered when extracting with acetone, as the TCT lowest value obtained (1.47 mg/g) was lower than the highest level extracted with methanol (1.70 mg/g) and ethanol (1.86 mg/g).

Table 19. Total condensed tannins (in mg/g) of pinto beans (*BaJa*) extracts under different extraction conditions and solvent systems using face centered cubic design

Std. Order	Methanol	Ethanol	Acetone
1	0.33 ± 0.01	0.50 ± 0.03	8.94 ± 0.57
2	0.66 ± 0.03	1.13 ± 0.06	12.57 ± 0.89
3	0.39 ± 0.03	1.35 ± 0.11	8.80 ± 0.62
4	0.53 ± 0.01	0.94 ± 0.05	8.45 ± 0.60
5	0.53 ± 0.02	0.68 ± 0.01	10.40 ± 0.61
6	0.80 ± 0.05	1.12 ± 0.05	6.71 ± 0.19
7	1.70 ± 0.18	1.27 ± 0.09	2.50 ± 0.15
8	0.63 ± 0.02	1.11 ± 0.08	8.43 ± 0.52
9	0.25 ± 0.01	0.55 ± 0.02	1.47 ± 0.08
10	0.31 ± 0.01	0.53 ± 0.02	8.04 ± 0.59
11	0.61 ± 0.05	1.44 ± 0.02	4.79 ± 0.25
12	0.26 ± 0.00	0.51 ± 0.02	1.95 ± 0.11
13	0.70 ± 0.03	1.07 ± 0.04	6.81 ± 0.23
14	0.34 ± 0.02	1.86 ± 0.07	3.35 ± 0.11
15	0.58 ± 0.01	1.17 ± 0.02	6.80 ± 0.40
16	0.32 ± 0.01	0.47 ± 0.03	7.45 ± 0.01
17	0.60 ± 0.06	1.01 ± 0.03	8.92 ± 0.26

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

Table 20. Ranges of total condensed tannins for each solvent system

Extraction Solvent	Total Phenols (mg/g)	Range (mg/g)
Methanol	0.25 – 1.70	1.45
Ethanol	0.47 – 1.86	1.39
Acetone	1.47 – 12.57	11.1

D.1.4.b *Fitting a Second-Order TCT model and test of its adequacy*:

Regression coefficients were once more determined using the LSM from the TCT results for each solvent system to predict the quadratic equations. The R^2 values for methanol, ethanol and acetone were 88.20, 72.64 and 79.37 percent, respectively, (Table 21). The *p*-value of the model for all three systems were also < 0.1. However, the model's adequacy via the lack of fit showed that ethanol did not fit the model as *p* > 0.05 (Table 21). For the TCT study, only acetone (*p*-value = 0.1324) and methanol (*p*-value =0.0703) passed the lack of fit test.

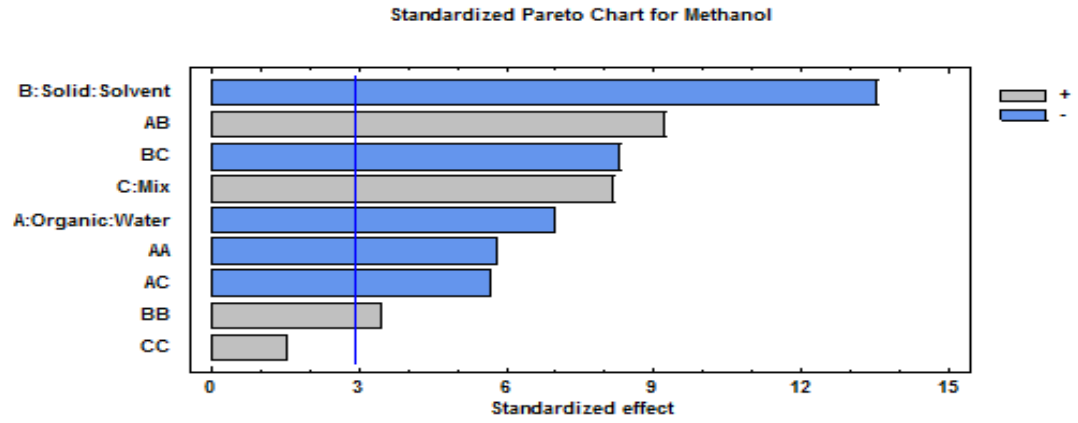
As ethanol failed the lack of fit test, a higher model (i.e., cubic) with more complex interactions between the different parameters could better explain the extractions. Interestingly, this scenario has occurred multiple times with ethanol in our lab when applied to different types of beans, even for TP and/or TF extraction. This phenomenon has yet to be demonstrated in the literature to our knowledge. A different RSM design other than the CCD may be needed to fit ethanol to a second-order model and/or that includes more sample analyses to substantiate a cubic model (Myers & Montgomery, 2002). Moreover, other independent factors, such as temperature, different mixing apparatus and matrix partial size may also affect the responses of the ethanol extraction, which could have affected its fitness for this FCCD model.

Figurer 13a-b shows the Pareto charts for methanol and acetone, respectively. All coefficient parameters form methanol were statistically significant with the notable exception of mixing time (Table, 21 Figure 13a). Most of the factors were inversely proportional with TCT yields, except for the linear effect of mixing time, the quadratic effect of solid:solvent ratio and the interaction between solvent polarity and solid:solvent ratio (Figure 13a).

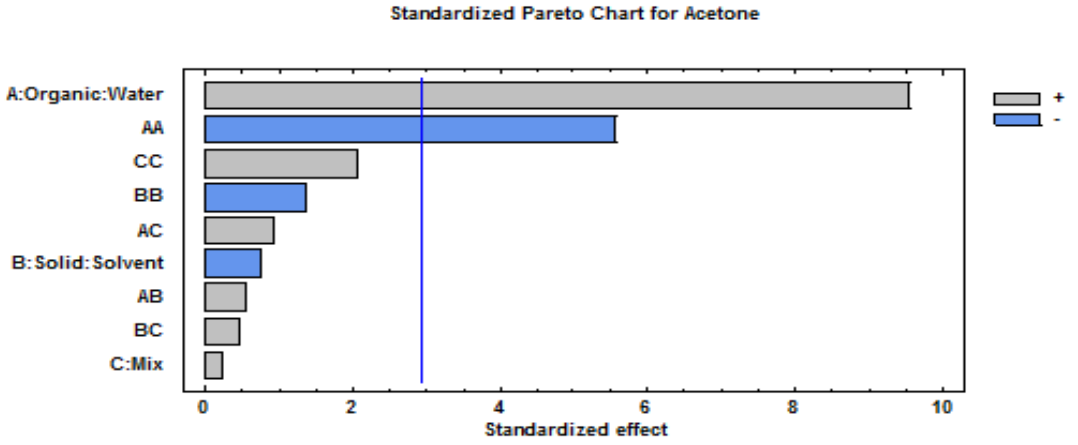
Table 21. Regression coefficients of the predicted quadratic model for TCT when extracted with the cited solvent systems for pinto bean (*BaJa*)

Coefficient	Methanol	Ethanol	Acetone
b_o	0.584	1.110	8.482
Linear			
b_1 (SP)	-0.127**	-0.186*	2.825**
b_2 (S:S)	-0.247*	-0.352*	-0.223
b_3 (MT)	0.150**	-0.025	0.071
Quadratic			
b_{11} (SP x SP)	-0.205**	0.072	-3.194**
b_{22} (S:S x S:S)	0.121^	-0.208**	-0.779
b_{33} (MT x MT)	0.053	-0.070	1.174
Cross product			
b_{12} (SP x S:S)	0.189**	0.048	0.184
b_{13} (SP x MT)	-0.116**	-0.044	0.308
b_{23} (S:S x MT)	-0.170**	0.051^	0.157
R^2	88.20	72.64	79.37
p values			
Model	0.0150	0.1757	0.0813
Lack of Fit	0.0703	0.0161	0.1324

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



(a)



(b)

Figure 13: Pareto charts show relative effects of regression coefficients for accepted TCT models for methanol (a) and acetone (b) for pinto bean (*BaJa*). The vertical line represents $p < 0.1$.

Considering that methanol did not extract high levels of TCT, the equation is rather complex. This could be due to the tannins-protein complexes that may have obtained in the methanol based extractions (Hagerman & Butler, 1980; Hagerman & Robbins, 1987).

D.1.4.c *Regression coefficients equations and Pareto charts*: The TCT regression equations for the acetone and methanol systems are shown in Table 22. These generated equations, as cited earlier, are based only on the significance of individual regression coefficients ($p < 0.1$). The Pareto charts summarizing the main effects for each regression coefficient for each solvent system are shown in Figures 13a and 13b. The acetone model (Table 22 and Figure 13b) shows two statistically significant effects. The main effect was linear solvent:water ratio and a lesser effect by the quadratic coefficient of the same factor (Tables 21, 22 and Figure 13b). The linear coefficient of the factor, solvent:water ratio was directly proportional with TCT yields; although, its quadratic effect was inversely proportional with TCT yields (Figure 13b).

D.1.4.d *Final optimized TCT values and processing factors*: The optimal processing factors were determined based on the generated acetone model, which predicted the highest TCT yields (Table 23). TCT were extracted most effectively in the acetone most likely because acetone inhibits the interaction between tannins and proteins. (Hagerman & Robins, 1987; Hagerman, 1988). The optimum factors using the acetone system for TCT (uncoded) are: solvent:water ratio (≈ 62:38), solid:volume ratio (≈ 20 percent) and mixing time (180 minutes) (Table 23). For the solvent:water ratio, a moderate proportion of acetone:water was required (62:38) to obtain the highest level of TCT. Yields of TCT were more dependent on solvent polarity as compared to TP and TF.

Table 22: Regression equations' fit to the models (passed the lack of fit test) for TCT

TCT methanol = 0.584 – 0.127**Xsp* – 0.247**Xss* + 0.150**Xmt* – 0.205**XspXs*p + 0.189**XspXss* – 0.116**XspXmt* + 0.121**XssXss* – 0.170**XssXmt*

TCT acetone = 8.482 + 2.825**Xsp* – 3.194**XspXsp*

Xsp: solvent polarity, Xss: solid:solvent, Xmt: mixing time.

Table 23: Optimized factors required to generating optimum TCT yield for the acetone system for pinto bean (*BaJa*)

Optimum value = 10.497 mg/g of beans

Factor	*Low*	*High*	*Optimum (coded)*	*Optimum (uncoded)*
Solvent:Water	-1.0	1.0	0.490	≈ 62:38
Solid:Volume	-1.0	1.0	0.016	≈ 20%
Mixing Time	-1.0	1.0	1.0	180 min

As shown in Figure 14b, the main effect of solvent:water ratio increased sharply from the lowest point (-1) until it reaches the highest point (≈0.5), then started to decrease showing a quadratic curve. Kallithraka et al. (1995) indicated that 70:30 acetone:water was able to extract the highest TCT from grape seeds (particularly, procyanidin trimer C1). Also, acetone:water (80:20) was more effective than methanol:water (80:20) or ethanol:water (80:20) for the extraction of TP and TCT at 80°C from beach pea seeds (*Lathyrus maritimus L.*) (Chavan & Amarowicz, 2013). Moreover, the extraction of TCT using 80 percent acetone was superior than acidic 70 percent acetone and/or 50 percent acetone for yellow pea, green pea, chickpea, yellow soybean, lentil, black soybean and red kidney bean (Xu & Chang, 2007). Also, 70 percent aqueous acetone was able to extract higher TCT from the leaves of three species of trees (burr oak, sugar maple and shagbark hickory) compared to aqueous or acidic methanol (Hagerman, 1988).

The main effect of solid:volume ratio for the acetone system was (coded; 0.01) (uncoded; ≈ 20 percent) (Figure 14a), which is higher than that for both TP and TF extractions (10 percent). As shown in Figure 14b, the curve of TCT yields increased from the lowest point (codded; -1) until it reaches the optimum point (codded; 0.01), then started to decent staidly until the highest point (codded; +1). As cited previously, research from several sources has shown that lower solid:volume ratio yielded higher polyphenols in multiple food systems (Pinelo et al., 2004; Al-Farsi & Lee 2008; Rajaei et al., 2010; Pompeu et al., 2009). However, none of the above studies measured TCT.

Despite the insignificant effect of mixing time for the acetone, the main curve in the main effect plot (Figures 14b) shows the estimated change in TCT yield as mixing time variable is moved from its low level to its high level, with all other

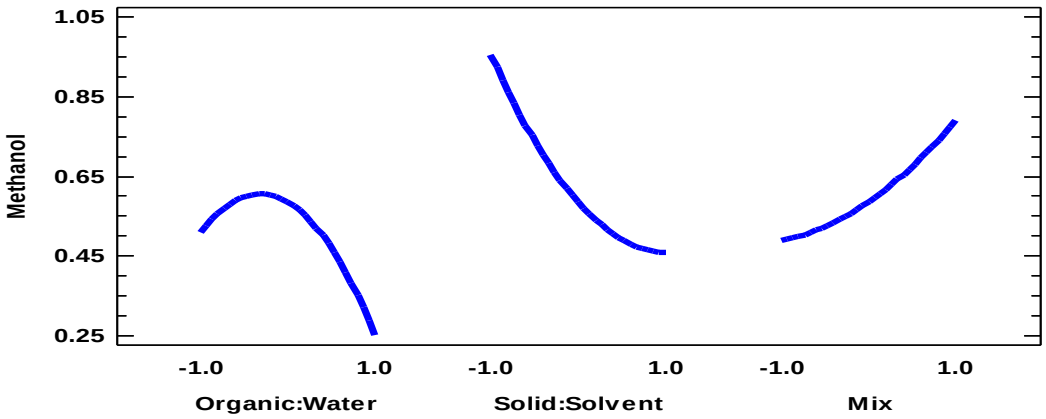


Figure 14a. Main effects plot for methanol (TCT).

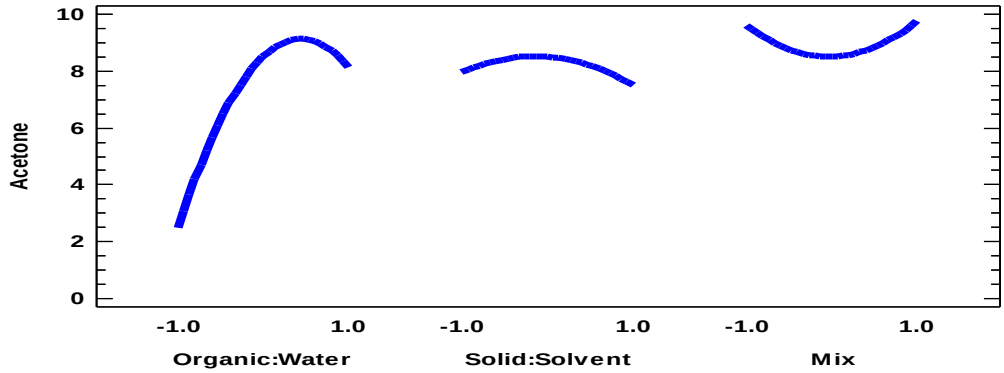


Figure 14b. Main effects plot for acetone (TCT).

factors held constant at a middle value between the lowest and the highest response (StatsGraphics, 2014). Remarkably, lower mixing time may also result in high TCT for pinto beans as the curve was at a high point for both -1 and +1 coded points (Figures 14b). Xu et al. (2007) extracted TCT along with other phenolics from common beans (black, navy, pinto, red kidney and pink beans) using acetone:water:acetic acid (70:29.5:0.5, v/v/v), solid:volume ratio (10 percent) and a mixing time of 3 hours, followed by another 12 hours. However, their TCT content from pinto bean (unspecified class) was 3.23 mg/g (Table 3). On the other hand, some studies reported that shorter mixing time improved polyphenols extraction as discussed earlier (Al-Farsi & Lee, 2008; Liyana-Pathirana, 2005; Rajaei et al., 2010). Although, none of the previous studies measured TCT.

D.2 *Specific Aim 2:*

D.2.1. *Adequacy of RSM to α-amylase and α-glucosidase data:* The initial objective of this Specific Aim was to determine whether optimal RSM (FCCD) procedures could be achieved for obtaining extracts from methanol, ethanol and acetone that are able to optimally inhibit α-amylase and α-glucosidase and thus generate predictive model equations. However, the statistical analyses of the RSM data did not provide such a model, as the data did not comply with any of the parameters stated in the Materials and Methods section: (R^2 >75) and the lack of fit test ($p>0.05$). Still, it was our intent to whether or not the bean extract could inhibit either or both of these enzymes, and if the recovered extracts differed in their inhibitory properties. As such a better understanding of using pinto beans for managing glucose would be realized. In addition, a comparison was made between the percent inhibition normalized to percent per mg of extract and the TP, TF and TCT levels in each extract to ascertain if a correlation existed. The evidence showed that no correlation occurred for TP, TF or

TCT levels and enzymes inhibition with the methanol extraction, but a weak to strong correlation resulted for TP and TF content and α-amylase and α-glucosidase inhibition for certain parameters, especially for the acetone extracts.

D.2.2. *Inhibition of α-amylase and α-glucosidase for the methanol extraction:* There were large differences between the inhibition results between these 17 extracts, again emphasizing that the extraction procedures play a very significant role when analyzing for specific health promoting properties of a food system (Table 24). (Total phenols, TF and TCT, expressed now as µg /mg of extract instead of mg/g of bean as used in Specific Aim 1, are shown in the same order of the RSM extraction procedures cited in the Materials and Methods section (Table 8).

Extract 14 exerted the highest inhibitory effect of alpha-amylase (12.57 percent / mg extract), which also contain the highest TP content (4.27 µg /mg of extract) (Table 24). Conversely, Extract 3 had high levels of TP (3.82 µg /mg of extract), but no inhibition properties were exhibited for either α-amylase or α-glucosidase. In addition, Extract 10 had the second highest levels of TP (3.99 µg /mg of extract), but exhibited low α-amylase inhibition (1.96 percent /mg extract). On the other hand, the highest inhibition of α-glucosidase (5.44 percent / mg extract) occurred with Extract 7. In general, the methanol extracted samples were able to obtain relatively higher inhibitions of α-amylase compared to α-glucosidase. Additionally, Extracts 3, 6, 13 and 16 showed no inhibitory effect of α-glucosidase.

These graphs on Figure 15 and 16 were constructed by plotting each replication of α-amylase and α- glucosidase inhibition against its associated TP, TF and TCT concentration pre mg of extract. As cited earlier, some extracts were positively correlated with the corresponding phenol level, such as Extract 14, which also exerted highest α-amylase inhibition and contained high TP levels.

Table 24. Alpha-amylase and α-glucosidase inhibition of pinto beans (*BaJa*) extracts using FCCD for the methanol solvent system

NO.	TP[a]	TF[a]	TCT[a]	Amylase[b]	Glucosidase[b]	Solv.: Water	Solid: Vol.	Time (min)
1	2.37 ± 0.29	1.29 ± 0.23	2.46 ± 0.26	1.14 ± 0.27	4.76 ± 0.50	75:25	20%	120
2	2.56 ± 0.66	1.84 ± 0.49	3.17 ± 0.81	1.59 ± 0.30	5.44 ± 1.09	50:50	20%	180
3	3.82 ± 0.06	2.83 ± 0.43	2.43 ± 0.37	NI	NI	75:25	10%	60
4	3.12 ± 0.82	2.76 ± 0.66	2.85 ± 0.75	1.75 ± 0.46	1.09 ± 0.05	50:50	20%	60
5	2.82 ± 0.93	2.32 ± 0.73	2.71 ± 0.90	4.59 ± 0.55	1.43 ± 0.52	50:50	30%	120
6	3.38 ± 1.00	2.90 ± 0.85	3.03 ± 0.86	2.43 ± 1.18	NI	50:50	10%	120
7	1.85 ± 0.46	1.62 ± 0.38	3.60 ± 0.96	2.99 ± 0.14	5.22 ± 0.17	25:75	10%	180
8	3.04 ± 0.11	2.78 ± 0.19	3.07 ± 0.10	2.42 ± 0.27	2.83 ± 0.26	50:50	20%	120
9	2.95 ± 1.14	1.37 ± 0.53	0.94 ± 0.37	5.12 ± 1.25	4.22 ± 0.66	25:75	30%	180
10	3.99 ± 0.61	2.49 ± 0.42	3.44 ± 0.53	1.96 ± 0.25	1.92 ± 0.27	75:25	30%	60
11	2.84 ± 0.38	2.32 ± 0.28	2.17 ± 0.16	6.19 ± 0.01	NI	25:75	10%	60
12	1.44 ± 0.72	1.00 ± 0.52	0.53 ± 0.28	2.20 ± 0.19	2.21 ± 0.24	25:75	30%	60
13	3.32 ± 0.67	2.48 ± 0.47	3.56 ± 0.67	1.17 ± 0.25	NI	50:50	20%	120
14	4.27 ± 0.27	2.79 ± 0.19	1.85 ± 0.13	12.57 ± 0.18	2.27 ± 0.38	25:75	20%	120
15	2.85 ± 0.15	2.04 ± 0.02	2.63 ± 0.07	2.50 ± 0.48	0.62 ± 0.04	50:50	20%	120
16	2.14 ± 1.12	1.41 ± 0.76	1.92 ± 1.04	0.40 ± 0.22	3.31 ± 0.64	75:25	30%	180
17	1.31 ± 1.18	0.89 ± 0.88	1.75 ± 1.77	1.41 ± 0.40	NI	75:25	10%	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).
NI: No inhibition.

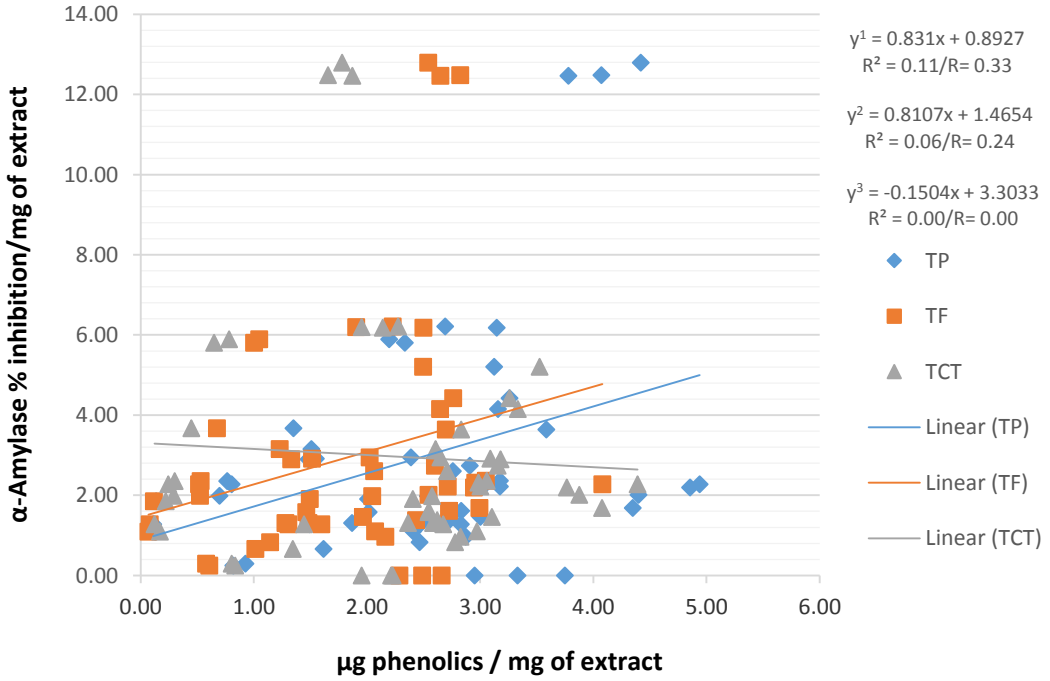


Figure 15: Effect of TP, TF and TCT concentrations on α-amylase inhibitory activities for the methanol solvent. The scatter plot contains data of each replication (trendline equations[1-3] are for TP, TF and TCT, respectively).

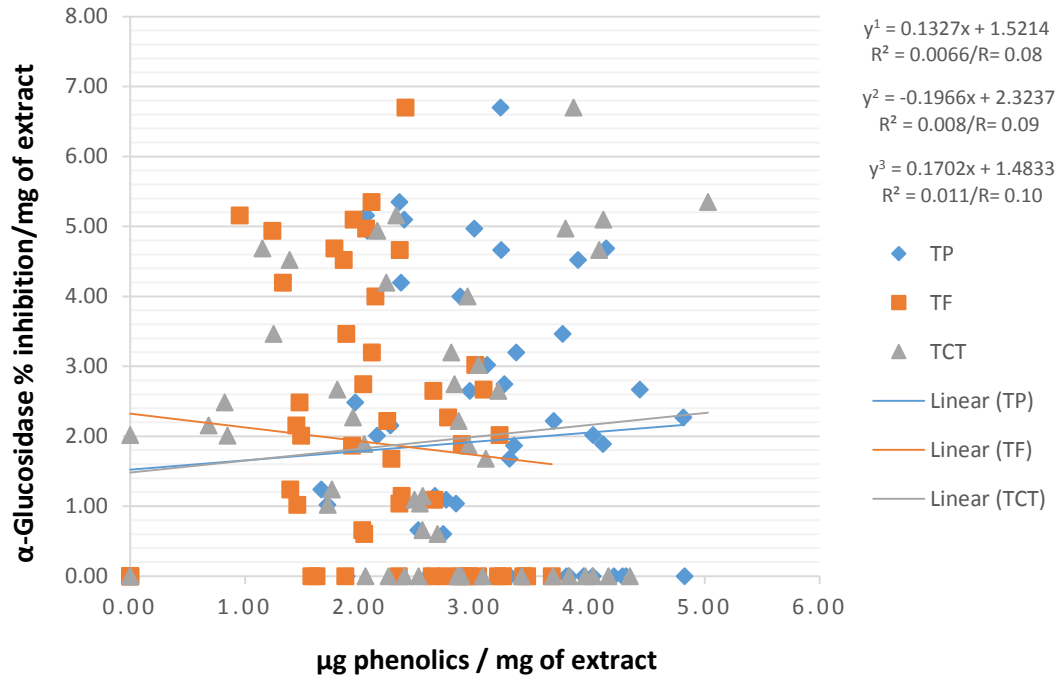


Figure 16: Effect of TP, TF, and TCT concentrations on α-glucosidase inhibitory activities for the methanol solvent. The scatter plot contains data of each replication (trendline equations[1-3] are for TP, TF and TCT, respectively).

Extract 7 also exhibited high α-glucosidase inhibition (5.22 percent /mg extract) correlation with a high TCT (3.60 µg /mg of extract) (Table 24). Nonetheless, the scatter plots (Figures 15 and 16) clearly demonstrate that the overall results of the methanol extract did not correlate TP, TF and TCT levels with either α-amylase or α-glucosidase inhibition. This statement is supported by the small R values of each phenolic group, as well by visual observation of Figures 15 and 16. For α-amylase, the R values were as follows: (R =0.33) for TP, (R =0.24) for TF and (R =0.00) for TCT (Figure 15). In the case of α-glucosidase, the R values were (R =0.08) for TP, (R =0.09) for TF and (R =0.10) for TCT (Figure 16).

In contrary, the inhibition of pancreatic α-amylase was positively correlated with the concentration of phenol extracts from strawberry, raspberry, blueberry, blackcurrant, red cabbage and green tea (McDougall et al., 2005), again initially using only one extraction followed by more steps to release and isolate the bound phenols. The researchers attributed the inhibition of pancreatic α-amylase mainly to the tannins (ellagitannins) content present in strawberry and raspberry extracts. It must be emphasized that these authors suggested that the phenolics in the tested food systems might be exerting a synergistic effect (McDougall et al., 2005).

For this study, the extracts were not purified to eliminate other types of components, such as peptides, amino acids, complex lipid and sugars. These components could also have contributed to the inhibition of these enzymes as well as to act as antagonists. This would be particularly true for the methanol based extractions where many small molecules and hydrophilic proteins are extracted from a food matrix in addition to the phenols. For example, studies have identified and purified the protein-type α-amylase inhibitors, including (α-AI-1) and (α-AI-2) (Ranilla et al., 2010) from different common bean classes (*Phaseolus vulgaris L.*),

including kidney bean, red kidney bean, white kidney bean, black kidney bean (Lajolo & Finardi Filho, 1985; Wilcox & Whitaker, 1984; Ishimoto & Kitamura, 1989; Ishimoto & Chrispeels, 1996; Berre-Anton et al., 1997) and from wild beans (Suzuki et al. 1993). To elaborate, (α-AI-1) selectively inhibits porcine pancreatic α-amylase and (α-AI-2) inhibits the α-amylase of *Z. subfasciatus* (Ishimoto & Chrispeels, 1996; de Sa et al., 1997).

D.2.3. *Inhibition of α-amylase and α-glucosidase for the ethanol extraction:* The ethanol extracts were better able to inhibit α-amylase and α-glucosidase compared to the methanol. The highest inhibition for α-amylase occurred with Extract 16 (42.94 percent /mg extract), which also contained the highest TP, TF and TCT levels at 7.55, 6.91, and 2.49 µg/mg of extract, respectively (Table 25). Yet, Extract 11 contained 7.51 TP µg/mg of extract and the corresponding α-amylase inhibition was only 1.68 percent /mg extract. Additionally, the extract was even unable to inhibit α-glucosidase (Table 25).The highest inhibition of α-glucosidase occurred with Extract 1 (9.67/mg extract). This extract contained moderate levels of TP, TF and TCT, i.e., 5.07, 5.57 and 1.22 µg /mg of extract, respectively (Table 25). Furthermore, Extracts 6, 9, 11, 12 and 15 did not exhibit any inhibitory properties against α-glucosidase. Notably, Extract 6 did not inhibit α-glucosidase for both the ethanol and methanol extraction. These results suggest that the methanol extracts that did not inhibit α-glucosidase but did so when ethanol served as the extraction solvent indicates that each had a different composition, not only in amounts of phenols, but possibly types, as well as other types of nonephenolic components.

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 17 and 18.

Table 25. Alpha-amylase and α-glucosidase inhibition of pinto beans (*BaJa*) extracts using FCCD for the ethanol solvent system

No.	TP[a]	TF[a]	TCT[a]	Amylase[b]	Glucosidase[b]	Solv.: Water	Solid: Vol.	Time (min)
1	5.07 ± 1.12	5.57 ± 1.04	1.22 ± 0.24	2.31 ± 0.66	9.67 ± 0.94	75:25	20%	120
2	3.33 ± 1.89	3.58 ± 2.08	1.19 ± 0.72	1.25 ± 0.05	5.16 ± 2.22	50:50	20%	180
3	2.30 ± 0.09	2.91 ± 0.10	1.12 ± 0.08	1.50 ± 0.18	5.66 ± 0.95	75:25	10%	60
4	5.46 ± 0.27	4.84 ± 0.30	1.60 ± 0.12	5.19 ± 0.06	1.76 ± 0.11	50:50	20%	60
5	4.86 ± 0.08	4.41 ± 0.26	1.07 ± 0.02	3.97 ± 0.19	3.66 ± 0.41	50:50	30%	120
6	5.83 ± 2.68	4.95 ± 2.31	1.46 ± 0.65	6.89 ± 1.67	NI	50:50	10%	120
7	2.13 ± 1.36	1.52 ± 0.76	0.66 ± 0.40	2.45 ± 0.16	3.20 ± 1.54	25:75	10%	180
8	5.21 ± 1.51	4.79 ± 1.43	1.61 ± 0.55	4.38 ± 0.06	3.12 ± 1.36	50:50	20%	120
9	3.11 ± 0.86	1.96 ± 0.53	0.55 ± 0.15	9.83 ± 0.20	NI	25:75	30%	180
10	5.54 ± 1.16	5.65 ± 1.13	1.03 ± 0.20	1.05 ± 0.38	7.08 ± 0.21	75:25	30%	60
11	7.51 ± 0.60	4.79 ± 0.47	1.35 ± 0.10	1.68 ± 0.27	NI	25:75	10%	60
12	4.09 ± 0.16	2.60 ± 0.24	0.69 ± 0.02	2.70 ± 0.15	NI	25:75	30%	60
13	5.45 ± 0.45	4.63 ± 0.54	1.86 ± 0.14	5.41 ± 0.55	5.37 ± 0.42	50:50	20%	120
14	4.45 ± 0.27	2.66 ± 0.31	1.00 ± 0.09	3.53 ± 0.35	2.00 ± 0.19	25:75	20%	120
15	3.01 ± 0.12	2.62 ± 0.07	1.15 ± 0.02	2.91 ± 0.63	NI	50:50	20%	120
16	7.55 ± 2.87	6.91 ± 2.59	2.49 ± 0.98	42.94 ± 5.64	5.94 ± 3.53	75:25	30%	180
17	0.72 ± 0.03	0.80 ± 0.06	0.26 ± 0.01	0.53 ± 0.13	1.02 ± 0.14	75:25	10%	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).
NI: No inhibition.

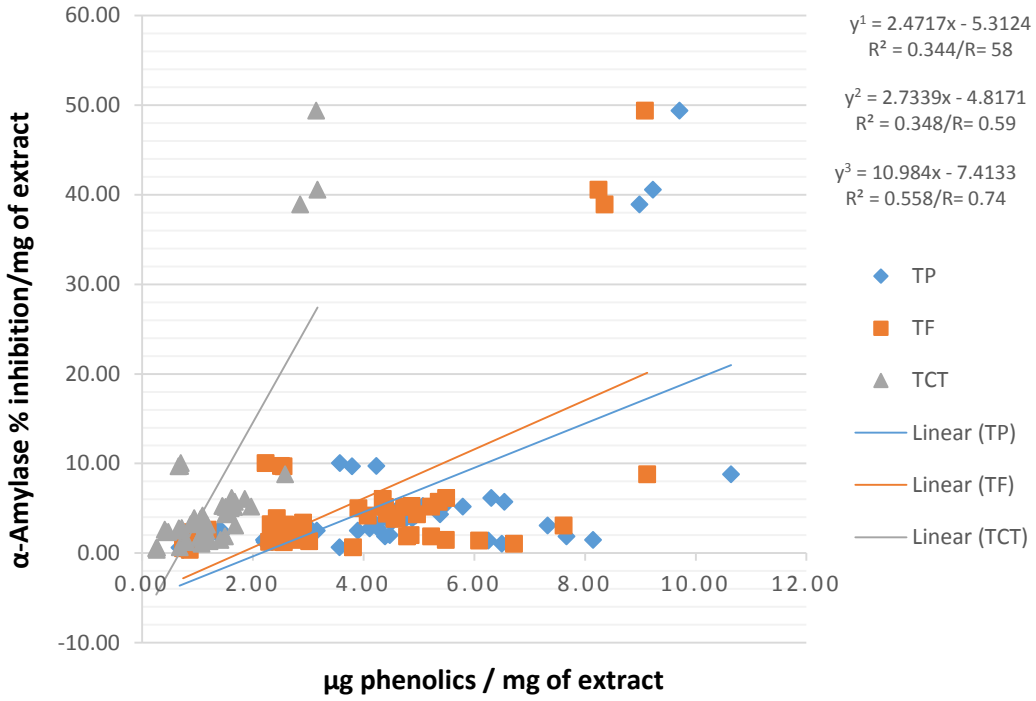


Figure 17: Effect of TP, TF and TCT concentrations on α-amylase inhibitory activities for the ethanol solvent. The scatter plot contains data of each replication (trendline equations[1-3] are for TP, TF and TCT, respectively).

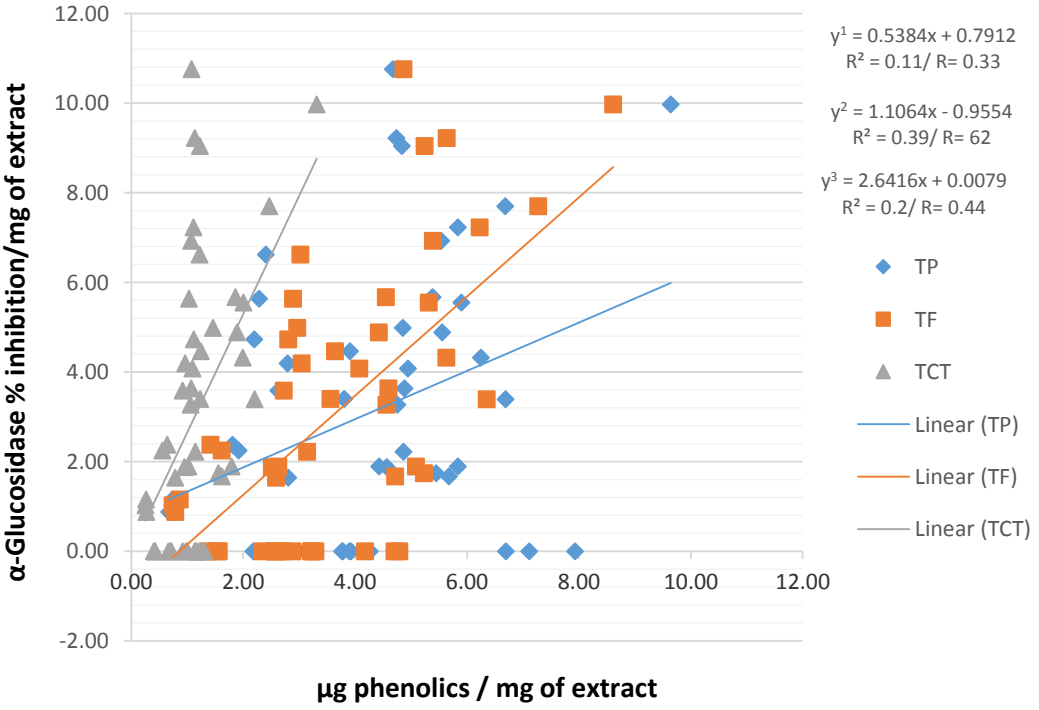


Figure 18: Effect of TP, TF and TCT concentrations on α-glucosidase inhibitory activities for the ethanol solvent. The scatter plot contains data of each replication (trendline equations[1-3] are for TP, TF and TCT, respectively).

The scatter plot (Figure 17) show low to moderate correlations between the phenolics (TP, TF and TCT) and α-amylase inhibition, as the R values were 0.58 for TP, 0.59 for TF and TCT producing the highest R value of 0.74. As cited earlier, several studies have attributed α-amylase inhibition to tannins in multiple food systems including strawberry, raspberry, berries, black tea and black tea pomace (Boath et al., 2012; Striegel et al., 2015; McDougall et al., 2005). These studies support the high R value for TCT of the present study. The extraction procedures that the other researchers used are as follows, which again were only one or two methods. First, Striegel et al. (2015) extracted the polyphenols from black tea using 10 percent (w/v) with 100 percent distilled water for 30 minutes at 90 °C, and 10 percent (w/v) with aqueous 70 percent acetone for 2 hours at room temperature for black tea pomace. Second, McDougall et al. (2005) used 100 percent (w/v) with 0.5 percent aqueous glacial acetic acid as an initial extraction step. Third, Boath et al. (2012) used 100 percent (w/v) with 0.2 percent (v/v) formic acid in ultra-pure water for polyphenols extraction. In the case of the α-glucosidase inhibitory correlation generated by this study, the R values were low to moderate, but improved compared to those produced by the methanol extracts, and are as follows: (R =0.33) for TP, (R =0.62) for TF and (R =0.44) for TCT (Figure 18).

D.2.4. *Inhibition of α-amylase and α-glucosidase for the acetone extraction:* The acetone extracts were able to inhibit α-amylase and α-glucosidase at much higher levels than either methanol or ethanol (Table 26). The three highest inhibitions of α-amylase occurred with Extracts 17 >3 >1 at 57.83, 52.21 and 47.64 percent /mg extract, respectively. Even though Extract 1 contained the highest TP, TF and TCT levels i.e., 30.7, 48.87 µg/mg and 11.21 µg/mg of extract, respectively (Table 26).

Table 26. Alpha-amylase and α-glucosidase inhibition of pinto beans (*BaJa*) extracts using FCCD for the acetone solvent system

No	TP[a]	TF[a]	TCT[a]	Amylase[b]	Glucosidase[b]	Solv.: Water	Sol.: Vol.	Time min
1	30.76 ± 1.39	48.87 ± 3.25	11.21 ± 0.65	47.64 ± 1.70	17.50 ± 2.23	75:25	20%	120
2	8.50 ± 0.32	9.27 ± 0.58	4.75 ± 0.35	0.88 ± 0.04	5.45 ± 0.12	50:50	20%	180
3	16.87 ± 7.04	26.30 ± 11.13	4.79 ± 1.97	52.21 ± 0.26	5.14 ±0.42	75:25	10%	60
4	8.39 ± 5.64	9.46 ± 6.38	3.20 ± 2.16	7.81 ± 0.31	1.31 ± 0.04	50:50	20%	60
5	5.11 ± 0.19	7.27 ± 0.79	2.58 ± 0.13	1.05 ± 0.05	2.23 ± 0.31	50:50	30%	120
6	11.23 ± 3.03	12.84 ± 3.80	8.66 ± 2.27	3.71 ± 0.47	NI	50:50	10%	120
7	3.53 ± 0.24	2.72 ± 0.04	1.58 ± 0.08	0.77 ± 0.17	NI	25:75	10%	180
8	11.36 ± 2.95	15.06 ± 3.76	4.24 ± 1.02	6.98 ± 0.35	2.35 ± 0.61	50:50	20%	120
9	4.16 ± 0.56	3.35 ± 0.37	1.31 ± 0.15	6.09 ± 0.53	1.20 ± 0.06	25:75	30%	180
10	18.39 ± 12.06	35.54 ± 23.53	7.56 ± 4.98	10.78 ± 0.32	13.92 ± 1.51	75:25	30%	60
11	3.59 ± 1.76	3.02 ± 1.39	0.72 ± 0.33	1.55 ± 0.37	1.04 ± 0.20	25:75	10%	60
12	6.75 ± 0.22	4.40 ± 0.43	0.78 ± 0.05	8.82 ± 0.26	NI	25:75	30%	60
13	11.41 ± 1.89	15.20 ± 2.51	3.00 ± 0.52	2.78 ± 0.09	4.13 ± 0.05	50:50	20%	120
14	5.26 ± 1.57	4.07 ± 1.22	0.88 ± 0.27	10.24 ± 0.13	0.65 ± 0.24	25:75	20%	120
15	7.55 ± 0.43	10.40 ± 0.75	2.12 ± 0.11	10.51 ± 0.77	6.97 ± 0.60	50:50	20%	120
16	19.98 ± 10.38	38.50 ± 20.02	6.57 ± 3.41	14.96 ± 1.06	17.59 ± 1.95	75:25	30%	180
17	22.91 ± 6.23	37.55 ± 10.18	5.92 ± 1.58	57.83 ± 6.12	10.40 ± 0.82	75:25	10%	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).
NI: No inhibition.

Alternatively, Extract 17 contained 22.91 µg TP, 37.55 µg TF and 5.92 µg TCT per mg of extract. Still, and contrary to the methanol and ethanol, the acetone solvent system resulted in relatively strong correlations between TF and TP α-amylase inhibition as evidenced by R =0.91 and R =0.89, respectively (Figure 19). The high R value of the TF supports the results from of previous studies where isolated flavonoids exerted high α-amylase inhibitory activates (Kim et al., 2000; Tadera al., 2005, Piparo et al., 2008).

In general, the inhibitory abilities of the acetone extracts were again lower for α-glucosidase than α-amylase. Extracts 16 >1 >10 had the greatest inhibitory effects at 17.59, 17.50, 13.92 percent mg extract, respectively. It must be noted that Extract 6 again did not inhabit α-glucosidase nor did Extracts 7 or 12 (Table 26). Yet, the scatter plot (Figure 20) shows strong correlation of the phenolics (TP, TF and TCT) with α-glucosidase inhibition, i.e., (R =0.92) for TP, (R =0.94) for TF and (R =0.78) for TCT. Several studies highlighted the effect of isolated flavonoids on the inhibition of α-glucosidase (Kim et al., 2000; Tadera al., 2005; Li et al., 2009; Matsui et al., 2007). Moreover, Yao et al. (2011) examined two isolated flavones from adzuki beans extracts, vitexin and isovitexin, which exhibited high inhibitory activities of α-glucosidase. Furthermore, Sheliya et al. (2015) studied the inhibitory effect of α-glucosidase in vitro and in vivo and determined that specific pernylated flavonoids from (*euohorbia hirta L.*) herb were more effective in inhibiting α-glucosidase than non-pernylated flavonoids (Sheliya et al., 2015). The extraction procedure in the previous study (for the in vitro part) was achieved with methanol using Soxhlet for 18 hours at 80 °C (Sheliya et al., 2015).

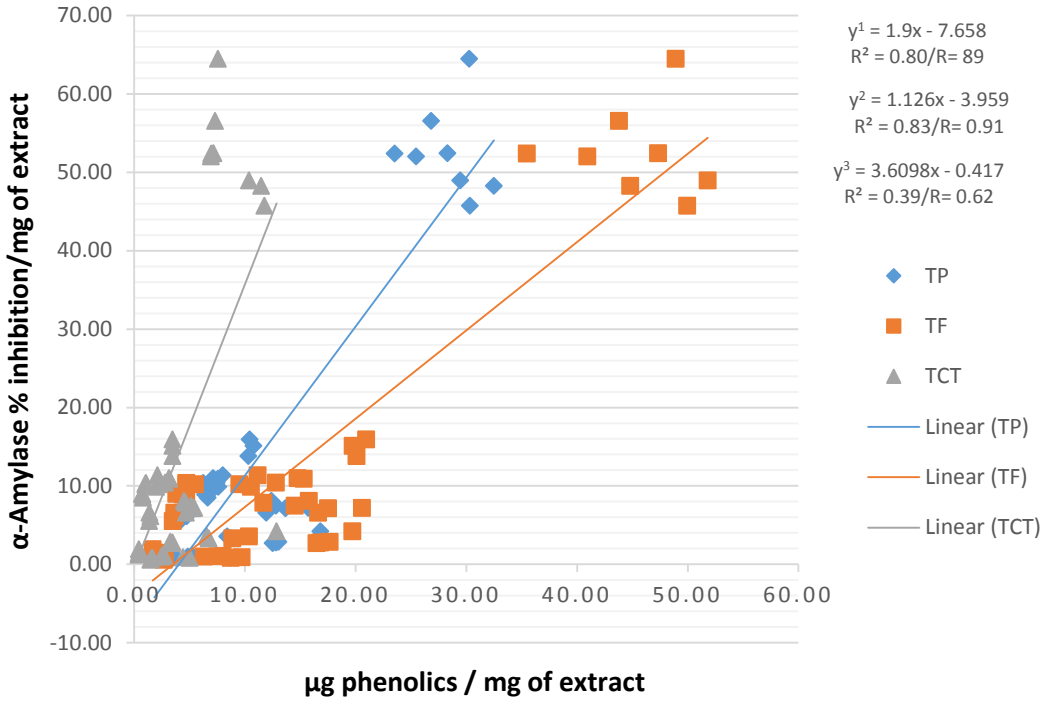


Figure 19: Effect of TP, TF and TCT concentrations on α-amylase inhibitory activities for the acetone solvent. The scatter plot contains data of each replication (trendline equations[1-3] are for TP, TF and TCT, respectively).

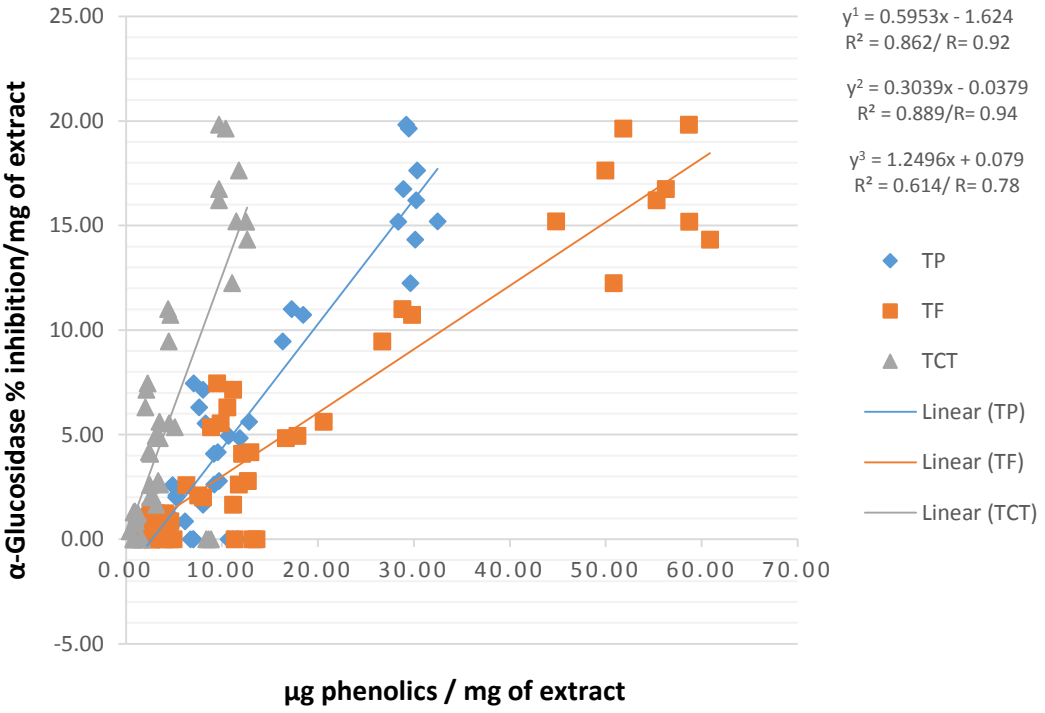


Figure 20: Effect of TP, TF and TCT concentrations on α-glucosidase inhibitory activities for the acetone solvent. The scatter plot contains data of each replication (trendline equations[1-3] are for TP, TF and TCT, respectively).

D.2.5. *Overall highest inhibitions of α-amylase and α-glucosidase:* A review of the literature has yet to demonstrate the use of different extraction procedures to assess the inhibitory activities of α-amylase and α-glucosidase from food system extracts. However, most of the phenol rich pinto bean extracts were effective on inhibiting α-amylase and α-glucosidase (Table 27) with a few exceptions. Yet, the inhibitory activities were varied according to the solvent type used in the extraction, the different extraction parameters and the initial extract concentrations. The highest inhibition of α-amylase and α-glucosidase was 57.83 percent /mg extract, 17.59 percent /mg extract, respectively, under the acetone extracting conditions (Table 27). Alternatively, the ethanol extraction produced a high α-amylase inhibition of 42.94 percent /mg extract and 9.67 percent /mg extract for α-glucosidase. The methanol extracts exhibited the lowest inhibitory activities by 12.57 percent /mg extract, 5.44 percent /mg extract for α-amylase and α-glucosidase, respectively.

These results correlate with Specific Aim 1 in that the acetone not only was the most effective solvent system for extracting polyphenols, but also exerted the highest inhibition properties for both enzymes. In addition, the difference between the highest α-amylase inhibition for the optimal acetone and ethanol extracts was 17 percent.

The overall results shows that α-amylase inhibitory activities was mostly higher than for α-glucosidase for all the solvents used (Table 27). Similarly, McCue et al. (2005) examined α-glucosidase (baker's yeast) inhibition of different food extracts (i.e., green pepper, string beans, broccoli sprouts, red pepper, fresh carrot, red grape, tomato, basil leaves, ginger, cinnamon, turmeric and cardamom powder). These researchers demonstrated that high total soluble phenolic content does not always result in high α-glucosidase (baker's yeast) inhibition.

Table 27. Highest inhibitory activities of α-amylase and α-glucosidase for each solvent

Extraction Solvent	α-Amylase[a]	α-Glucosidase[a]
Methanol	12.57 ± 0.18 (14)[b]	5.44 ± 1.09 (2)[b]
Ethanol	42.94 ± 5.64 (16)[b]	9.67 ± 0.94 (1)[b]
Acetone	57.83 ± 6.12 (17)[b]	17.59 ± 1.95 (16)[b]

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

Interestingly, the antioxidant activity extracts were positively correlated with α-amylase (porcine pancreas) inhibition (McCue et al., 2005). Yet, the pervious study used only distilled water for extracting TP. Ranilla et al. (2010) also examined the in vitro anti-diabetic and anti-hypertension potential effect of ten selected dry bean cultivars (raw and thermally treated).

Alpha-amylase (porcine pancreas) and α-glucosidase (baker's yeast) inhibitory activities were higher than 90 percent / 20-100 mg of raw sample weight and 16-31 percent / 2-10 mg of raw sample weight, respectively, but the inhibitions was significantly reduced after thermal treatment (autoclaving). It must be noted that authors determined that phenol levels did not correlate with α-amylase or α-glucosidase inhibitory activities, signifying that non phenolic compounds may also be have involved (Ranilla et al., 2010). Yet, the pervious study also used only distilled water for the extraction of TP so the inhibitory properties may have varied with different extraction factors. Moreover, Mojica et al. (2015) conducted in vitro research to examine the inhibitory activities of α-glucosidase (baker's yeast) and α-amylase (porcine pancreas) on 15 market classes of dry beans hulls, including 4 cultivars of pinto beans. Their results showed that the highest inhibition of α-amylase (74.2 percent /g coat) occurred with the pinto bean (*saltillo*), whereas the black bean (*Otomi*) had the greatest effect on α-glucosidase inhibition (82.5 percent /g coat). Again, the previous study used one extraction method (100 percent of methanol). Taken all together, the results from Specific Aim 2 confirm the importance of characterizing different food extracts to better understand the effects of food matrices composition and their influences on their health promoting properties, such as inhibiting these two significant carbohydrate–hydrolysis enzymes.

E. CONCLUSION

The following conclusions can be drawn from the present study:

- RSM was successfully used for obtaining critical information relative to the recovery of phenolic-rich extracts from pinto beans (*BaJa*).
- Acetone was the most effective solvent for extracting TP, TF and TCT.
- The optimum observed yields for TP was 2.31 mg/g of beans, which was the same as the predicted value by the analysis of RSM of the quadratic model. The most effective factors that resulted in the optimum yields (predicted) were 75:25 acetone:water, 10 percent solid:solvent and 87 minutes of mixing.
- For optimal TF extraction, the maximum observed response was 3.79 mg/g of beans and the maximum predicted was 3.71 mg/g of beans. The most effective factors as predicted by the generated RSM model were 75:25 acetone:water, 10 percent solid:solvent and 119 minutes of mixing.
- The Maximum observed TCT value was 12.57 mg/g of beans and the optimum predicted was 10.49 mg/g of beans. This predicted response should be achieved with 62:38 acetone:water, 20 percent solid:solvent and mixing time of 180 minutes.
- In most cases, a second-order polynomial model could be used to optimize the extraction of TP, TF and TCT from pinto beans with the exception of ethanol for TCT as the data did not fit the model, which could be due to variability in the assay. Alternatively, a higher order model may better to explain the complex interactions occurring.
- This study has shown that extracts originating from pinto beans (*BaJa*) were capable of inhibiting key carbohydrate-hydrolysis enzymes, but this property

depends on the extract, most likely due to different phenol levels / types, and the presence of other nonephenolic components.

- Acetone extracts were also the most effective for inhibiting α-amylase and α-glucosidase (57.83, 17.59 percent /mg extract, respectively).
- Alpha-amylase and α-glucosidase inhibition did not correlate with TP, TF or TCT for the methanol extracts, but the correlation increased with the highest occurring in the acetone extracts.
- For α-amylase and α-glucosidase inhibitions with the acetone, the highest correlation detected was with TF.
- Lastly, consumption of a nutrient-rich food such as pinto beans may reduce or delay the absorption of glucose due to the chemical diverse polyphenols, thus can aid in the management or regulation of type 2 diabetes.

F. FURTHER RESEARCH

The results from this research provided the following insights for future work:

- Applying different ratios of solvent:water, solid:volume and mixing time to determine if other extraction factors affect phenols extracts, which may create even better second degree models. (Specific Aim 1 shows that some parameters were not optimized, as several of the uncoded values in the optimized tables were either 1 or -1. At some point, a number between these two values is expected.
- Characterization of phenolics (groups and individuals) using chromatographic/spectrometric-based techniques (i.e., HPLC, GC-MS and LC-MS/MS) in pinto beans. This is a very important need, especially for those extracts that showed high and low phenols content against the high or low

enzymatic inhibitions, as other components, such as proteins, minerals and sugars might also be accessed during the extraction.

- Identifying the most effective phenolic isolates from the literature and test them as individuals and in combinations to better understanding the synergistic or additive effect of these phenolics against α-amylase and α-glucosidase.
- Assessment of polyphenols in processed pinto beans (i.e., soaked, steamed and/or boiled) and their effect on α-amylase and α-glucosidase.
- Studying the influence of other carbohydrate-hydrolysis enzymes (i.e., glucoamylse and isomaltas) to better understand the mechanism of starch degradation, which may involve developing new methods.
- In vivo assessment of α-amylase and α-glucosidase (glucose uptake) using pinto bean to better understand the properties and mechanism of these enzymes at the cellular level.

REFERENCES

Adom, K. K., & Liu, R. H. (2002). Antioxidant activity of grains. *Journal of Agricultural and Food Chemistry, 50*(21), 6182-6187.

Agricultural Research Service, United States Department of Agriculture. (2015). *National nutrient database for standard reference.* (Release 28). Online: http://www.ars.usda.gov/ba/bhnrc/ndl: USDA.

Aldawsari, M. (2014). *Characterization of extraction methods to recover phenolic rich extracts from pinto beans that exert high antioxidative activities using response surface approach* (M.S. Thesis).

Al-Farsi, M. A., & Lee, C. Y. (2008). Optimization of phenolics and dietary fibre extraction from date seeds. *Food Chemistry, 108*(3), 977-985.

Ali, H., Houghton, P., & Soumyanath, A. (2006). α-Amylase inhibitory activity of some malaysian plants used to treat diabetes; with particular reference to phyllanthus amarus. *Journal of Ethnopharmacology, 107*(3), 449-455.

American Diabetes Association. (2014). Diagnosis and classification of diabetes mellitus. *Diabetes Care, 37 Suppl 1*, S81-90. [doi:10.2337/dc14-S081]

American Diabetes Association. (2016). Diabetes basics. Retrieved from http://www.diabetes.org/diabetes-basics/?loc=db-slabnav

Andersen, O. M., & Markham, K. R. (2006). *Flavonoids: Chemistry, biochemistry and applications* CRC Press.

Anderson, J. W., Smith, B. M., & Washnock, C. S. (1999). Cardiovascular and renal benefits of dry bean and soybean intake. *The American Journal of Clinical Nutrition, 70*(3), 464s-474s.

Anderson, R. A., Broadhurst, C. L., Polansky, M. M., Schmidt, W. F., Khan, A., Flanagan, V. P., . . . Graves, D. J. (2004). Isolation and characterization of polyphenol type-A polymers from cinnamon with insulin-like biological activity. *Journal of Agricultural and Food Chemistry, 52*(1), 65-70.

Appel, H. M., & Schultz, J. C. (1992). Activity of phenolics in insects: The role of oxidation. *Plant polyphenols* (pp. 609-620) Springer.

Ballard, T. S., Mallikarjunan, P., Zhou, K., & O'Keefe, S. F. (2009). Optimizing the extraction of phenolic antioxidants from peanut skins using response surface methodology. *Journal of Agricultural and Food Chemistry, 57*(8), 3064-3072.

Bennink, M. (2002). Consumption of black beans and navy beans (phaseolus vulgaris) reduced azoxymethane-induced colon cancer in rats. *Nutrition and Cancer, 44*(1), 60-65.

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta, 76*(5), 965-977.

Bhagwat, S., Haytowitz, D., & Holden, J. (2014). USDA database for the flavonoid content of selected foods, release 3.1. *Beltsville: US Department of Agriculture, Agricultural Research Service.,* Retrieved from Nutrient Data Laboratory Home Page: http://www.ars.usda.gov/nutrientdata/flav

Bhandari, M. R., Jong-Anurakkun, N., Hong, G., & Kawabata, J. (2008). α-Glucosidase and α-amylase inhibitory activities of nepalese medicinal herb pakhanbhed (bergenia ciliata, haw.). *Food Chemistry, 106*(1), 247-252.

Bhat, R., Sridhar, K. R., & Tomita-Yokotani, K. (2007). Effect of ionizing radiation on antinutritional features of velvet bean seeds (mucuna pruriens). *Food Chemistry, 103*(3), 860-866.

Boath, A. S., Grussu, D., Stewart, D., & McDougall, G. J. (2012). Berry polyphenols inhibit digestive enzymes: A source of potential health benefits? *Food Digestion, 3*(1-3), 1-7.

Boschin, G., & Arnoldi, A. (2011). Legumes are valuable sources of tocopherols. *Food Chemistry, 127*(3), 1199-1203.

Broadhurst, C. L., Polansky, M. M., & Anderson, R. A. (2000). Insulin-like biological activity of culinary and medicinal plant aqueous extracts in vitro. *Journal of Agricultural and Food Chemistry, 48*(3), 849-852.

Buisson, G., Duee, E., Haser, R., & Payan, F. (1987). Three dimensional structure of porcine pancreatic alpha-amylase at 2.9 A resolution role of calcium in structure and activity. *The EMBO Journal, 6*(13), 3909-3916.

Burns, D. T., Danzer, K., & Townshend, A. (2009). A tutorial discussion of the use of the terms "Robust" and "Rugged" and the associated characteristics of "Robustness" and "Ruggedness" as used in descriptions of analytical procedures. *Journal of the Association of Public Analysts (Online), 37*, 40-60.

Butterfield, D. A., Castegna, A., Pocernich, C. B., Drake, J., Scapagnini, G., & Calabrese, V. (2002). Nutritional approaches to combat oxidative stress in Alzheimer's disease. *The Journal of Nutritional Biochemistry, 13*(8), 444-461.

Cannas, A. (2010). Tannins: Fascinating but sometimes dangerous molecules. Retrieved from http://poisonousplants.ansci.cornell.edu/toxicagents/tannin.html

Centers for Disease Control and Prevention. (2014). National diabetes statistics report: Estimates of diabetes and its burden in the United States, 2014. *Atlanta, Ga: US Department of Health and Human Services,*

Chauhan, B., & Gupta, R. (2004). Application of statistical experimental design for optimization of alkaline protease production from bacillus sp. RGR-14. *Process Biochemistry, 39*(12), 2115-2122.

Chavan, U., & Amarowicz, R. (2013). Effect of various solvent systems on extraction of phenolics, tannins and sugars from beach pea (lathyrus maritimus L.). *International Food Research Journal, 20*(3)

Chaves, M. M., Maroco, J. P., & Pereira, J. S. (2003). Understanding plant responses to drought—from genes to the whole plant. *Functional Plant Biology, 30*(3), 239-264.

Chen, F., Liu, C., Tschaplinski, T. J., & Zhao, N. (2009). Genomics of secondary metabolism in populus: Interactions with biotic and abiotic environments. *Critical Reviews in Plant Science, 28*(5), 375-392.

Chew, K., Khoo, M., Ng, S., Thoo, Y., Wan Aida, W., & Ho, C. (2011). Effect of ethanol concentration, extraction time and extraction temperature on the recovery of phenolic compounds and antioxidant capacity of orthosiphon stamineus extracts. *International Food Research Journal, 18*(4)

Cheynier, V. (2012). Phenolic compounds: From plants to foods. *Phytochemistry Reviews, 11*(2-3), 153-177.

Chung, K., Wong, T. Y., Wei, C., Huang, Y., & Lin, Y. (1998). Tannins and human health: A review. *Critical Reviews in Food Science and Nutrition, 38*(6), 421-464.

Clifford, M., Brown, J., Andersen, Ø., & Markham, K. (2006). Dietary flavonoids and health-broadening the perspective. *Flavonoids: Chemistry, Biochemistry and Applications*, 319-370.

Clifford, M. N. (2004). Diet-derived phenols in plasma and tissues and their implications for health. *Planta Medica, 70*(12), 1103-1114. [doi:10.1055/s-2004-835835]

Crozier, A., Clifford, M. N., & Del Rio, D. (2012). Bioavailability of dietary monomeric and polymeric flavan-3-ols. *Bioavailability and Function of Flavonoids: Oxidative Stress and Disease, 30*, 45-78.

Dabhi, A. S., Bhatt, N. R., & Shah, M. J. (2013). Voglibose: An alpha glucosidase inhibitor. *Journal of Clinical and Diagnostic Research: JCDR, 7*(12), 3023-3027. [doi:10.7860/JCDR/2013/6373.3838]

Damodaran, S., Parkin, K. L., & Fennema, O. R. (2007). *Fennema's food chemistry* CRC press.

Dannehl, D., & Josuttis, M. (2014). Cultivar and production effects on bioactive polyphenols. In R. Watson (1st Ed.), *Polyphenols in plants: Isolation, purification and extract preparation* (pp. 3-9) Academic Press.

Dastjerdi, Z. M., Namjoyan, F., & Azemi, M. E. (2015). Alpha amylase inhibition activity of some plants extract of teucrium species. *European Journal of Biological Sciences, 7*(1), 26-31.

De Pascual-Teresa, S., Santos-Buelga, C., & Rivas-Gonzalo, J. C. (2000). Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. *Journal of Agricultural and Food Chemistry, 48*(11), 5331-5337.

De Sa, M Fatima Grossi, Mirkov, T. E., Ishimoto, M., Colucci, G., Bateman, K. S., & Chrispeels, M. J. (1997). Molecular characterization of a bean α-amylase inhibitor that inhibits the α-amylase of the mexican bean weevil zabrotes subfasciatus. *Planta, 203*(3), 295-303.

Di Mascio, P., Murphy, M. E., & Sies, H. (1991). Antioxidant defense systems: The role of carotenoids, tocopherols, and thiols. *The American Journal of Clinical Nutrition, 53*(1 Suppl), 194S-200S.

Durling, N. E., Catchpole, O. J., Grey, J. B., Webby, R. F., Mitchell, K. A., Foo, L. Y., & Perry, N. B. (2007). Extraction of phenolics and essential oil from dried sage (salvia officinalis) using ethanol–water mixtures. *Food Chemistry, 101*(4), 1417-1424.

Earp, C., Akingbala, J., Ring, S., & Rooney, L. (1981). Evaluation of several methods to determine tannins in sorghums with varying kernel characteristics. *Cereal Chemistry (USA),*

Eberhardt, M. V., Lee, C. Y., & Liu, R. H. (2000). Nutrition: Antioxidant activity of fresh apples. *Nature, 405*(6789), 903-904.

Espinosa-Alonso, L. G., Lygin, A., Widholm, J. M., Valverde, M. E., & Paredes-Lopez, O. (2006). Polyphenols in wild and weedy mexican common beans (phaseolus vulgaris L.). *Journal of Agricultural and Food Chemistry, 54*(12), 4436-4444.

Food and Agriculture Organization of the United Nations. (2014). *Production of dry beans* Available from FAOSTAT database.

Food and Drug Administration. (2014). *Guidance for Industry: A Food Labeling Guide (14.Appendix F: Calculate the Percent Daily Value for the Appropriate Nutrients,*

Galili, S., & Hovav, R. (2014). Chapter 16 - determination of polyphenols, flavonoids, and antioxidant capacity in dry seeds. In R. R. Watson (Ed.), *Polyphenols in plants* (pp. 305-323). San Diego: Academic Press. doi: http://dx.doi.org/10.1016/B978-0-12-397934-6.00016-4

Giada, Maria de Lourdes Reis. (2013). Food phenolic compounds: Main classes, sources and their antioxidant power. *Oxidative Stress and Chronic Degenerative diseases—A Role for Antioxidants.InTech*, 87-112.

Gómez-Caravaca, A., Verardo, V., Segura-Carretero, A., Fernández-Gutiérrez, A., Caboni, M., & Watson, R. (2013). Phenolic compounds and saponins in plants grown under different irrigation regimes. *Stress and Polyphenols in Plants*, 37-48.

Gu, L., Kelm, M. A., Hammerstone, J. F., Beecher, G., Holden, J., Haytowitz, D., . . . Prior, R. L. (2004). Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *The Journal of Nutrition, 134*(3), 613-617.

Hagerman, A. E. (1988). Extraction of tannin from fresh and preserved leaves. *Journal of Chemical Ecology, 14*(2), 453-461.

Hagerman, A. E., & Butler, L. G. (1980). Determination of protein in tannin-protein precipitates. *Journal of Agricultural and Food Chemistry, 28*(5), 944-947.

Hagerman, A. E., & Robbins, C. T. (1987). Implications of soluble tannin-protein complexes for tannin analysis and plant defense mechanisms. *Journal of Chemical Ecology, 13*(5), 1243-1259.

Han, X., Shen, T., & Lou, H. (2007). Dietary polyphenols and their biological significance. *International Journal of Molecular Sciences, 8*(9), 950-988.

Hanhineva, K., Törrönen, R., Bondia-Pons, I., Pekkinen, J., Kolehmainen, M., Mykkänen, H., & Poutanen, K. (2010). Impact of dietary polyphenols on carbohydrate metabolism. *International Journal of Molecular Sciences, 11*(4), 1365-1402.

Hanrahan, G., & Lu, K. (2006). Application of factorial and response surface methodology in modern experimental design and optimization. *Critical Reviews in Analytical Chemistry, 36*(3-4), 141-151.

Harborne, J. B., & Baxter, H. (1999). *The handbook of natural flavonoids. Volume 1 and volume 2.* John Wiley & Sons.

Hill, W. J., & Hunter, W. G. (1966). A review of response surface methodology: A literature survey. *Technometrics, 8*(4), 571-590.

Hogan, S., Zhang, L., Li, J., Sun, S., Canning, C., & Zhou, K. (2010). Antioxidant rich grape pomace extract suppresses postprandial hyperglycemia in diabetic mice by specifically inhibiting alpha-glucosidase. *Nutrition & Metabolism, 7*(1), 1.

Horwitt, M. K. (1986). The promotion of vitamin E. *The Journal of Nutrition, 116*(7), 1371-1377.

Hughes, J. S., Ganthavorn, C., & Wilson-Sanders, S. (1997). Dry beans inhibit azoxymethane-induced colon carcinogenesis in F344 rats. *The Journal of Nutrition, 127*(12), 2328-2333.

Hutzler, P., Fischbach, R., Heller, W., Jungblut, T. P., Reuber, S., Schmitz, R., . . . Schnitzler, J. (1998). Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. *Journal of Experimental Botany, 49*(323), 953-965.

ICH. (2005). Validation of analytical procedures: text and methodology. *Q2 (R1), 1*.

Inglett, G. E., Rose, D. J., Chen, D., Stevenson, D. G., & Biswas, A. (2010). Phenolic content and antioxidant activity of extracts from whole buckwheat (fagopyrum esculentum möench) with or without microwave irradiation. *Food Chemistry, 119*(3), 1216-1219.

ISHIMOTO, M., & KITAMURA, K. (1989). Growth inhibitory effects of an Alpha-amylase inhibitor from the kidney bean, phaseolus vulgaris (L.) on three species of bruchids (coleoptera: Bruchidae). *Applied Entomology and Zoology, 24*(3), 281-286.

Ishimoto, M., & Chrispeels, M. J. (1996). Protective mechanism of the Mexican bean weevil against high levels of alpha-amylase inhibitor in the common bean. *Plant Physiology, 111*(2), 393-401. [doi:111/2/393]

Jiang, Q., Christen, S., Shigenaga, M. K., & Ames, B. N. (2001). Gamma-tocopherol, the major form of vitamin E in the US diet, deserves more attention. *The American Journal of Clinical Nutrition, 74*(6), 714-722.

Jones, A. L. (1999). *PHASEOLUS BEAN: Post-harvest.* (Document). Food and Agriculture Organization.

Kallithraka, S., Garcia-Viguera, C., Bridle, P., & Bakker, J. (1995). Survey of solvents for the extraction of grape seed phenolics. *Phytochemical Analysis, 6*(5), 265-267.

Kalogeropoulos, N., Chiou, A., Ioannou, M., Karathanos, V. T., Hassapidou, M., & Andrikopoulos, N. K. (2010). Nutritional evaluation and bioactive microconstituents (phytosterols, tocopherols, polyphenols, triterpenic acids) in cooked dry legumes usually consumed in the mediterranean countries. *Food Chemistry, 121*(3), 682-690.

Kannan, S., Nielsen, S. S., & Mason, A. C. (2001). Protein digestibility-corrected amino acid scores for bean and bean-rice infant weaning food products. *Journal of Agricultural and Food Chemistry, 49*(10), 5070-5074.

Karacabey, E., & Mazza, G. (2010). Optimisation of antioxidant activity of grape cane extracts using response surface methodology. *Food Chemistry, 119*(1), 343-348.

Kelly, G. S. (1999). Squalene and its potential clinical uses. *Alternative Medicine Review: A Journal of Clinical Therapeutic, 4*(1), 29-36.

Khuri, A. I., & Mukhopadhyay, S. (2010). Response surface methodology. *Wiley Interdisciplinary Reviews: Computational Statistics, 2*(2), 128-149.

Kim, J., Kwon, C., & Son, K. H. (2000). Inhibition of alpha-glucosidase and amylase by luteolin, a flavonoid. *Bioscience, Biotechnology, and Biochemistry, 64*(11), 2458-2461.

Kim, Y., Wang, M., & Rhee, H. (2004). A novel α-glucosidase inhibitor from pine bark. *Carbohydrate Research, 339*(3), 715-717.

Klein, S., Sheard, N. F., Pi-Sunyer, X., Daly, A., Wylie-Rosett, J., Kulkarni, K., . . . American Society for Clinical Nutrition. (2004). Weight management through lifestyle modification for the prevention and management of type 2 diabetes: Rationale and strategies: A statement of the american diabetes association, the north american association for the study of obesity, and the american society for clinical nutrition. *Diabetes Care, 27*(8), 2067-2073. [doi:27/8/2067]

Koehler, H., CHANG, C., SCHEIER, G., & Burke, D. (1987). Nutrient composition, protein quality, and sensory properties of Thirty-Six cultivars of dry beans (phaseolus vulgaris L.). *Journal of Food Science, 52*(5), 1335-1340.

Krygier, K., Sosulski, F., & Hogge, L. (1982). Free, esterified, and insoluble-bound phenolic acids. 1. Extraction and purification procedure. *Journal of Agricultural and Food Chemistry, 30*(2), 330-334.

Kutoš, T., Golob, T., Kač, M., & Plestenjak, A. (2003). Dietary fibre content of dry and processed beans. *Food Chemistry, 80*(2), 231-235.

Labarbe, B., Cheynier, V., Brossaud, F., Souquet, J., & Moutounet, M. (1999). Quantitative fractionation of grape proanthocyanidins according to their degree of polymerization. *Journal of Agricultural and Food Chemistry, 47*(7), 2719-2723.

Lajolo, F. M., & Finardi Filho, F. (1985). Partial characterization of the amylase inhibitor of black beans (phaseolus vulgaris), variety rico 23. *Journal of Agricultural and Food Chemistry, 33*(1), 132-138.

Lattanzio, V., Kroon, P. A., Quideau, S., & Treutter, D. (2008). Plant phenolics—secondary metabolites with diverse functions. *Recent Advances in Polyphenol Research, 1*, 1-35.

Law, M. R. (2000). Plant sterol and stanol margarines and health. *The Western Journal of Medicine, 173*(1), 43-47.

Le Berre-Anton, V., Bompard-Gilles, C., Payan, F., & Rouge, P. (1997). Characterization and functional properties of the α-amylase inhibitor (α-AI) from kidney bean (phaseolus vulgaris) seeds. *Biochimica Et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology, 1343*(1), 31-40.

Le Man, H., Behera, S., & Park, H. (2010). Optimization of operational parameters for ethanol production from korean food waste leachate. *International Journal of Environmental Science & Technology, 7*(1), 157-164.

Li, Y. Q., Zhou, F. C., Gao, F., Bian, J. S., & Shan, F. (2009). Comparative evaluation of quercetin, isoquercetin and rutin as inhibitors of α-glucosidase. *Journal of Agricultural and Food Chemistry, 57*(24), 11463-11468.

Lin, L., Harnly, J. M., Pastor-Corrales, M. S., & Luthria, D. L. (2008). The polyphenolic profiles of common bean (phaseolus vulgaris L.). *Food Chemistry, 107*(1), 399-410.

Liyana-Pathirana, C., & Shahidi, F. (2005). Optimization of extraction of phenolic compounds from wheat using response surface methodology. *Food Chemistry, 93*(1), 47-56.

Lo Piparo, E., Scheib, H., Frei, N., Williamson, G., Grigorov, M., & Chou, C. J. (2008). Flavonoids for controlling starch digestion: Structural requirements for inhibiting human α-amylase. *Journal of Medicinal Chemistry, 51*(12), 3555-3561.

Luna, B., & Feinglos, M. N. (2001). Oral agents in the management of type 2 diabetes mellitus. *American Family Physician, 63*(9), 1747-1756.

Luthria, D. L., & Pastor-Corrales, M. A. (2006). Phenolic acids content of fifteen dry edible bean (phaseolus vulgaris L.) varieties. *Journal of Food Composition and Analysis, 19*(2), 205-211.

Madhujith, T., Izydorczyk, M., & Shahidi, F. (2006). Antioxidant properties of pearled barley fractions. *Journal of Agricultural and Food Chemistry, 54*(9), 3283-3289.

Madhujith, T., & Shahidi, F. (2005). Antioxidant potential of pea beans (phaseolus vulgaris L.). *Journal of Food Science, 70*(1), S85-S90.

Manach, C., Scalbert, A., Morand, C., Remesy, C., & Jimenez, L. (2004). Polyphenols: Food sources and bioavailability. *The American Journal of Clinical Nutrition, 79*(5), 727-747.

Maran, J. P., Manikandan, S., Nivetha, C. V., & Dinesh, R. (2013). Ultrasound assisted extraction of bioactive compounds from nephelium lappaceum L. fruit peel using central composite face centered response surface design. *Arabian Journal of Chemistry*,

Maritim, A., Sanders, a., & Watkins, 3. J. (2003). Diabetes, oxidative stress, and antioxidants: A review. *Journal of Biochemical and Molecular Toxicology, 17*(1), 24-38.

Matsui, T., Tanaka, T., Tamura, S., Toshima, A., Tamaya, K., Miyata, Y., . . . Matsumoto, K. (2007). α-Glucosidase inhibitory profile of catechins and theaflavins. *Journal of Agricultural and Food Chemistry, 55*(1), 99-105.

Matsui, T., Ueda, T., Oki, T., Sugita, K., Terahara, N., & Matsumoto, K. (2001). α-Glucosidase inhibitory action of natural acylated anthocyanins. 1. survey of natural pigments with potent inhibitory activity. *Journal of Agricultural and Food Chemistry, 49*(4), 1948-1951.

Matsui, T., Yoshimoto, C., Osajima, K., Oki, T., & Osajima, Y. (1996). In vitro survey of α-glucosidase inhibitory food components. *Bioscience, Biotechnology, and Biochemistry, 60*(12), 2019-2022.

Mattila, P., Astola, J., & Kumpulainen, J. (2000). Determination of flavonoids in plant material by HPLC with diode-array and electro-array detections. *Journal of Agricultural and Food Chemistry, 48*(12), 5834-5841.

Mazid, M., Khan, T., & Mohammad, F. (2011). Role of secondary metabolites in defense mechanisms of plants. *Biology and Medicine, 3*(2), 232-249.

Mazur, W. M., Duke, J. A., Wähälä, K., Rasku, S., & Adlercreutz, H. (1998). Isoflavonoids and lignans in legumes: Nutritional and health aspects in humans. *The Journal of Nutritional Biochemistry, 9*(4), 193-200.

Mccue, P., KWON, Y., & Shetty, K. (2005). Anti-amylase, anti-glucosidase and anti-angiotensin i-converting enzyme potential of selected foods. *Journal of Food Biochemistry, 29*(3), 278-294.

McDougall, G. J., Shpiro, F., Dobson, P., Smith, P., Blake, A., & Stewart, D. (2005). Different polyphenolic components of soft fruits inhibit α-amylase and α-glucosidase. *Journal of Agricultural and Food Chemistry, 53*(7), 2760-2766.

McGinnis, L., & Suszkiw, J. (2006). Breeding better beans. *Agricultural Research, 54*(6), 12.

Mensack, M. M., McGinley, J. N., & Thompson, H. J. (2012). Metabolomic analysis of the effects of edible dry beans (phaseolus vulgaris L.) on tissue lipid metabolism and carcinogenesis in rats. *British Journal of Nutrition, 108*(S1), S155-S165.

Messina, M. J. (1999). Legumes and soybeans: Overview of their nutritional profiles and health effects. *The American Journal of Clinical Nutrition, 70*(3 Suppl), 439S-450S.

Mitchell, D. C., Lawrence, F. R., Hartman, T. J., & Curran, J. M. (2009). Consumption of dry beans, peas, and lentils could improve diet quality in the US population. *Journal of the American Dietetic Association, 109*(5), 909-913.

Moghadasian, M. H., & Frohlich, J. J. (1999). Effects of dietary phytosterols on cholesterol metabolism and atherosclerosis: Clinical and experimental evidence. *The American Journal of Medicine, 107*(6), 588-594.

Mojica, L., Meyer, A., Berhow, M. A., & de Mejía, E. G. (2015). Bean cultivars (phaseolus vulgaris L.) have similar high antioxidant capacity, in vitro inhibition of α-amylase and α-glucosidase while diverse phenolic composition and concentration. *Food Research International, 69*, 38-48.

Mukhopadhyay, S., Luthria, D. L., & Robbins, R. J. (2006). Optimization of extraction process for phenolic acids from black cohosh (cimicifuga racemosa) by pressurized liquid extraction. *Journal of the Science of Food and Agriculture, 86*(1), 156-162.

Myers, R. H., & Montgomery, D. C. (2002). *Response surface methodology: Process and product optimization using designed experiments* (2nd Ed.) John Wiley & Sons, INC.

Naczk, M., Nichols, T., Pink, D., & Sosulski, F. (1994). Condensed tannins in canola hulls. *Journal of Agricultural and Food Chemistry, 42*(10), 2196-2200.

Naczk, M., & Shahidi, F. (1989). The effect of methanol-ammonia-water treatment on the content of phenolic acids of canola. *Food Chemistry, 31*(2), 159-164.

Nakatani, N., Kayano, S., Kikuzaki, H., Sumino, K., Katagiri, K., & Mitani, T. (2000). Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (prunus d omestica L.). *Journal of Agricultural and Food Chemistry, 48*(11), 5512-5516.

NDL, BHNRC, ARS, USDA. (2004). USDA database for the proanthocyanidin content of selected foods. *Washington, DC: US Department of Agriculture,*

Nelson, K. M., Reiber, G., Boyko, E. J., & NHANES III. (2002). Diet and exercise among adults with type 2 diabetes: Findings from the third national health and nutrition examination survey (NHANES III). *Diabetes Care, 25*(10), 1722-1728.

Oki, T., Matsui, T., & Osajima, Y. (1999). Inhibitory effect of α-glucosidase inhibitors varies according to its origin. *Journal of Agricultural and Food Chemistry, 47*(2), 550-553.

Okuda, T., Yoshida, T., & Hatano, T. (1995). Hydrolyzable tannins and related polyphenols. *Fortschritte der chemie organischer Naturstoffe/Progress in the chemistry of organic natural products* (pp. 1-117) Springer.

Önal, S., Timur, S., Okutucu, B., & Zihnioğlu, F. (2005). Inhibition of α-glucosidase by aqueous extracts of some potent antidiabetic medicinal herbs. *Preparative Biochemistry and Biotechnology, 35*(1), 29-36.

Oomah, B. D., Corbé, A., & Balasubramanian, P. (2010). Antioxidant and anti-inflammatory activities of bean (phaseolus vulgaris L.) hulls. *Journal of Agricultural and Food Chemistry, 58*(14), 8225-8230.

Osorio-Díaz, P., Bello-Pérez, L. A., Sáyago-Ayerdi, S. G., Benítez-Reyes, M. d. P., Tovar, J., & Paredes-López, O. (2003). Effect of processing and storage time on in vitro digestibility and resistant starch content of two bean (phaseolus vulgaris L) varieties. *Journal of the Science of Food and Agriculture, 83*(12), 1283-1288.

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity, 2*(5), 270-278. [doi:10.4161/oxim.2.5.9498]

Peterson, D. W. (1951). Effect of soybean sterols in the diet on plasma and liver cholesterol in chicks. *Proceedings of the Society for Experimental Biology and Medicine.Society for Experimental Biology and Medicine (New York, N.Y.), 78*(1), 143-147.

Pinelo, M., Rubilar, M., Sineiro, J., & Nunez, M. (2004). Extraction of antioxidant phenolics from almond hulls (prunus amygdalus) and pine sawdust (pinus pinaster). *Food Chemistry, 85*(2), 267-273.

Pompeu, D., Silva, E., & Rogez, H. (2009). Optimisation of the solvent extraction of phenolic antioxidants from fruits of euterpe oleracea using response surface methodology. *Bioresource Technology, 100*(23), 6076-6082.

Rajaei, A., Barzegar, M., Hamidi, Z., & Sahari, M. (2010). Optimization of extraction conditions of phenolic compounds from pistachio (pistachia vera) green hull through response surface method. *Journal of Agricultural Science and Technology, 12*, 605-615.

Ranilla, L. G., KWON, Y., Genovese, M. I., LAJOLO, F., & Shetty, K. (2010). Effect of thermal treatment on phenolic compounds and functionality linked to type 2 diabetes and hypertension management of peruvian and brazilian bean cultivars (phaseolus vulgaris L.) using in vitro methods. *Journal of Food Biochemistry, 34*(2), 329-355.

RCSB, P. (2015). Images of alpha-amylase and alpha-glucosidase. Retrieved from http://www.rcsb.org/pdb

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*(7), 933-956.

Ross, K., Beta, T., & Arntfield, S. (2009). A comparative study on the phenolic acids identified and quantified in dry beans using HPLC as affected by different extraction and hydrolysis methods. *Food Chemistry, 113*(1), 336-344.

Rostagno, M., Palma, M., & Barroso, C. (2004). Pressurized liquid extraction of isoflavones from soybeans. *Analytica Chimica Acta, 522*(2), 169-177.

Rothwell, J. A., Urpi-Sarda, M., Boto-Ordonez, M., Knox, C., Llorach, R., Eisner, R., . . . Scalbert, A. (2012). Phenol-explorer 2.0: A major update of the phenol-explorer database integrating data on polyphenol metabolism and pharmacokinetics in humans and experimental animals. *Database: The Journal of Biological Databases and Curation, 2012*, bas031. doi:10.1093/database/bas031

Saari, J. T., Reeves, P. G., Johnson, W. T., & Johnson, L. K. (2006). Pinto beans are a source of highly bioavailable copper in rats. *The Journal of Nutrition, 136*(12), 2999-3004. [doi:136/12/2999]

Sakihama, Y., Cohen, M. F., Grace, S. C., & Yamasaki, H. (2002). Plant phenolic antioxidant and prooxidant activities: Phenolics-induced oxidative damage mediated by metals in plants. *Toxicology, 177*(1), 67-80.

Salamatullah, A. (2014). *Characterization of extraction methods to recover phenolic-rich antioxidants from blue green algae (spirulina) using response surface approaches* (M.S. Thesis).

Sarwar, G., & McDonough, F. E. (1990). Evaluation of protein digestibility-corrected amino acid score method for assessing protein quality of foods. *Journal - Association of Official Analytical Chemists, 73*(3), 347-356.

Schaafsma, G. (2000). The protein digestibility-corrected amino acid score. *The Journal of Nutrition, 130*(7), 1865S-7S.

Schober, T. J., Messerschmidt, M., Bean, S. R., Park, S., & Arendt, E. K. (2005). Gluten-free bread from sorghum: Quality differences among hybrids. *Cereal Chemistry, 82*(4), 394-404.

Shahidi, F., & Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects–A review. *Journal of Functional Foods, 18*, 820-897.

Sheliya, M. A., Rayhana, B., Ali, A., Pillai, K. K., Aeri, V., Sharma, M., & Mir, S. R. (2015). Inhibition of α-glucosidase by new prenylated flavonoids from euphorbia hirta L. herb. *Journal of Ethnopharmacology, 176*, 1-8.

Shrestha, G., & St Clair, L. L. (2014). Chapter 4 - lichen phenolics: Environmental effects. In R. R. Watson (1st Ed.), *Polyphenols in plants: Isolation, purification and extract preparation* (pp. 53-62). San Diego: Academic Press. Retrieved from http://dx.doi.org/10.1016/B978-0-12-397934-6.00004-8

Sigal, R. J., Kenny, G. P., Wasserman, D. H., Castaneda-Sceppa, C., & White, R. D. (2006). Physical activity/exercise and type 2 diabetes: A consensus statement from the american diabetes association. *Diabetes Care, 29*(6), 1433-1438. [doi:29/6/1433]

Silva, E., Pompeu, D., Larondelle, Y., & Rogez, H. (2007). Optimisation of the adsorption of polyphenols from inga edulis leaves on macroporous resins using an experimental design methodology. *Separation and Purification Technology, 53*(3), 274-280.

Singleton, V., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*(3), 144-158.

Sreerama, Y. N., Takahashi, Y., & Yamaki, K. (2012). Phenolic antioxidants in some vigna species of legumes and their distinct inhibitory effects on α-Glucosidase and pancreatic lipase activities. *Journal of Food Science, 77*(9), C927-C933.

Statgraphics, C. (2014). Statgraphics® centurion XVI user manual. Retrieved from http://www.statgraphics.com/statistics-library

Stephen, A. M., Phillips, G. O., & Williams, P. A. (2006). *Food polysaccharides and their applications* (2nd Ed.). Boca Raton, FL: CRC Press.

Strack, D. (1997). Phenolic metabolism. In P. M. Dey, & J. B. Harborne (Eds.), *Plant biochemistry* (pp. 387-416). San Diego, CA: Academic Press.

Striegel, L., Kang, B., Pilkenton, S. J., Rychlik, M., & Apostolidis, E. (2015). Effect of black tea and black tea pomace polyphenols on α-glucosidase and α-amylase inhibition, relevant to type 2 diabetes prevention. *Frontiers in Nutrition, 2*

Subramanian, R., Asmawi, M. Z., & Sadikun, A. (2008). In vitro alpha-glucosidase and alpha-amylase enzyme inhibitory effects of andrographis paniculata extract and andrographolide. *Acta Biochim Pol, 55*(2), 391-398.

Sultana, B., Anwar, F., & Ashraf, M. (2009). Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. *Molecules, 14*(6), 2167-2180.

Sun, T., & Ho, C. (2005). Antioxidant activities of buckwheat extracts. *Food Chemistry, 90*(4), 743-749.

Suzuki, K., Ishimoto, M., Kikuchi, F., & Kitamura, K. (1993). Growth inhibitory effect of an ALPHA-amylase inhibitor from the wild common bean resistant to the mexican bean weevil (zabrotes subfasciatus). *Japanese Journal of Breeding, 43*(2), 257-265.

Tadera, K., Minami, Y., Takamatsu, K., & Matsuoka, T. (2006). Inhibition of. ALPHA.-glucosidase and. ALPHA.-amylase by flavonoids. *Journal of Nutritional Science and Vitaminology, 52*(2), 149-153.

Tappy, L. (2012). Q&A:'toxic'effects of sugar: Should we be afraid of fructose? *BMC Biology, 10*(1), 1. [doi:10.1186/1741-7007-10-42]

Thilagam, E., Parimaladevi, B., Kumarappan, C., & Mandal, S. C. (2013). α-Glucosidase and α-amylase inhibitory activity of senna surattensis. *Journal of Acupuncture and Meridian Studies, 6*(1), 24-30.

Timoracká, M., Vollmannová, A., & Ismael, D. (2011). Minerals, trace elements and flavonoids content in white and coloured kidney bean. *Potravinárstvo, 5*(1), 56-60.

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. *Nutrients, 2*(12), 1231-1246.

Udani, J. K., Singh, B. B., Barrett, M. L., & Preuss, H. G. (2009). Lowering the glycemic index of white bread using a white bean extract. *Nutrition Journal, 8*(1), 1.

United States Dry Bean Council, USDBC. (2015). Production facts. Retrieved from http://www.usdrybeans.com/resources/production/production-facts/

US Department of Health and Human Services, & US Department of Agriculture. (2015). *2015-2020 dietary guidelines for Americans.* (8th Ed.). Washington, DC: DHHS.

USDA, National Agricultural Statistics Service. (2016). *Crop production 2015 summary* (No. 1057-7823).USDA.

Van Huynh, A., & Bevington, J. M. (2014). MALDI-TOF MS analysis of proanthocyanidins in two lowland tropical forest species of cecropia: A first look at their chemical structures. *Molecules, 19*(9), 14484-14495.

Vermerris, W., & Nicholson, R. (2008). Families of phenolic compounds and means of classification. *Phenolic compound biochemistry* (pp. 1-34) Springer.

Vinson, J. A., Kharrat, H. A., & Shuta, D. (2009). Investigation of an amylase inhibitor on human glucose absorption after starch consumption. *The Open Nutraceuticals Journal, 2*(1)

WILCOX, E. R., & WHITAKER, J. R. (1984). Structural features of red kidney bean a-amylase inhibitor important in binding with a-amylase. *Journal of Food Biochemistry, 8*(3), 189-213.

Wilson, T. C., & Hagerman, A. E. (1990). Quantitative determination of ellagic acid. *Journal of Agricultural and Food Chemistry, 38*(8), 1678-1683.

Winham, D., Webb, D., & Barr, A. (2008). Beans and good health. *Nutrition Today, 43*(5), 201-209.

World Health Organization. (2001). *The world health report 2001: Mental health: New understanding, new hope* World Health Organization.

World Health Organization. (2014). The 10 leading causes of death in the world, 2000 and 2012. Retrieved from http://www.who.int/mediacentre/factsheets/fs310/en/

Xu, B., & Chang, S. K. (2009). Total phenolic, phenolic acid, anthocyanin, flavan-3-ol, and flavonol profiles and antioxidant properties of pinto and black beans (phaseolus vulgaris L.) as affected by thermal processing. *Journal of Agricultural and Food Chemistry, 57*(11), 4754-4764.

Xu, B., & Chang, S. (2007). A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. *Journal of Food Science, 72*(2), S159-S166.

Xu, B., Yuan, S., & Chang, S. (2007). Comparative analyses of phenolic composition, antioxidant capacity, and color of cool season legumes and other selected food legumes. *Journal of Food Science, 72*(2), S167-S177.

Yao, Y., Cheng, X., Wang, L., Wang, S., & Ren, G. (2011). A determination of potential α-glucosidase inhibitors from azuki beans (vigna angularis). *International Journal of Molecular Sciences, 12*(10), 6445-6451.

Yilmaz, Y., & Toledo, R. T. (2006). Oxygen radical absorbance capacities of grape/wine industry byproducts and effect of solvent type on extraction of grape seed polyphenols. *Journal of Food Composition and Analysis, 19*(1), 41-48.

Zadernowski, R., Naczk, M., & Nesterowicz, J. (2005). Phenolic acid profiles in some small berries. *Journal of Agricultural and Food Chemistry, 53*(6), 2118-2124.

Zahniser, S., & Wells, H. F. (2014). *Vegetables and pulses outlook: Special article commodity highlight: Dry beans.* (No. VGS-354-SA1). Retrieved from http://www.ers.usda.gov/media/1680744/vgs-354-sa1.pdf: Economic Research Service.

Zhao, H., Dong, J., Lu, J., Chen, J., Li, Y., Shan, L., . . . Gu, G. (2006). Effects of extraction solvent mixtures on antioxidant activity evaluation and their extraction capacity and selectivity for free phenolic compounds in barley (hordeum vulgare L.). *Journal of Agricultural and Food Chemistry, 54*(19), 7277-7286.