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CHARACTERIZATION OF EXTRACTION METHODS TO RECOVER PHENOLIC RICH EXTRACTS FROM PINTO BEANS THAT EXERT HIGH ANTIOXIDATIVE ACTIVITIES USING RESPONSE SURFACE APPROACH

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CHARACTERIZATION OF EXTRACTION METHODS TO RECOVER PHENOLIC RICH EXTRACTS FROM PINTO BEANS THAT EXERT HIGH ANTIOXIDATIVE ACTIVITIES USING RESPONSE SURFACE APPROACH

by

Mohammed S. Aldawsari

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May, 2014

CHARACTERIZATION OF EXTRACTION METHODS TO RECOVER PHENOLIC RICH EXTRACTS FROM PINTO BEANS THAT EXERT HIGH ANTIOXIDATIVE ACTIVITIES USING RESPONSE SURFACE APPROACH

Mohammed S. Aldawsari, M.S

University of Nebraska, 2014

Advisor: Vicki Schlegel

The pinto bean has been linked to the prevention of multiple diseases due in large part to the presence of phenolic antioxidants, which are higher in beans than in many fruits and vegetables. These components deliver health properties beyond basic nutritional characteristics by scavenging free oxygen radicals. However, these benefits are most likely due to the ability of these chemically diverse phenols to impart greater protective properties as additives or synergists acting in combination. However, optimal parameters to isolate these compounds (in terms of ratios and types) from a given natural source are not known. Without this knowledge, understanding the responsible components and their possible synergistic / additive / potentiate mechanistic interactions is problematic at best.

Therefore, the *objective of this project*, which is a step toward achieving the long-term goal, was to apply response surface techniques to obtain pinto bean extracts with high total phenols (TP) flavonoids (TF) and antioxidative capacities (AC). This project was completed by using a three factor face centered composite - response surface design

that consisted of adjusting the solvent polarity ratio (organic vs water) of six different solvents (methanol, methanol – HCl, ethanol, ethanol – HCL, acetone, acetone – HCl), while also modifying for mix time and solid / solvent ratio. The most effective factors relative to maximum TF and TP yields were a solvent composition of 50% and solid ratio of 10% and a 60 min mix time, although the solvents were different. 50% Acetone: water without HCL was the most effective for extracting TP, while 50% methanol:water without HCl yielded the highest TPs and AC values. Other solvents produced even higher values, but the data did not fit the models, which could be due to variability in the assays or pinto bean particle size, or that a higher order model is needed. Still, the data suggest that HCl is not needed for these extractions, as it did not aid and often resulted in lower AC and phenolic values. Furthermore, compositional analysis of select samples shows that slight changes in the processing parameters, as well as the solvent used, resulted in different profiles.

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A. LITERATURE REVIEW

A.1 Dry Edible Beans:

Dry edible beans (*Phaseolus vulgaris*) are members of the legume family that consist of several market classes, including black beans, kidney beans, navy beans and pinto beans. Dry beans are grown all over the world with Brazil being the world's leading producer followed by India and China (FAOSTAT, 2011). Figure 1 depicts the overall dry bean production grown by the leading countries in 2011, whereas Table 1 shows the actual amount of beans produced by the top nations. The US world-wide ranking for dry bean production fluctuates between 5th and 6th place depending upon the year. Of the dry beans grown in the US, pinto beans account for the vast majority (FAOUN, 2012).

Dry bean production is scattered across 19 states with North Dakota, Michigan, Nebraska, Minnesota and Idaho being the top producers in terms of total yields. However, Nebraska is the No. 1 producer of great northern beans in the United States, while pinto beans rank between No. 2-3, depending on the year (USDA, 2010-2012). According to the Continuing Survey of Food Intakes by Individuals (CSFII, 2013; Lucier et al., 2000), nearly 14 percent of Americans consume at least one food containing cooked dry beans on any given day with pinto beans being used by nearly 4 percent of the population each day (CSFII, 2013; Lucier et al., 2000). Pinto bean intake is particularly prevalent in the US West and South accounting for approximately 45 percent of the total consumption (Lucier et al., 2000).

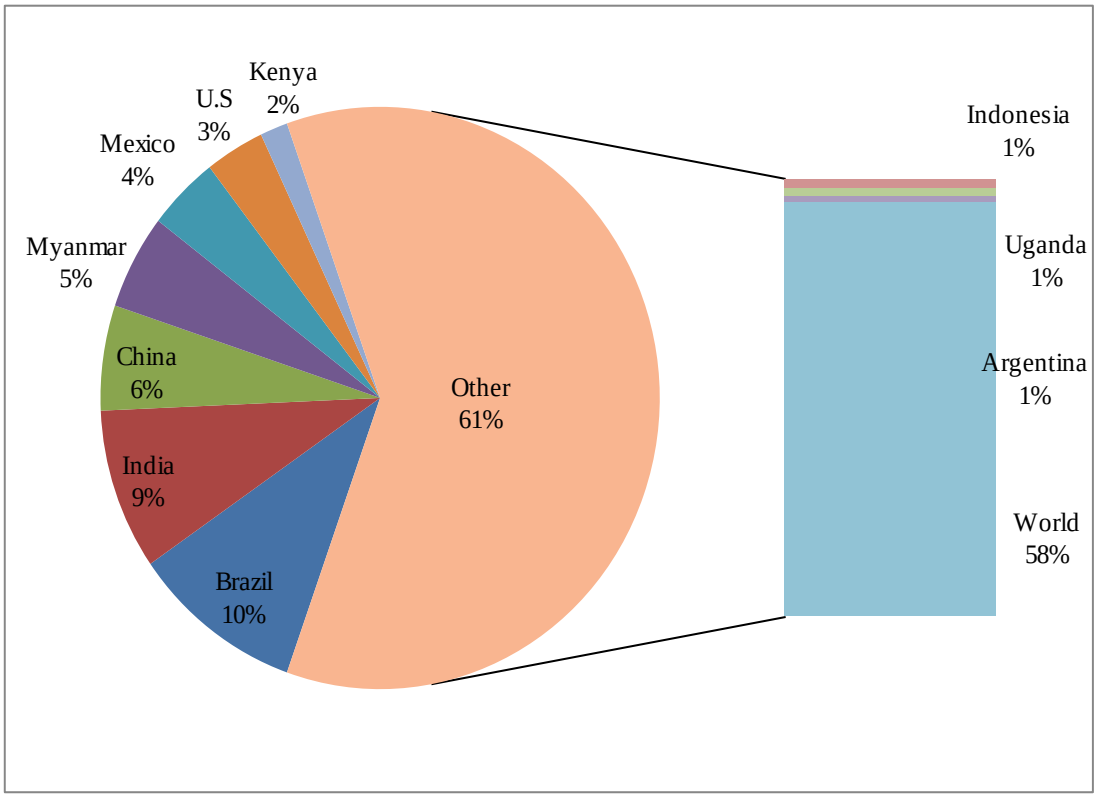


Figure 1: Pie chart showing world wide percentage of dry bean grown in ranking countries. (USDAa Data Base, 2011).

Table 1: Production amounts of top 10 countries (FAOUN, 2011).

Countries	Amount (tons)
India	4,870,000
Brazil	3,202,150
Burma	3,029,800
China	1,538,693
United States	1150808
Mexico	1156250
Tanzania	950,000
Uganda	460000
Kenya	390598
Argentina	338120
World	**23,230,000**

A.2 Basic Dry Bean Composition:

Pinto beans and other common beans have been characterized as a nearly perfect food due to their high levels of protein, essential vitamins, minerals, fiber and complex carbohydrates (Baojunand Chang, 2009) (Table 2). In addition, dry beans contain eight of the nine essential amino acids in relatively high quantities barring methionine (Bressani et al, 1963; FAO, 1957)**.** Dry beans even contain the amino acid, lysine, which is most commonly found in animal based products. As a result, nutritionists have recommended a 2 ounce-equivalent of dry bean consumption for a 1000 calorie diet to a 7 ounce-equivalents for a >/= 2800 calorie diet (USDA, 2012-2014). Due to these high protein levels, dry beans hold a position in the protein group of the USDA "my plate guide" (Sath et al., 1984; Deshpande and Damodaran, 1989).

Furthermore, the fiber content in dry bean has been linked to the lower risks for heart disease and cancer (Table 2) (Rimm et al., 1996; Wolk et al., 1999; Bazzano et al., 2003; Pereira et al., 2004; Eshak et al., 2010). Based on the fiber levels, recommended levels of dry bean consumption is minimally 20-30 gram a day (Prosky et al., 1988). Dry beans are also part of the vegetable group on the USDA "my plate guide" **(**USDA, 2012-2014**).** Additionally, dry beans contain various types of prebiotics, such as resistant starch, and fructooligosaccharides (e.g., stachyose and raffinose) (Reyes-Moreno,et al., 1993; USDA, 2012) (data not shown in Table 2). Research has shown digestion of such carbohydrates positively influence the gastrointestinal tract by enriching for certain bacteria, (e.g., Lactobacillus and Bifobacteria), which in turn have been linked to multiple health benefits (Blaut, 2002; Finley et al., 2007).

Table 2. Basic composition of raw pinto bean (adopted from USDAb)

Nutrient	Unit	Value Per 100 g (Raw)
Main Components		
Water	g	11.33
Energy	kcal	347
Protein	g	21.42
Total lipid (fat)	g	1.23
Carbohydrate	g	62.55
Fiber, total dietary	g	15.5
Sugars, total	g	2.11
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	2.28
Vitamins		
Vitamin C, total ascorbic acid	mg	6.3
Thiamin	mg	0.713
Riboflavin	mg	0.212
Niacin	mg	1.174
Vitamin B-6	mg	0.474
Folate, DFE	mg	525
Vitamin E (alpha-tocopherol)	mg	0.21
Vitamin K (phylloquinone)	mg	5.6
Lipids		
Fatty acids, total saturated	g	0.235
Fatty acids, total monounsaturated	g	0.229
Fatty acids, total polyunsaturated	g	0.407

Dry beans are also contain of a variety of essential minerals, which are difficult to obtain in other food systems, such iron, and calcium. An average serving size (100 g) of beans contains 5.07 mg of iron and 133 mg of calcium (USDA, 2013-2014). Lastly, dry beans also contain multiple other components that are essential for maintaining human nutrition and health, including the B vitamins (thiamine, riboflavin, niacin, B6) and the E vitamin isomers (α, γ, δ tocopherols) (Augustin et al., 1981).

Although beans contain relatively low levels of fat, the predominant triacylglyceride fraction are mainly of mono or polyunsaturated fatty acid chains (Table 2) with the essential lipid, linolenic acid C18:3, accounting for the highest overall percentage (USDA, 1988; Messina, 1999; Schlegel lab, unpublished data). Several studies have shown that C18:3 intake lowers the incidences of cardiovascular disease, cancer, and obesity most likely due to its anti-inflammatory properties (Pandalai et al., 1995; Harper and Jacobson, 2001; Buckley and Howe, 2009).

A.3 <u>Pinto Beans and Phenolic Compounds</u>:

All the dry beans contain the phytochemicals, phenolic acids and flavonoids, but the amount and type differ between market classes (Reynoso-Camacho et al., 2006). As secondary metabolites are ubiquitous throughout the plant kingdom, phenols perform various endogenous functions but primarily protect the plant from environmental stressors, such as pathogens and insect pressure, via their potent antioxidative properties (Wildman, 2006). Phenolic compounds are categorized into different classes due to their complex chemical diversity (Figure 2), but are all tied together by one or more phenol groups in their structural backbone (Figure 3). For example, phenolic acids contain one

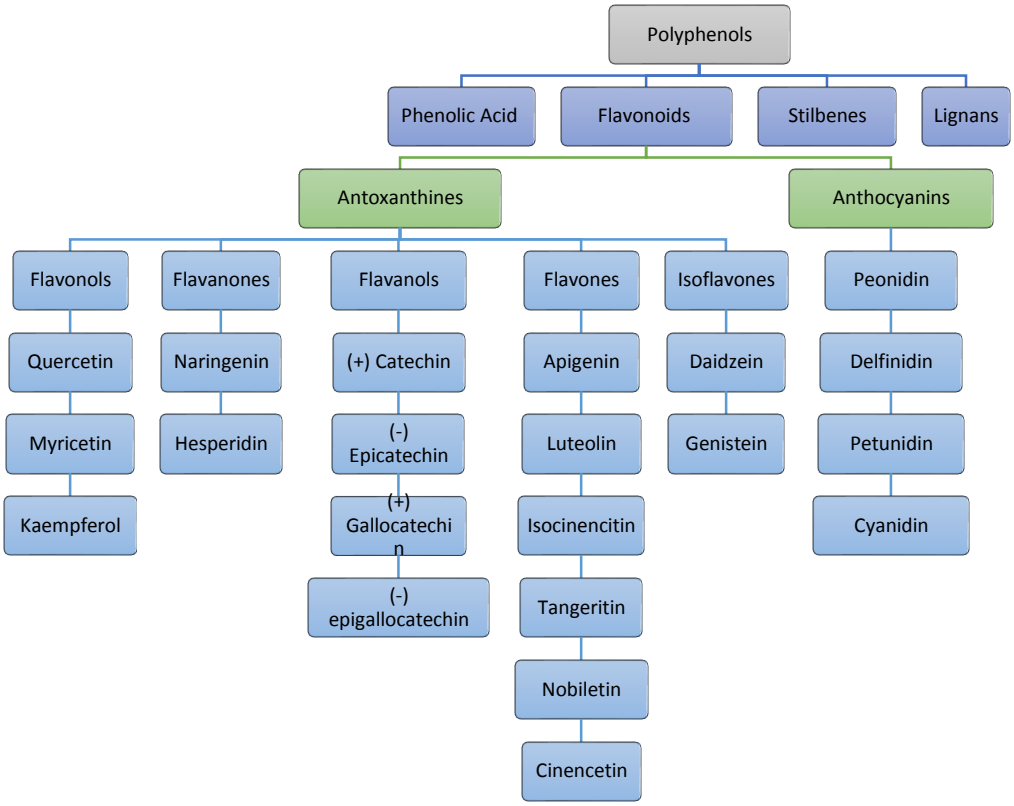


Figure 2: Classification of the main polyphenols .(Robards, 1999; Morton et al., 2000; Aherne and O'Brien, 2002; Tsao, 2010).

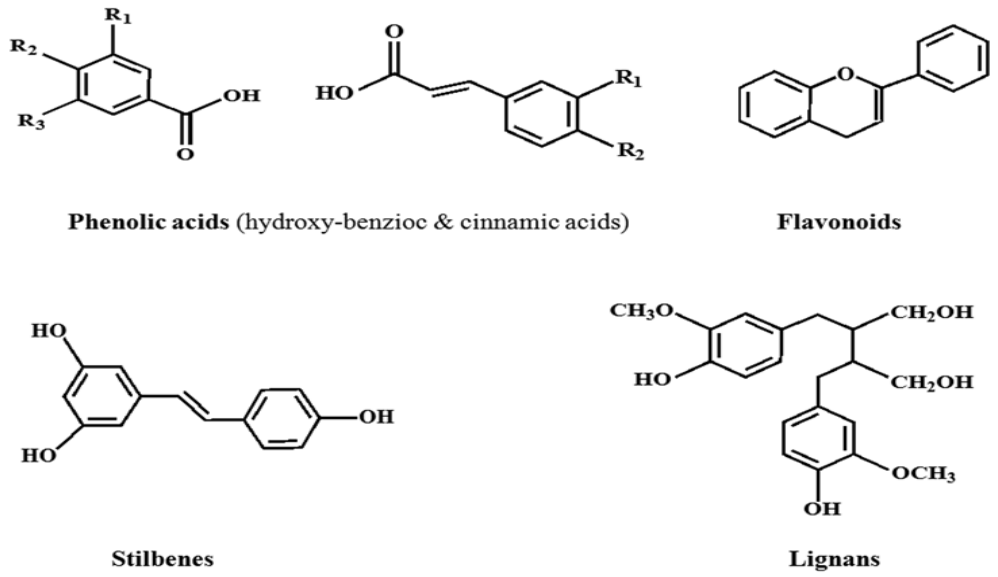


Figure 3: Chemical structures of the different classes of polyphenols. (Adapted from Pandey et al., 2009).

aromatic ring, a carboxyl acid group and one or more hydroxyl groups. On the other hand, flavonoids are unique phenols in that each contains three heterocyclic rings in their backbone, but are further separated into different classes based upon the position of the rings relative to one another, their degree of conjugation or the presence / position of their hydroxyl groups (Figure 3) (Shahidi and Naczk, 1995). Of the phenols, the flavonoids are considered to be particularly potent antioxidants, more specifically the anthocyanins and tannins (Beecher, 2003). Anthocyanins are known for their red, blue or purple color depending on the pH, whereas condensed tannins are basically polymers of anthocyanins.

Pinto beans contain phenols at levels higher than most other types of legumes, or at comparable or higher amounts than other types of bean market classes, depending on the extraction methods used (Tables 3-5) (Wu et al., 2004; Luthria and Pastor-Corrales, 2006). From a compositional perspective, pinto beans contain tannins and other flavonoids that belong to the anthoxanthins sub-category (flavonols, flavanones, flavanols, or isoflavones). More specifically, the primary phenolic acids in pinto beans are ferulic acid, gallic acid, p-coumeric acid and sinapic acid (Table 4 and Figure 4) (Luthria & Pastor-Corrales, 2006). Relative to the flavonoids, pinto beans contain keampferol 3-O-glucoside and kaempferol 3-O-acetyl glucoside (Figure 4) (Xu and Chang, 2009). Furthermore, pinto beans are composed of other non-flavonoid polyphenols, such as stilbenes and lignans (Figure 3).

A.4 Phenols and Oxidative Stress:

Free radicals and other reactive oxygen or nitrogen species (RS) (Table 6) generated during oxidative stress as by-products of normal cellular metabolism, or by

Table 3. Phenolic content of pinto beans vs other select legumes.

Natural System	Total Phenol (mg/g)	Total Flavonoid (mg/g)	Condensed Tannin (mg/g)	Anthocyanin (μg/g)
Pinto Bean	3.76 ± 0.06	2.99 ± 0.12	3.23 ± 0.11	6.70. ± 06
Chickpea	0.98 ± 0.01	0.72 ± 0.02	0.52 ± 0.02	ND*
Green pea	0.65 ± 0.04	0.14 ± 0.01	0.24 ± 0.00	ND
Yellow pea	1.14 ± 0.03	0.10 ± 0.01	0.29 ± 0.01	ND

*Not Detected (ND). Results are shown as the mean +/- standard deviation (n=3) weight basis. The varieties of cultivars conducted on this study listed respectively as shown in the table (Phaseolus vulgaris L., Cicer arietinum L., Pisum sativum L., Pisum sativum L.) .The legume flour (0.5 g each) were extracted with different solvent ratio, acetone/water (50:50, v/v) extraction solvent were added to peas, chickpeas; acetone/water/acetic acid (70:29.5:0.5, v/v/v) extraction solvent were added to pinto bean. The mixture were extracted for 3 hours under horizontal shake for 300 rpm follow by another 12 hours of overnight incubation. The volume of the extract was 5 mL. Total phenol and Flavonoid determined using a colorimetric method. (Xu et al., 2007)

Table 4. Phenolic acid content for different market clases dry beans.

Bean Market Class	Phenolic acid concentration (mg/100 g)				Total phenolic acid content (mg/100 g)
	Caffeic acid	*P*coumaric acid	Ferulic acid	Sinapic acid	
Pinto	ND*	4.86	18.03	7.8	30.69
Great Northern	ND	5.15	17.1	9.2	31.45
Navy	ND	12.4	26.6	9.2	48.2
Black	1.1	9.42	20.62	7.2	37.25
Dark Red Kidney	ND	1.8	15.3	3.8	20.9
Pink	ND	6.8	19.4	8.2	34.4

Not Detected (ND) * Results are shown as the average of different cultivars. (Ground beans were with MeOH containing 0.2% TBH (2,3-tertbutyl- 4-hydroxy anisole) and 10% acetic acid (85:15). The mixture was sonicated for 30 min and the volume of the extract was adjusted to 10 mL with distill water. Individual phenolic acids were quantitated via C18 HPLC (Luthria and Pastor-Corrales, 2006).

Table 5. Phenolic acid content for different market classes

Bean Market Class	Total phenolic content (mg/100 g)*
Pinto	41.99
Black	33.84
Dark Red Kidney	31.13

Summation of individual phenolic acid as detected by C18 HPLC.
Ground samples were combined with 2 N NaOH with 13.4 mM EDTA and 2% ascorbic acid) was added to 0.2 g of ground sample. The mixture was flushed with nitrogen and allowed to hydrolyse for 30 min at 40–45_C. After 30 min, the sample was allowed to cool and the reaction mixture was acidified by adding 1.4 mL of 7.2 N HCl. The liberated phenolic acids were extracted with diethyl ether / ethyl acetate at 1:1 (v/v) ratio (1:1, v/v). (Ross and Beta, 2009)

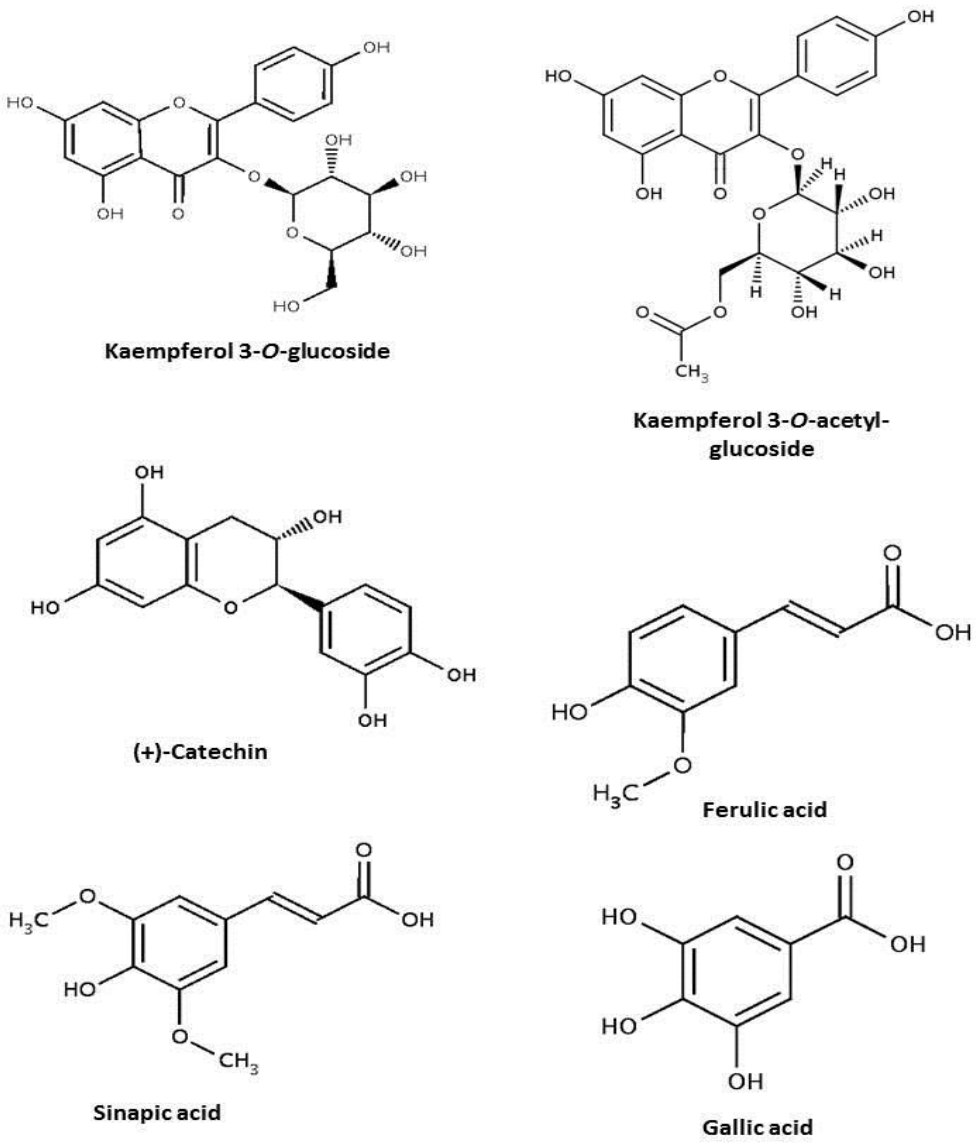


Figure 4: Main polyphenolic compounds presents in pinto beans (from CHEBI database).

Table 6: List of reactive molecules present in the cells and can cause oxidative stress, if left unchecked.

Estimated half-life of ROS

ROI		Half-life (sec)
$HO^{\cdot}$	Hydroxyl radical	10^{-9}
$RO^{\cdot}$	Alkoxyl radical	10^{-6}
$ROO^{\cdot}$	Peroxyl radical	$7 - 10^{-2}$
H_2O_2	Hydrogen peroxide	enzymatic
$O_2^{\cdot -}$	Superoxide anion	10^{-6}
$NO^{\cdot}$	Nitric oxide	1-10
$ONOO^-$	Peroxynitrite	0.05 - 1

(Modified from Yu BP (1994) Physiol. Rev. 74: 139)

environmental exposures including, (radiation, smoking, diet, aging), may damage multiple cellular molecules, including proteins, fatty acids, and deoxynucleotides if not kept in check (Finkel and Holbrook, 2000; Paracha et al., 2013). Major damage to any of these components can cause cellular dysfunction leading to several serious diseases (Barnham et al., 2004; Halliwell, 2007; Madamanchi et al., 2005; Sun and Chen, 1998; Mate´ et al., 1999). To protect against cellular oxidation imbalances, several protective enzymes are produced, (e.g., superoxide dismutase (SOD) and catalase (CAT)), which are able to deactivate RS, (e.g., superoxide and hydrogen peroxide) (Mate´, 2000) (Figure 5). Alternatively, oxidation enzymes catalyze RS formation (Figure 5). For example, xanthine oxidase catalyzes the formation of superoxides by reacting with oxygen or xanthine (Yahaya and Don, 2012). Lipid peroxyl radicals and superoxides can be formed when lipoxygenase reacts with oxygen and a lipid, such arachidonic acid (Figure 6) (Catalano and Procopio, 2005).

Phenols can protect against oxidative stress by either scavenging free radicals or inhibiting / activating redox related enzymes (Luigi Casella, 2006). Relative to the former method, which is the scope of this study, phenolic compounds are able to donate hydrogen atoms to rapidly stabilize the free radicals. The shift of the charge in the aromatic ring then stabilizes the phenol compound (Leja et al.,2007; Kurek-Górecka et al., 2013). The position, as well as the quantity, of the hydroxyl groups on the phenolic groups allows certain phenols to be more potent scavengers than others. The flavonoids have been identified as strong antioxidants, particularly the anthocyanins, due to their ability to stop the oxidative chain reaction of lipid peroxidation (Narayansingh and Hurta , 2009).

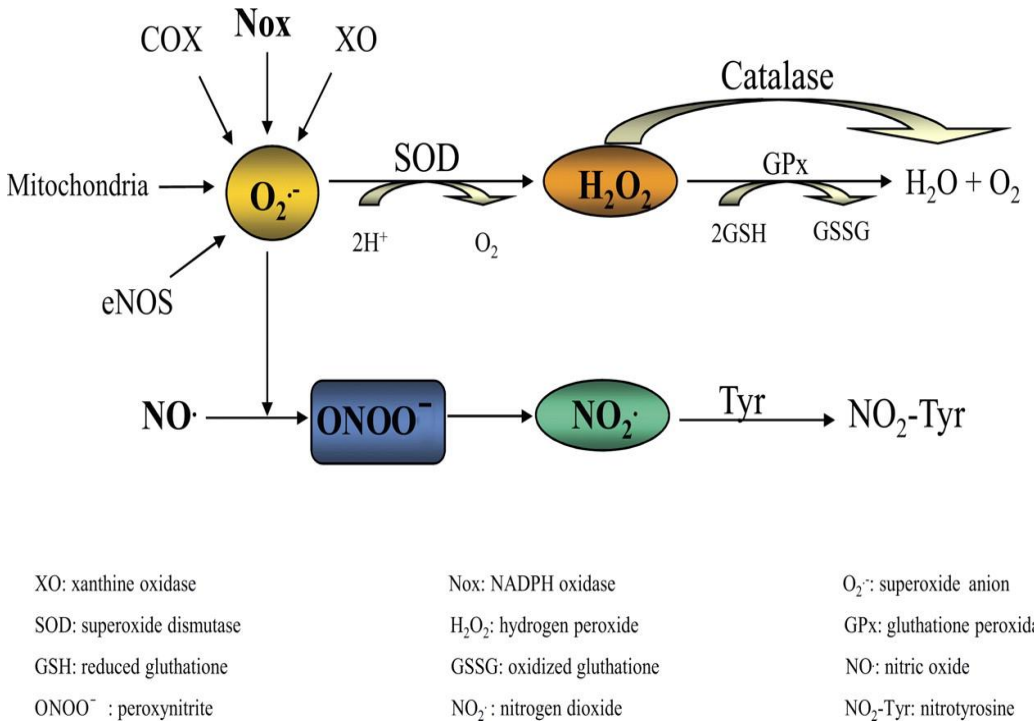


Figure 5: Schematic showing oxidative enzymes and the reactive molecules produced and their conversion to other species through antioxidative enzymes.

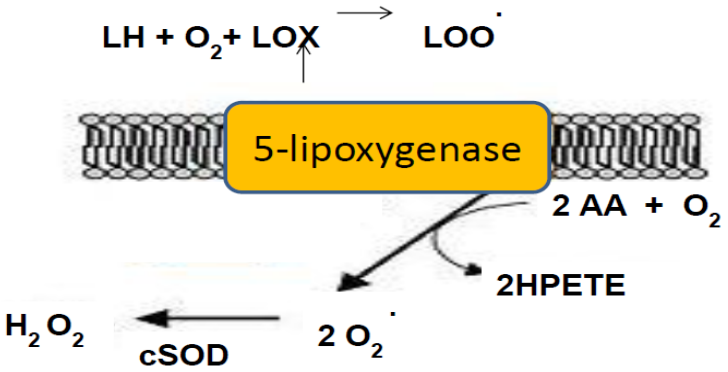


Figure 6: Schematic showing the oxidative enzyme 5-lipoxygenase and the reactive molecules produced.

Reports have shown that pinto beans have the highest antioxidant capacity compared to other types of beans analyzed (Xu and Chang, 2012). For example, the total concentration of phenolics in the seed coats of pinto beans was higher than that detected in other beans, which in turn positively correlated with antioxidant activities (Anton et al., 2008). After thermal processing of several types of legumes, pinto beans and black beans contained the highest total phenolic content and exhibited the highest antioxidant capacity as measured by the diphenylpicrylhydrazyl (DPPH) method (Rocha-Guzman, 2013). Moreover, Change et al. (1993) and others showed that flavonoids isolated from *Bougainvillea spectabillis* Wild had strong activity and induced competitive inhibition of the xanthine enzyme (Chang et al., 1993). Nagao et al. (1999) determined that quercetin and other flavonoids inhibited the xanthine oxidase activity at low concentrations (Nagao et al., 1999). Sadik et al. (2003) also demonstrated that flavonoids combine both lipoxygenase-inhibitory activities and free radical-scavenging properties in one agent. Toyokuni et al. (2003) reported that total phenols and flavonoids increased the activities of Cu, Zn-SOD enzymes (superoxide dismutase), which inhibit oxidative stress.

These beneficial properties may result from different interactions or combinations of the chemically diverse phenols that impart greater protective properties on one biological response relative to another. As such, different types and ratios of the phenols may be responsible for a given health promoting properties, which together may act as synergists, additives or potentiates. The optimal parameters to isolate these compounds (quantities, types, and ratios) relative to a given natural system and their overall oxidative protective benefits as a whole food are not known. This lack of knowledge impedes our

ability to produce consistently safe and efficacious pinto beans targeted at specific cellular stressor diseases.

A.5 Response Surface Method (RSM):

Analyte extraction efficiency can be significantly influenced by a number of factors, such as solvent composition, matrix composition, extraction time, extraction temperature, solvent to solid ratio, extraction pressure, and particle size, to name a few (Wettasinghe & Shahidid, 1999; Cacace and Mazza, 2003a). Characterizing certain extracts based on their chemical and biological responses is limited by the traditional one factor at a time approach due to the time requirements needed, potential interactions between variables can be ignored. Considering the chemical diversity of phenolic compounds, however, an interactive influence among the variables is expected. Thus, to obtain extracts that are either chemically diverse or exert a potent biological response, (and mostly likely both), and to ultimately understand the phenolic composition of the product as a whole, a more comprehensive extraction approach must be applying and characterized.

Response surface methodology (RSM) is a modeling strategy that allows the optimization of a given response, such as obtaining an extract with the highest levels of phenols, based on evaluation of several different experimental parameters concurrently (Myers and Montomery, 2002). Moreover, RSM is based on a statistical approach that is able to generate informative results using small sample sizes, which in turn can be used to build a model for predicting the optimal, but combined experimental parameters. RSM has many advantages, such as providing information to characterize interactions between multiple processes, determining kinetic constants, and investigating enzyme stability and

kinetics (Cheynier et al., 1983). To easily visualize the output from the RSM, the response can be represented graphically by three dimensional space or contour plots. With respect to extracting phenols from natural systems, RSM has been applied to wheat (Chandrika and Shahidia, 2005), fruits of Euterpe oleracea (Pompeu et al, 2009), and peanut skins (Ballard et al, 2009). The response surface has not been used to characterize phenolic compounds in pinto beans in terms of amount or links to antioxidative benefits. This information is needed to ultimately understand the phenolic composition in the whole bean as affected by environmental/genetic effectors, and to identify the components responsible for eliciting a given health benefit.

B. OBJECTIVE and SPECIFIC AIMs:

The *objective of this project* was to determine the optimal parameters to recover phenolic rich antioxidants from pinto beans (PB) using response surface methods (RSM), which was fulfilled by completing the following Specific Aims.

Specific Aim 1: *To determine the optimal procedures to recover phenolic / flavonoid rich PB antioxidative extracts with 6 solvent systems (methanol, ethanol, acetone +/- HCl) by using a RSM approach that incorporated a three factor cube centered strategy for solvent polarity, mixing time, and solid/solvent ratio.*

Specific Aim 2: *To determine predictive equations from the RSM data (Specific Aim 1) and then to determine optimal levels.*

Specific Aim 3: *To characterize the phenolic compositional profiles of the extracts that contained the highest levels of phenols / flavonoids (Specific Aim 1) and/or exhibited the greatest anti-oxidative responses (Specific Aim 2).*

C. MATERIALS and METHODS

C.1 Chemicals and Reagents:

Extraction solvents, including methanol, ethanol, and acetone were purchased from Fisher Scientific Co. (Fair Lawn, New Jersey). Other reagents used for the study that were provided by Fisher Scientific included sodium carbonate, sodium nitrite, and potassium phosphate. Other reagents were purchased from various vendors, including Folin-Ciocalteu (MP Biomedical Inc.; Solon, OH), aluminum chloride (Acros Organics Inc.; Fair Lawn, NJ), sodium hydroxide (BD, West Chester, PA), Fluorescein (J.T. Baker: Center Valley, PA), and 2-2′-azobis (2-amino-propane) dihydrochloride (AAPH) (Sigma-Aldrich., ST. Louis, MO). The standards used for the phenolic (gallic acid), flavonoid (catechins) and oxygen radical absorbance capacity (Trolox) assays were obtained from Sigma-Aldrich, ST. Louis, MO.

C.2 Sample Information:

Pinto beans (LaPaz cultivar, 2009) were provided as a generous gift from Dr. Carlos Urrea (University of Nebraska Panhandle Research and Extension Center). Upon arrival, the beans were maintained at -20 ^{o}C until prepared for analysis.

C.3 Extraction Procedures:

The beans were homogenized into a fine ground powder with an electric grinder. The effects of six different solvent systems (methanol, ethanol, and acetone +/- 1.2 N HCl) on phenols, flavonoids and antioxidative capacity were monitored while also adjusting for solvent polarity ratio (25-75%), solid / solvent ratio (10-30%), and mix time (60-180 min). The optimization procedures were based on a response surface design (three factor), i.e., a three-factor-three-level face-centered cube design. The cited solvent

systems and other parameters were selected because multiple studies have used combinations of these conditions, but as single factor extractions, to obtain phenols from various natural systems (Karacabey and Mazza 2010; Silvaet al 2007; Liyana-Pathirana and Fereidoon Shahidi , 2004). For this study, the solid amounts were adjusted accordingly to maintain a 3-5 ml final extraction volume. The suspension was mixed horizontally under steady rocking for the designated time period at room temperature. The samples were then centrifuged at 8000 rpm at 25 C for at least 10 minutes and supernatant collected. The supernatant was collected and analyzed for total phenols, flavonoids, and antioxidant capacity. Each extraction was performed in triplicate.

C.4 Total Phenolic Assay:

The Folin-Ciocalteu method was used to determine total phenol levels in the collected supernatant as described by Singleton and Rossi, **(**1965). Briefly, a sample aliquot (100 µL) was reacted with 100 µL of Folin-Ciocalteu reagent and 4.5 mL of nanopure water. After 3 minutes of shaking at room temperature, 0.3 mL of 2% (w/v) sodium carbonate was added to the samples followed by a reaction time of 2 hours at room temperature with intermittent shaking. Detection of the phenols was achieved with a UV-Vis spectrometer (Beckman Coulter, Brea, CA) at a wavelength of 760 nm. A standard calibration curve using gallic acid was plotted to calculate the results. Total phenols were thus expressed in mg *gallic acid* / g pinto bean powder.

C.5 Total Flavonoid Assay:

To quantify total flavonoids, 125 µL of the sample supernatant prepared was combined with 37.5 µL of 5% (w/v) sodium nitrite and 0.625 mL of nanopure water according to (Adom and Liu, 2002). After allowing the reagent to react with the sample

for 4-6 minutes at room temperature, 75 µL of 10% (w/v) aluminum chloride was added to each sample, followed by 0.25 mL of 1.0 M sodium hydroxide. Nanopure water (0.4 mL) was added after allowing the sample to mix for 5-7 minutes. The mixture was vortexed and then measured by photometric detection at 510 nm. Total flavonoids were expressed as mg *catechin* / g pinto bean powder.

C.6 Oxygen Radical Absorbance Capacity:

To measure antioxidant capacity, the oxygen radical absorbance capacity (ORAC) was completed as described by Huang et al. (2002). A standard stock solution was prepared by dissolving 0.010 g of Trolox (a water derivative of Vitamin E) in 10 mL of 75 mM potassium phosphate buffer, pH 7.4. Standard dilution concentrations were prepared that that ranged from 0.46–62.50 µg/mL. Fluorescein (8.16×10^{-5} mM) was incubated with the diluted standards and test samples for 10 minutes. The reaction was then activated by adding the radical initiator, 153 mM 2, 2'-azobis (2-amidinopropane) hydrochloride to generate peroxyl radicals. All samples/standards were prepared in 96 well plates and monitored with a fluorescent microplate reader (BMG LABTECH GmbH, Offenburg, Germany). Fluorescence was measured every 1.5 minutes at excitation and emission wavelength of 485 nm and 520 nm, respectively, until the values plateaued. The area under the curve (AUC) and Net AUC were calculated to plot Net AUC vs. Trolox (µg/mL) calibration curves. The results were expressed as µmol Trolox / g pinto bean flour.

C.7 Composition Analysis of Select Extracts:

Phenolic compositional analyses were completed on select samples by initially passing the extracts through a 0.45 µm filter before injection onto 5 µm, 4.6 mm x 250

mm C18 reversed phase column (Vydac, Deerfield, IL). Separation was achieved using a Waters 600 HPLC system set at a flow rate of 1.0 ml/min and gradient elution (t = 0 min 100% A, t = 4min 92% A and 8% B, t = 10 min 14% B and 86% C, t = 22.5 min 16.5% B and 83.5% C, t = 27.5 min 25% B and 75% C, t = 50 min 80% B and 20% C, t = 55 min 100% A and t = 60 min 100% A) with 50 mM ammonium phosphate at pH 2.6 (A), 80:20 (v:v) acetonitrile, 50 mM ammonium phosphate at pH 2.6 (B) and 200 mM phosphoric acid at pH 1.5 (C) serving as the mobile phases. Detection of the phenols and flavonoids was accomplished with a Waters 2996 UV-Vis detector at 280 nm. Samples were identified and quantitated using external standards.

C.8 Experimental Design for RSM Analysis:

A three-factor and three level face center central composite design consisting of 16 extracts for total phenols, flavonoids, and antioxidative capacity was employed including two replicates at the center point (Table 7). The experimental design (coded and uncoded) is presented in Table 8. The behavior of each solvent system was explained by a second degree polynomial equation, as shown below:

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

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

Table 7: Levels of independent variables for extraction process based on central composite face centered design.

Independent Variable	Units	Factor	Coded Levels		
			-1	0	+ 1
Organic Solvent : Water	(v:v)	X_1	25:75	50:50	75:25
Solid : Volume	(%)	X_2	10%	20%	30%
Time	(min)	X_3	60	120	180

*** Methanol, Ethanol, or Acetone +/- HCl to Water Ratio**

Table 8: Three factor, three- level face-centered cube design with 2 center points used for RSM, (coded and uncoded) parameters.

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

C.9 Statistical Analysis and Verification of the Model:

All determinations were carried out in triplicate and the experimental results were expressed as the mean +/- SD. The statistical analysis was performed using StatsGraph Centerium (version 26, Warrenton, VA). The experimental data were analyzed by multiple regression analysis through the least squares method. Two different tests, i.e., the sequential sum of the squares and model summary statistics, were carried out on the experimental data in order to determine the adequacy of various models. The model and the regression coefficients involved in the model and their effect were analyzed by Pareto ANOVA and were considering significant at $p < 0.10$. The fitness of the regression curve was further evaluated by determining by the correlation coefficient for the model R^2 (>75), whereas the ability of the model to fit the experimental data was assessed by a lack of fit test ($p>0.05$).

C.10 Response Surface Curves:

Regression equations were formulated based on whether the data obtained from each solvent system complied with the criteria stated in C.9. Response surface plots based on these "accepted" equations were generated with R2 software (version 3.0.2, Kurt Hornik, 2013) by using the function of two factors and keeping the other constant.

D. RESULTS and DISCUSSION

D.1 Selection of Independent Variables:

Solid–liquid extractions use a solvent to remove a soluble fraction from an insoluble, but permeable solid. For bioactive compounds, such as the 8000 + phenols present in any given natural system, knowledge of the factors influencing their extraction is needed to ultimately link the responsible components to the health benefit. Efficient extraction of the bio-active compounds is mainly dependent on the type of phenolics present, the solvent system used and the ability of the bioactive agents to access that solvent. As this information has not been provided in the literature for pinto beans, a wide range of solvents were selected for this study that included methanol, ethanol, and acetone. A minimum of 25% water was added to each to an upper range of 75% as studies have shown that water promotes the solubility of phenolic compounds (Rostanogo et al., 2004). Similar experiments were conducted with the methanol, ethanol, and acetone at the different solvent to water ratios, but HCl was also added to each to facilitate cell rupture and bond breakage thereby allowing access of the solvent to phenols distributed close to cell wall (Pompeu, et al.; 2009 Kim et al., 2004; Ju and Howard, 2003).

Equilibrium and mass transfer rate are the two fundamental concepts that govern the extraction process (Cacace and Mazza, 2002). Although solvent composition is an important factor in achieving both equilibrium and mass transfer, (i.e., the rate that the compound dissolves and reaches equilibrium in the solvent), other influential factors include temperature, solid to liquid ratio, mixing time, and the type of mixing (Azizah et al., 1999; Wettasinghe and Shahidi, 1999; Pinelo et al., 2005). For the purposes of these

studies, solid to liquid ratio (10-30 (w/v)) and mixing time (60-180 min) were evaluated. Mixing was achieved with a rocker to ensure steady, yet full contact with the bean particles. Although elevated temperatures have shown increased phenolic yields, (Liyana-Pathiran and Shahidi, 2005; Spigno et al., 2007; Pompeu et al., 2009) **,** this factor was not applied for this study so as to prevent as much as possible phenolic degradation, considering that a biological response (antioxidative capacity) was being studied as well. Total phenols, and total flavonoids were also measure in response to the cited extraction process as a means to understand the phenolic composition of pinto beans and their interaction with typical solvent systems.

D.2 Total Phenols:

D.2.1. *Total phenol results obtained from FCCC-RSM:* Total phenolics (TP) levels for each solvent system (methanol, ethanol, and acetone +/- 1.2 N HCl) were determined using a face centered central composite (FCCC) design. Adjusting for three factors, (solvent: water ratio, solid: solvent ratio, and mix time), 16 different extractions were completed, two of which included the center points (Table 7). The results obtained for each solvent, without and with HCl, are shown in Tables 9 and 10, respectively, and are expressed as the mean +/- standard deviation of three replicates. From these results, the range in TP levels was also determined (Table 11). Total phenols extraction efficiencies were the greatest for both the acetone systems, and the lowest for the methanol, with a difference of ~ 2 mg/g. However, methanol and ethanol yielded similar levels with or without HCl, suggesting that acidified solvents are not needed for the extraction of pinto beans, at least for these two systems. Many studies have used either methanol, ethanol, or acetone to extract the phenolics from vegetables and fruits (Hertog et al.,

Table 9: Experimental data for total phenolic response (in mg/g) of pinto beans extracts under different extraction conditions and solvent systems.

Standard Order	Methanol	Ethanol	Acetone
1	0.29 ± 0.03	0.45 ± 0.06	0.29 ± 0.01
2	0.67 ± 0.03	1.01 ± 0.18	1.64 ± 0.09
3	0.56 ± 0.03	0.53 ± 0.05	0.46 ± 0.09
4	0.98 ± 0.03	1.02 ± 0.15	1.55 ± 0.09
5	0.80 ± 0.03	1.06 ± 0.03	1.93 ± 0.03
6	1.19 ± 0.02	0.56 ± 0.01	2.39 ± 0.01
7	0.93 ± 0.12	0.94 ± 0.02	1.25 ± 0.04
8	1.02 ± 0.04	1.31 ± 0.03	1.74± 0.08
9	0.56 ± 0.03	0.59 ± 0.02	1.14 ± 0.02
10	0.31 ± 0.05	0.29 ± 0.02c	0.91 ± 0.01
11	0.38 ± 0.09	0.73 ± 0.02	1.42 ± 0.02
12	1.08 ± 0.12	0.58 ± 0.03	0.75 ± 0.02
13	0.94 ± 0.03	1.28 ± 0.09	1.63 ± 0.02
14	1.14 ± 0.45	0.82 ± 0.03	1.08 ± 0.07
15	0.23 ± 0.02	0.33 ± 0.03	0.86 ± 0.01
16	0.50 ± 0.14	0.65 ± 0.06	0.42 ± 0.01

* Data are shown as the mean ± standard deviation.

Table 10: Experimental data for total phenolic response (in mg/g) of pinto beans extracts under different extraction conditions and solvent systems.

Standard Order	Methanol + HCl	Ethanol + HCl	Acetone + HCl
1	0.67 ± 0.03	1.37 ± 0.04	0.25 ± 0.02
2	0.51 ± 0.09	1.03 ± 0.19	0.24 ± 0.01
3	1.34 ± 0.06	1.86 ± 0.23	0.22 ± 0.01
4	0.91 ± 0.03	1.21 ± 0.04	0.23 ± 0.00
5	0.77 ± 0.01	0.90 ± 0.01	0.27 ± 0.01
6	1.25 ± 0.03	1.65 ± 0.07	0.45 ± 0.01
7	1.11 ± 0.03	0.83 ± 0.10	0.84 ± 0.00
8	1.03 ± 0.11	1.13 ± 0.02	0.86 ± 0.05
9	0.69 ± 0.02	0.47 ± 0.10	0.24 ± 0.01
10	0.74 ± 0.03	1.03 ± 0.03	1.17 ± 0.04
11	0.85 ± 0.01	1.28 ± 0.14	0.72 ± 0.06
12	0.45 ± 0.07	0.79 ± 0.05	0.25 ± 0.00
13	0.88 ± 0.04	1.13 ± 0.02	0.94 ± 0.02
14	0.56 ± 0.03	1.40 ± 0.02	1.92 ± 0.11
15	0.19 ± 0.02	0.88 ± 0.18	0.53 ± 0.02
16	0.96 ± 0.16	1.60 ± 0.12	2.14 ± 0.03

* Data are shown as the mean ± standard deviation.

Table 11: Ranges of total phenols for each solvent system.

Extraction Solvent	High and Low Total Phenols (mg/g)	Range (mg/g)
Methanol	0.23 – 1.19	0.96
Ethanol	0.29 – 1.31	1.02
Acetone	0.29 – 2.39	2.1
Methanol + HCl	0.19 – 1.34	1.15
Ethanol + HCl	0.47 – 1.86	1.39
Acetone + HCl	0.22 – 2.14	1.92

Sun & Ho, 2005; Xu and Chang, 2007), but very few have applied RSM to determine an appropriate solvent system for the food being studied in terms of total phenol levels. A preliminary study was performed by Liyana-Pathirana and Shahidi (2005), using antioxidant capacity as the response, to select an appropriate extraction medium. For each of three solvent systems (ethanol, methanol, and acetone), the researchers varied the range from 0-100% (v/v) with water (0-100%) in each of the solvents while maintaining the other extraction parameters, such as mix time, solid:solvent ratio, temperature, etc. As the highest antioxidative values were obtained with ethanol containing 50% water, the RSM experiments were conducted with only this solvent but varying the water ratio between 30-70%. Ballard et al. (2009) studied the extraction efficiencies of ethanol and methanol on total phenolics from peanut skins using RSM. Adjusting for solvent:water composition, temperature, and mix time, these researchers obtained the highest TP yields with aqueous ethanol. 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 reported using acetone (Eberhardt et al., 2000; Nakatani et al., 2000). It must be noted that the addition of HCl to any of the solvents provided no or only minimal improvement in the TP yields (Table 11).

D.2.2. *Fitting the TP model*: Although acetone showed optimal TP yields for pinto beans, the next step was to assure that the results fit the quadratic model. Therefore, multiple regression coefficients were determined by applying a least squares technique to the results obtained for each solvent system (Table 12 and 13). (The coefficients are related to coded values). Analysis of variance (ANOVA) of the quadratic model for the solvents without HCl showed a significant p value < 0.05 for the acetone only, while p values of 0.1489 and 0.1471 were obtained for methanol and ethanol, respectively (Table 12). Additionally, the R^2 value was high for acetone (96.4) indicating that most of the variability could be explained, which further supports the adequacy of this model for actone based TP extractions from pinto beans. According to Le, Behera and Park (2010) and Chauhan and Gupta (2004), an $R^2 > 75$ is sufficient to accept a model. On the other hand, the model fit for acetone + HCl was questionable. Even with an R^2 value of 80.3, the corresponding model p value was > 0.10. Conversely, ANOVA of the models for the methanol and ethanol + HCl extractions generated p values of <0.05 and R^2 above 90.

D.2.3. *Adequacy of the TP models*: Next, the ability of each model to fit experimental data was determined to provide assurance of obtaining predictable results. The model's adequacy was evaluated by comparing the differences between the residuals of the current model with that of observed data (Maren et al. 2013), which indicates the "lack of fit". If the model residuals correspond to that of the experimental a p value > 0.05 is expected. Methanol, ethanol, and acetone without HCl, and ethanol with HCL passed this test as the corresponding p values were all greater than 0.05. Alternatively,

Table 12: Regression coefficients (coded) predicted by the quadratic polynomial model for phenols when extracted with the cited solvent systems.

Coefficient	Methanol	Ethanol	Acetone
b_o	0.987	1.11	1.72
Linear			
b_1 (SP)	-0.214*	-0.095	-0.270^
b_2 (S:S)	-0.051	-0.011	-0.035
b_3 (MT)	-0.036	0.079	0.020
Quadratic			
b_{11} (SP x SP)	-0.271	-0.380*	-1.07^
b_{22} (S:S x S:S)	0.007	-0.217	0.406^
b_{33} (MT x MT)	-0.159	-0.006	-0.160
Cross product			
b_{12} (SP x S:S)	-0.111	-0.063	0.210*
b_{13} (SP x MT)	-0.028	-0.062	-0.037
b_{23} (S:S x MT)	-0.148	-0.090	0.070
R^2	78.3	78.4	96.4
p values			
Model	0.1489	0.1471	0.0011
Lack of Fit	0.1334	0.0515	0.3030

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

Table 13: Regression coefficients (coded) predicted by the quadratic polynomial model for phenols when extracted with the cited solvent systems.

Coefficient	Methanol + HCl	Ethanol + HCl	Acetone + HCl
b_o	0.854	1.2528	0.951
Linear			
b_1 (SP)	0.044	0.201^	0.198
b_2 (S:S)	-0.265^	-0.348^	-0.228
b_3 (MT)	-0.061	-0.154*	0.100
Quadratic			
b_{11} (SP x SP)	-0.184**	0.083	1.129*
b_{22} (S:S x S:S)	0.209*	-0.032	-0.607
b_{33} (MT x MT)	-0.856	-0.187	-0.738
Cross product			
b_{12} (SP x S:S)	-0.083	-0.096	0.013
b_{13} (SP x MT)	-0.169*	-0.022	0.107
b_{23} (S:S x MT)	-0.036	0.019	-0.373
R^2	92.4	92.1	80.3
p values			
Model	0.0096	0.0109	0.1916
Lack of Fit	0.5251	0.0064	0.0837

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

the test showed a lack of fit for ethanol and acetone + HCl with p value of 0.5251 and 0.0837, respectively.

D.2.4. *Regression coefficients equations and Pareto charts*: Based on the criteria for accepting a model, as described in the Material and Methods section, (Section C.9), TP regression equations for only the acetone and methanol + HCL were provided (Table 14). These equations are based on the significance of individual regression coefficients only ($p < 0.10$) (Table 12 and 13). However, Pareto charts were also shown to schematically show the overall contribution of each coefficient (Figure 7).

Comparison of both charts clearly shows that TP extraction from pinto beans is affected differently by the two solvents. TP extractions via acetone were mainly impacted by the solvent:water composition, with higher amounts negatively affecting TP yields for both the quadratic coefficient and the linear coefficient (Figure 7a). The next parameter that played a primarily role in TP yields was the solid:solvent ratio, which again showed a quadratic yet positive relationship between solid:solvent ratio and TP yields. Interesting, the solvent and solid ratio had an interactive yet positive effect on TP levels. For methanol-HCl, the reverse occurred for the linear solid:solvent coefficient as the higher solid amounts are expected to negatively and significantly impact overall yields, but yet exert a quadratic positive effect (Figure 7b). The extraction of pinto beans by methanol–HCl also showed a positive cross product interaction with solvent composition and mix times**.** Lastly, this chart shows that the solvent composition plays a role in positively affecting the response via a quadratic relationship.

Table 14: Regression equations that fit the model and passed lack of fit test for TP.

TPacetone	= 1.72 - 0.270*Xsp -1.07XspXsp* + 0.406*XsstXss +0.210 XspXss.*
TP methanol + HCl	= 0.854 - 0.265*Xss* -0.184*XspXsp* + 0.209*XssXss* -0.169*XspXmt.*

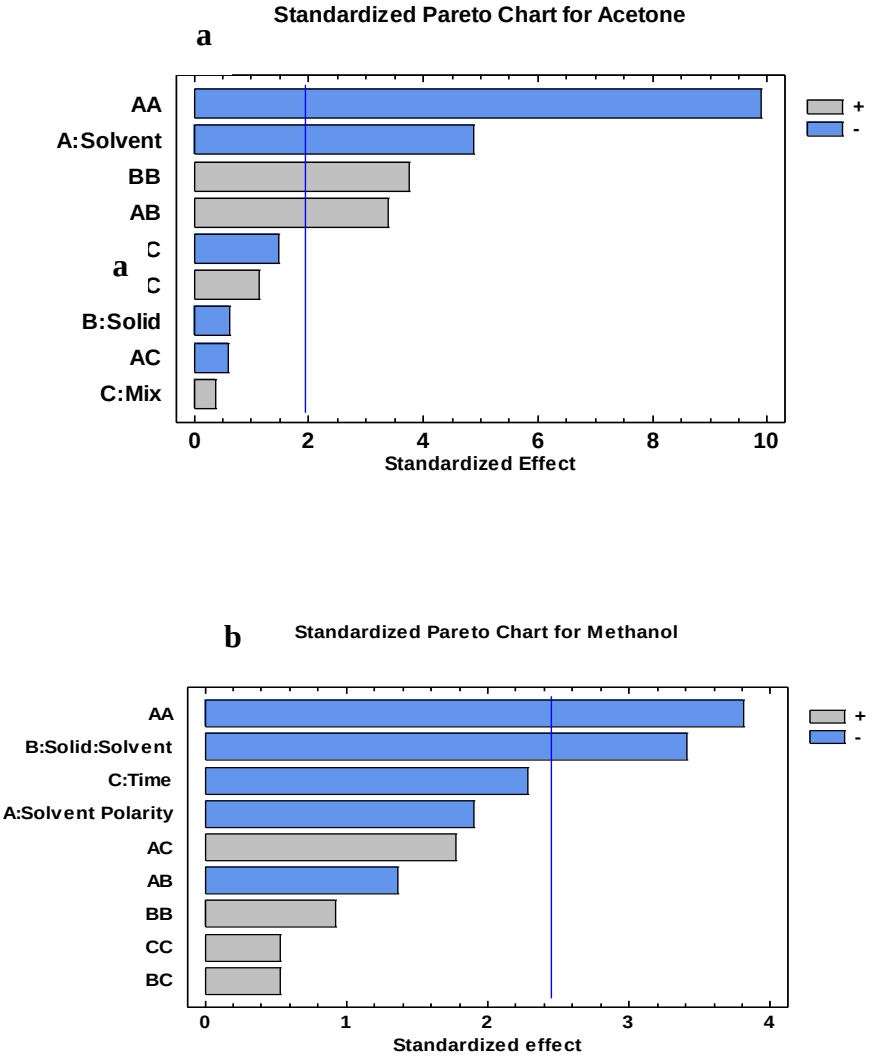


Figure 7: Pareto charts showing relative effects of regression coefficient for total phenols accepted models a.) acetone without HCl, b.) methanol + HCl. Vertical line represents $p < 0.10$.

D.2.4.a *TP response surface plots-**Acetone**.* A non-linear relationship for the acetone based TP extraction is clearly evident, as illustrated by the three 3D plots, especially for those shown in Figures 8a and b. The first two plots differ in that the center points show lower values (Figure 8a) and higher relative values when the solid:solvent is kept constant (Figure 8b). At a constant mixing time (Figure 8a), TP levels increased proportionately with acetone composition, but to a point (~50%) and then began to decrease again. The highest TP levels are predicted to be obtained at 50% composition but at the extremes of the solid:solvent ratios (10% and 30%). With solid amounts of ~15-25%, comparable TP levels cannot be reached, especially at the low and the high acetone composition. This relationship is to be expected as the Pareto charts shows significant interaction between solid:solid and solvent composition (Figure 7a). As shown in Figure 8b, the TP amounts are relatively high at the center points when the solvent composition is approximately 40-50% regardless of time. However, higher solvent composition results in substantially lower TP levels. This effect has been reported by a number of researchers (Xu et al. 2013; Radojkovic et al. 2012; Liyana-Pathirana et al., 2005). Cacace and Mazza, (2003b) proposed that this effect was due to the reduction in the dielectric constant, which results in lower energy, at higher solvent compositions thus allowing solute molecules to migrate between solvent molecules. This relationship was also expected for the pinto beans considering the significant negative influence of the quadratic solvent coefficient (Figure 7a).

Based on the response curves, the highest TP amounts can be attained by setting the composition to 50% acetone, which then will allow any mixing times to be

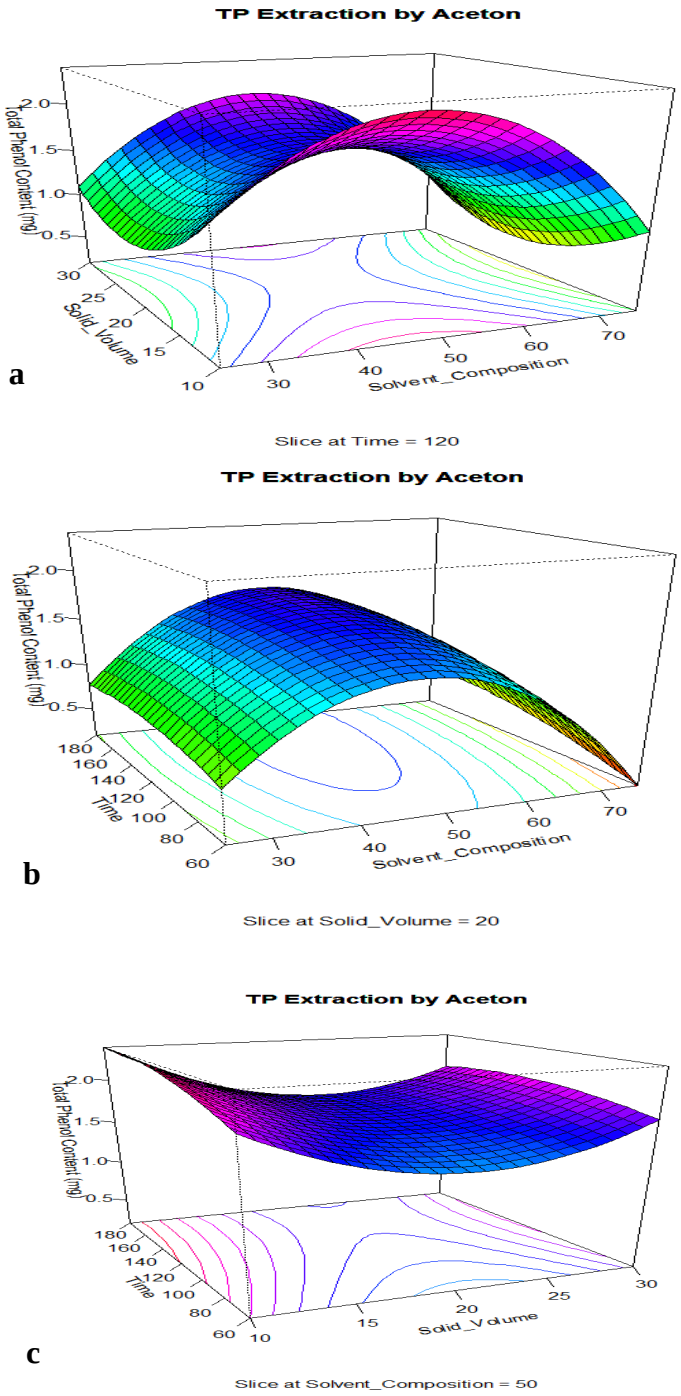


Figure 8: Response surface plots of the total phenols extracted with acetone as function of time, solvent composition and solid volume: (a) time of extraction was constant at 120 min, (b) solid:solvent kept constant at 20%, (c) solvent composition was kept constant at 50%.

used. However, optimal TPs can be obtained by avoiding solid:solvent ratios that fall in the mid range (15-25%). Based on the regression equation, the Pareto chart (Figure 7a) and the contour plots (Figures 7a,b,c), time has little or no effect on the TP outcome for this solvent process.

D.2.4.b *TP response surface plots-**Methanol-HCL***: According to the regression equation (Table 14) and the Pareto chart (Figure 7b), the main influencing factor on the methanol-HCl extraction of TPs from pinto beans is the solvent composition followed closely by an interactive cross product of solvent composition and mix time. It is interesting that mix time did affect the acetone based extractions, but is expected to play a major role in methanol-HCl based extractions. When time is held constant at 120 min, (Figure 9a), TP levels increased with higher solvent compositions but only with low solid:sovent ratio (~10%). Moreover, higher solid:solvent ratio resulted in decreasing TP levels regardless of the solvent composition, which could be due to the degradation of acid labile phenols ((Kim et al., 2004). By maintaining a solid:solvent of 20%, TP levels remained were fairly steady across many of the points (Figure 9b). With higher solvent compositions, shorter mix times resulted in only slightly higher TP levels. Despite this effect, at a constant solid:solvent ratio(20%), time again did not play a major role in the amount of TP recovered.

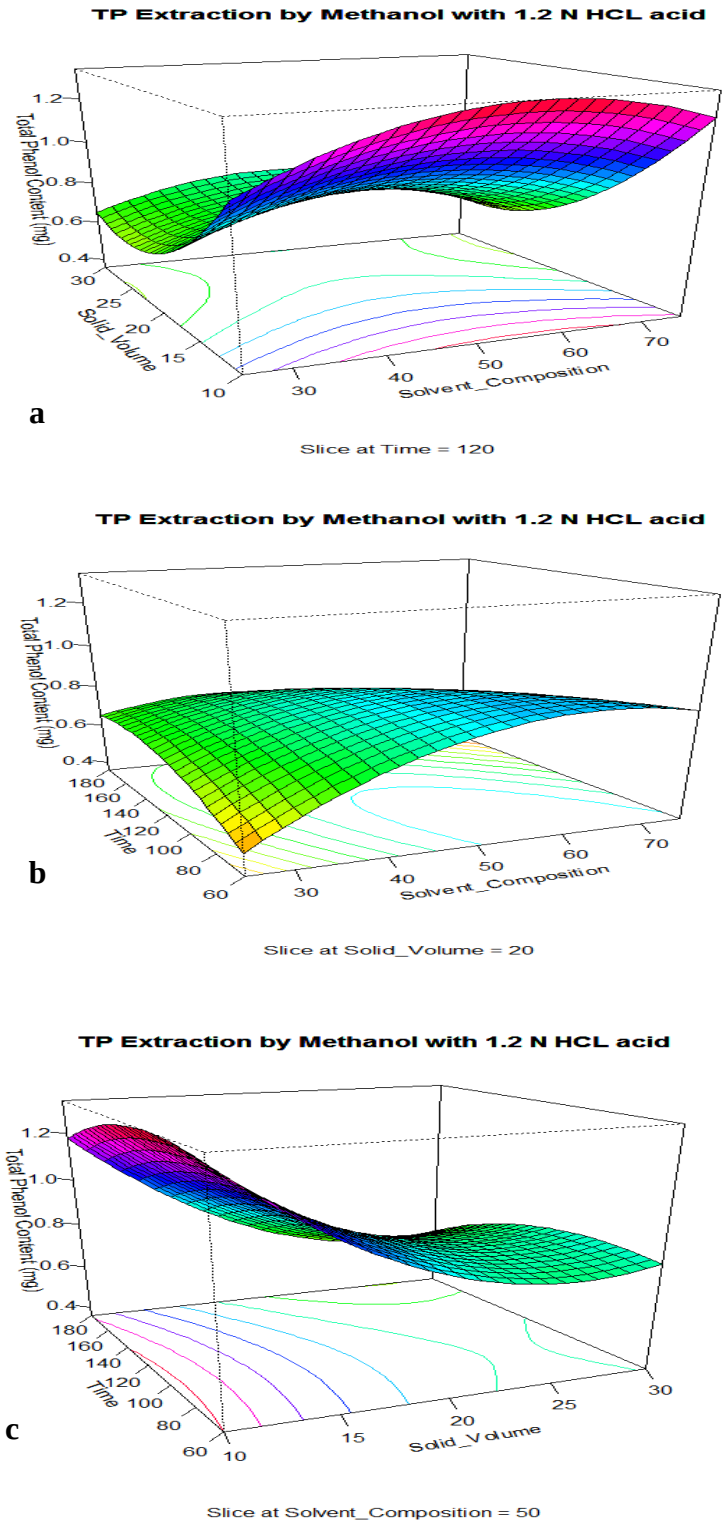


Figure 9: Response surface plots of the total phenols extracted with methanol + 1.2 N HCl as function of time, solvent composition and solid volume: (a) time of extraction was kept constant at 120 min, (b) solid:solvent kept constant at 20%, (c) solvent composition was kept constant at 50%.

As shown in Figure 9C, as the solid:solvent ratio declined to about 20%, but almost doubled by the 10% solid:solvent percentage. This phenomenon is actually fundamental to mass transfer principles (Ying et al. 2011). Basically, higher solvent volumes (or lower solid:solvent ratio) are better able to dissolve target compounds leading to higher yields.

D.2.5. *Final optimized TP values and processing factors*: Based on the model, and the factors tested, optimal processing factors were determined that are expected to produce the highest TP yields (Tables 15 a and b). A comparison of the optimum yields suggest that the phenols present in pinto beans are more non-polar as the acetone system was able to recover higher TP compared to the methanol + HCl. However, a high proportion of water was needed considering a coded value of -0.22 (or ~45:55 solvent:water). The HCl present in the methanol may be degrading acid labile phenols, and thus affecting overall yields. For both systems, optimal yields are expected for a solid ratio of 10%, which was confirmed by the associated regression equations (Table 14) and the response curves (Figures 8 and 9). Considering that the optimal coded value for this factor is -1, the lowest solid:solvent ratio actually tested, even lower solid:solvent ratios may increase TP yields. It should be noted that experimental data is not available as these conditions have yet to be applied to a real sample. However, these experiments are intended for future work.

Table 15: Optimized factors (in coded value) required to produce optimum TP yield for the cited system.

a.) Optimum value for acetone = 2.22 mg/g

Factor	***Optimum***
Solvent	-0.222463
Solid: Liquid	-1.0
Time	-0.126208

b.) Optimum value for methanol + HCl = 1.38 mg/g

Factor	***Optimum***
Solvent	0.752096
Solid:Liquid	-1.0
Time	-0.885965

D.3 Total Flavonoids (TF):

D.3.1 *TF results obtained by face centered composited design (FCCC):* The TF results for each extraction defined by the FCCC-RSM are shown in Table 16 (solvent without HCl) and Table 17 (solvents with HCl) as the mean +/- standard deviation of three replicates. TF levels in terms of high, low, and overall ranges are presented in Table 18. As shown by the latter table, the highest TF levels were extracted with acetone followed by acetone + HCl, whereas the methanol without HCl yielded the lowest TF levels followed by methanol + HCl with only an insignificant difference of 0.20 mg/g. The ethanol solvents with/without HCl were able to extract slightly higher TF levels compared to methanol. A study performed by Escribano-Bailon and Santos-Buelga (2003) indicated that the acidified solvents (methanol, ethanol, and acetone) could positively influence the recovery of TF. However, these results indicate that HCl added to any of the solvents provided no improvements in yields, but did not negatively affect the outcome either.

Table 16: Experimental data for total flavonoid response (in mg/g) of pinto beans extracts under different extraction conditions and solvent systems.

Standard Order	Methanol	Ethanol	Acetone
1	0.43 ± 0.06	0.54 ± 0.02	0.33 ± 0.03
2	0.52 ± 0.03	1.87 ± 0.07	2.25 ± 0.10
3	1.12 ± 0.18	1.93 ± 0.07	0.25 ± 0.07
4	0.72 ± 0.01	1.07 ± 0.02	2.15 ± 0.23
5	0.62 ± 0.03	0.73 ± 0.03	2.25 ± 0.07
6	0.81 ± 0.04	1.19 ± 0.05	2.63 ± 0.15
7	0.76 ± 0.08	1.10 ± 0.06	3.46 ± 0.04
8	0.74 ± 0.04	0.92 ± 0.01	0.32 ± 0.09
9	0.39 ± 0.05	0.52 ± 0.01	1.80 ± 0.08
10	0.72 ± 0.05	0.34 ± 0.02	0.92 ± 0.06
11	1.09 ± 0.08	1.27 ± 0.08	3.46 ± 0.07
12	0.46 ± 0.05	0.66 ± 0.02	0.49 ± 0.09
13	0.69 ±0.04	0.85 ± 0.02	0.41 ± 0.00
14	0.59 ± 0.25	0.58 ± 0.01	2.90 ± 0.020
15	0.17 ± 0.01	0.85 ± 0.02	0.86 ± 0.06
16	1.03 ± 0.10	1.62 ± 0.11	0.40 ± 0.05

* Data are shown as the mean ± standard deviation.

Table 17: Experimental data for total flavonoid response (in mg/g) of pinto beans extracts under different extraction conditions and solvent systems.

Standard Order	Methanol + HCl	Ethanol + HCl	Acetone + HCl
1	1.04 ± 0.05	1.03 ± 0.07	0.28 ± 0.08
2	0.30 ± 0.04	1.21 ± 0.04	0.31 ±0.01
3	1.42 ± 0.02	1.39 ± 0.09	0.14 ±0.03
4	0.89 ± 0.04	0.82 ± 0.02	0.23 ± 0.02
5	0.78 ± 0.02	0.54 ± 0.06	0.26 ± 0.01
6	0.96 ± 0.08	1.37 ± 0.02	0.59 ± 0.05
7	0.52 ± 0.04	0.78 ± 0.07	0.80 ± 0.02
8	0.86 ± 0.09	1.00 ± 0.07	0.43 ± 0.11
9	0.27 ± 0.02	0.39 ± 0.09	0.55 ± 0.04
10	0.69 ± 0.05	0.65 ± 0.01	0.20 ± 0.02
11	0.51 ± 0.09	0.67 ± 0.03	0.75 ± 0.02
12	1.18 ± 0.19	1.16 ± 0.05	0.57 ± 0.03
13	0.75 ± 0.06	0.99 ± 0.03	1.16 ± 0.01
14	0.42 ± 0.03	0.82 ± 0.17	0.77 ± 0.02
15	0.60 ±0.01	0.76 ± 0.04	0.22 ± 0.02
16	1.00 ± 0.02	1.70 ± 0.11	2.27 ± 0.08

* Data are shown as the mean ± standard deviation.

Table 18: Ranges of total flavonoid for each solvent system.

Extraction Solvent	High and Low Total Flavonoid (mg/g)	Range (mg/g)
Methanol	0.17 – 1.12	0.95
Ethanol	0.34 – 1.93	1.59
Acetone	0.25 – 3.46	3.21
Methanol + HCl	0.27 – 1.42	1.15
Ethanol + HCl	0.39 – 1.70	1.31
Acetone + HCl	0.14 – 2.27	2.13

D.3.2 *Fitting the TF models:* Multiple regression coefficients for the total flavonoids are summarized in Tables 19 and 20, for three solvent systems with and without HCl, respectively. The ANOVA of the quadratic model was adequate only for the ethanol (without/with HCl) as $p < 0.05$. In addition, the model coefficients R^2 indicated a low dispersion for both systems (85.8 for ethanol only, and 88.0 for ethanol + HCl). As the results for these solvents adequately described the model, the solvents were assessed for lack of fit (refer to Section D.3.3.) Moreover, methanol was also accepted for further evaluation as the model p value was > 0.10, and the corresponding R^2 was 83.2. None of the other solvent systems complied with the criteria of the model, and thus will not be discussed any further. However, analysis of more points or center points may account for the low R^2 value and failure to satisfy the model. One reason could be due to difference in particle sizes between difference samples, as reports have shown that this parameter affects extraction efficiencies (Stalikas, 2007; Luthria et al., 2011; Brewer et al., 2014). Although every attempt was made to maintain the particle size for each RSM experiment, there may have been inconsistencies.

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

Coefficient	Methanol	Ethanol	Acetone
b_o	0.631	0.935	1.49
Linear			
b_1 (SP)	0.016	0.115	-0.930*
b_2 (S:S)	-0.245^	-0.401^	-0.382
b_3 (MT)	-0.122**	0.069	0.155
Quadratic			
b_{11} (SP x SP)	-0.081	-0.402**	-0.439
b_{22} (S:S x S:S)	0.126	0.001	0.387
b_{33} (MT x MT)	0.032	0.509*	0.153
Cross product			
b_{12} (SP x S:S)	-0.033	-0.146	0.714
b_{13} (SP x MT)	-0.030	-0.065	-0.159
b_{23} (S:S x MT)	-0.024	0.106	0.132
R^2	83.2	85.8	74.6
p values			
Model	0.0798	0.0520	0.9385
Lack of Fit	0.1442	0.1310	0.0429

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

Table 20: Regression coefficients (coded) predicted by the quadratic polynomial model for flavonoids when extracted with the cited solvent systems.

Coefficient	Methanol + HCl	Ethanol + HCl	Acetone + HCl
b_o	0.765	1.00	0.563
Linear			
b_1 (SP)	0.191*	0.179*	0.035
b_2 (S:S)	-0.082	-0.250^	-0.273
b_3 (MT)	-0.193*	0.021	0.231
Quadratic			
b_{11} (SP x SP)	-0.001	-0.060	0.385
b_{22} (S:S x S:S)	0.140	-0.038	-0.060
b_{33} (MT x MT)	-0.137	0.041	-0.218
Cross product			
b_{12} (SP x S:S)	-0.200*	-0.229*	-0.193
b_{13} (SP x MT)	0.041	0.142**	0.272
b_{23} (S:S x MT)	-0.079	-0.141	-0.270
R^2	80.2	88.0	80.3
p values			
Model	0.1201	0.0336	0.1862
Lack of Fit	0.4144	0.2376	0.2323

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

D.3.3 *Adequacy of the TF models and corresponding regression equations:* The data obtained from methanol and ethanol (+/- HCl) also complied to the lack of fit test as each had p value > 0.05 (Table 18 and 19).

D.3.4 *Regression coefficients equations and Pareto charts:* Regression equations that fit the model (Section D.3.2) and passed the lack of fit test (Section D.3.3) are shown in Table 21, with only the coefficients that were significant (Table 18 and 19). Coupled with these equations, the influences of individual coefficients were evaluated against Pareto charts (Figure 10). The main effect for TF extraction for all the solvents was the linear solid:solvent coefficient, which showed a negative impact. Based on the Pareto chart for methanol, mixing time also had a negative influence (Figure 10a). Meanwhile, the solvent polarity of the ethanol extraction as well as mixing time significantly affected the TF of the extracts at the quadratic level, but the influences were positive and negative, respectively. In addition to the solid:solvent ratio, extractions with ethanol + HCl were affected by mixing time and solvent polarity (SP), with the coefficients being linear for SP, but cross product interaction for mixing time with solvent composition and solid:solvent ratio and mix times (Figure 10c). In general, these results demonstrate that the solid:solvent ratio significantly affect TF yields in linear relationship, resulting in higher yields with decreasing solid levels.

Table 21: Regression equations that fit the model and passed lack of fit test.

TF methanol	= 0.631- *0.245Xsp – 0.122mt*
TF ethanol	= 0.935 - 0.401*Xss* - *0.402XspXsp* + 0.509*XmttXmt.*
TF ethanol + HCl	= 1.00 + 0.179*Xsp* - 0.250*Xss* - 0.229*XspXss* + 0.142*XspXmt.*

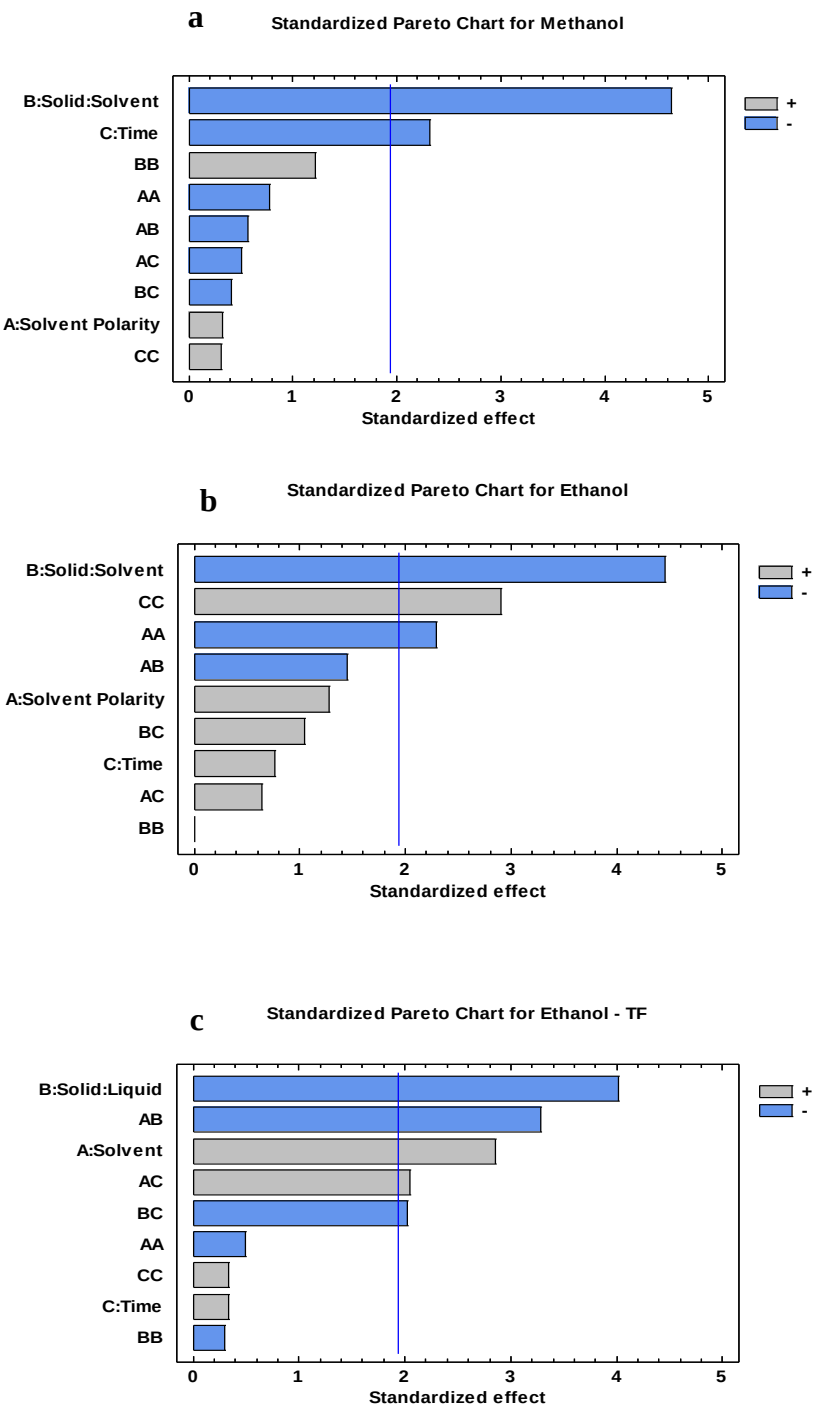


Figure 10: Pareto charts showing relative effects of regression coefficient for total flavonoid accepted models for (a) acetone without HCl, (b) methanol + HCl, and (c) ethanol + HCL. Vertical line represents $p < 0.10$.

D.3.4.a *TF response surface plots-**Methanol**:* The three 3D plots of the data obtained from the methanol extraction are relatively planar with only slight curvature contours (Figures 11a, b, c), which clearly show the primary linear relationship between the factors and TF values, as discussed previously. When the mixing time was held constant (Figure 11a), TF levels increased rapidly as the solid:solvent ratio decreased starting at the center point (~ 20%). Furthermore, the highest yields occurred at the lowest ratio (10%) with only slightly higher TP values at a 70% methanol:water composition. These results indicate that the higher methanol composition is not able to promote solute migration into the solvent molecules when pinto bean solids are relatively low, at least for extracting TF. A similar contour plot was generated when TP was extracted with methanol + HCl (Figure 9A), which again demonstrates that HCl has very little impact on the extraction efficiency of phenolic compounds from this based matrix.

The second and third plots (Figures 11 b,c) show the effects of mixing time on TF levels with respect to solvent composition and solid:solvent ratio, respectively. TF values were inversely proportional with mixing times and inclined slightly with higher solvent composition up to approximately 50% (Figure 11b). Alternatively, the lowest TF values were extracted with 75% solvent composition and a mixing time of ~180 min. When the solvent composition was maintained at 50% (Figure 11c), the strong influence of the solid:solvent ratio is again apparent for methanol based extractions of pinto beans, as TF values increased dramatically with decreasing solid:solvent ratios with only minimal effects with respect to mixing time. The lowest TF yields occurred at the extreme highs of the extraction process, i.e., 30% solid:solvent ratio and 180 min mixing time.

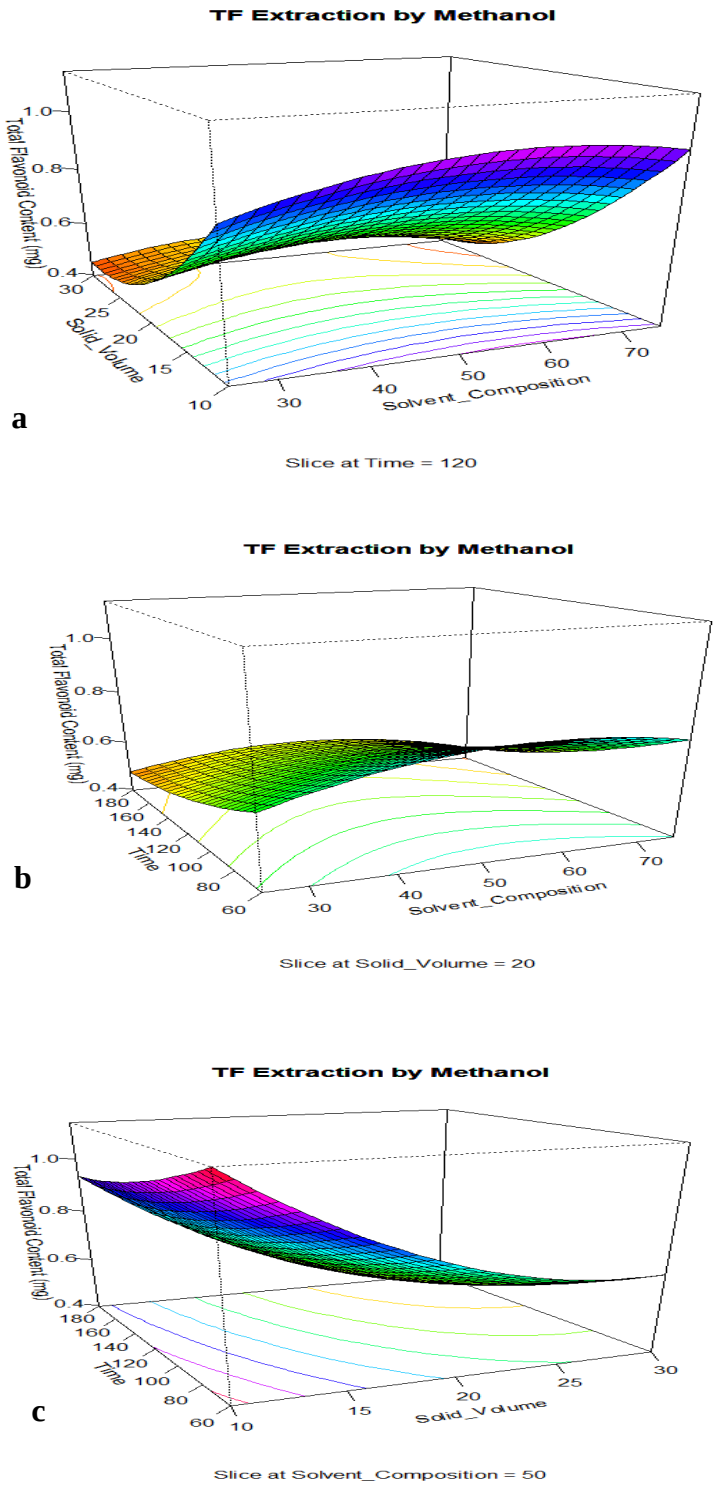


Figure 11: Response surface plots of the total flavonoids extracted with methanol as function of time, solvent composition and solid volume: (a) time of extraction was kept constant at 120 min, (b) solid:solvent kept constant at 20%, (c) solvent composition was kept constant at 50%.

A review of the literature has also shown that a longer extraction time has minimal effects on phenolic levels from various types of natural products. For example, water extraction of phenolics from pistachio hulls showed a dramatic increase in TP levels from 5-20 min but then plateaued from 20 min to 100 min (Rajaei et al., 2010). When extracting TPs from the fruit peel of *Nephelium lappacium L.* using ultrasound assisted methodology, only 20 min was required to obtain optimal yields with no substantial gains after this time point (Maran et al., 2012). In this case, temperature was the most influential factor on total phenolic levels. Liyana-Pathirana (2005) also reported that mix time had no significant effect on phenolic compound extractions, but rather ethanol composition and temperature.

D.3.4.b *TF response surface plots-**Ethanol**:* The response surface plots of the ethanol based extractions (Figure 12) show more clearly the quadratic effect of this solvent on TF levels, which was briefly discussed with respect to the regression equations and Pareto charts (Section D.3.3). Relative to the regression response curve generated by the TF data (Figure 11), these plots exhibit the typical curve contour expected from a quadratic relationship. Yet more dramatic quadratic based curves have been reported by other researchers when extracting flavonoids from other natural systems using ethanol (Radojkovic et al., 2012; Xu et al., 2013), which supports the need to develop product based extraction process as from a product specific perspective.

Nonetheless, Figures 12a and 12c again demonstrate the influence of the solid:solvent ratio on TF levels, particularly when the solvent composition is held at 50% (Figure 12c). TF values again increased with decreasing solid:solvent ratios. For this

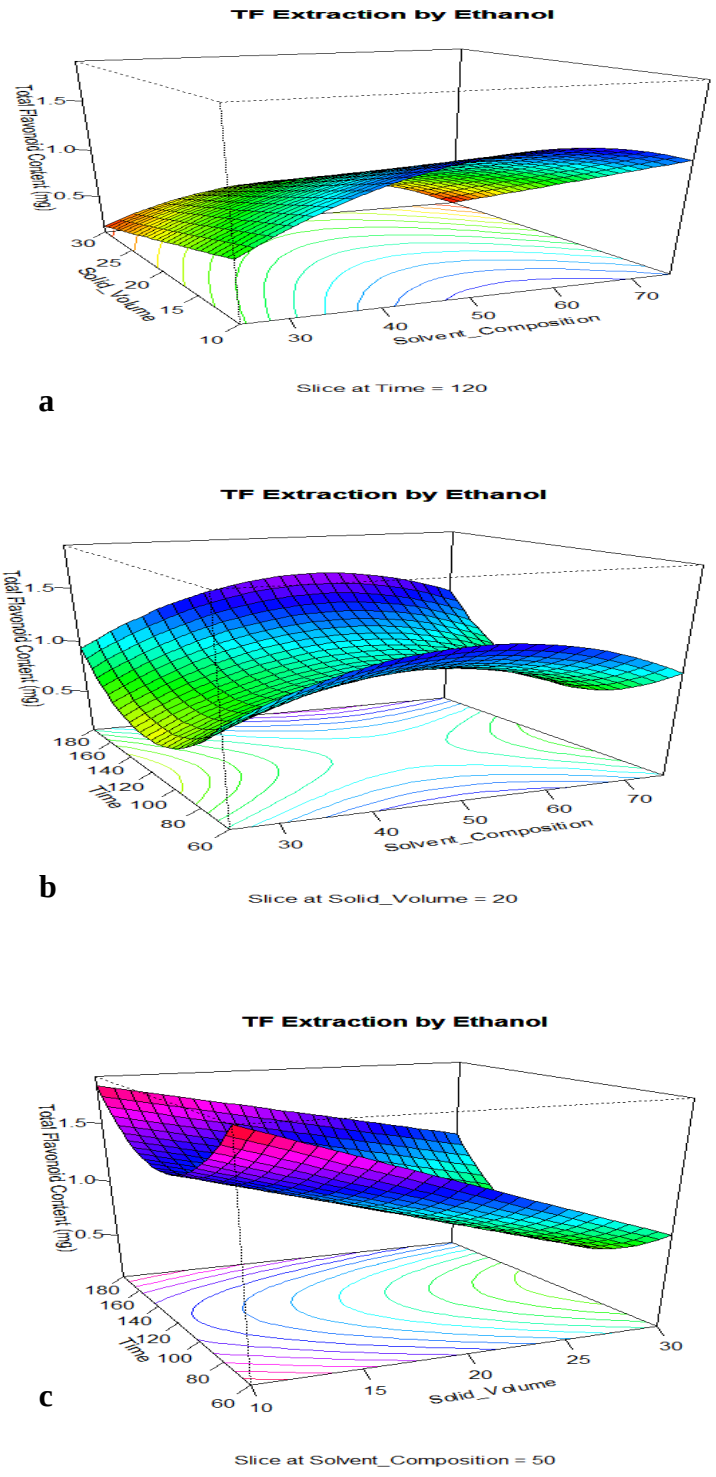


Figure 12: Response surface plots of the total flavonoids extracted with ethanol as function of time, solvent composition and solid volume: (a) time of extraction was kept constant at 120 min, (b) solid:solvent kept constant at 20%, (c) solvent composition was kept constant at 50%.

solvent system, mixing time impacted on TF levels, which increased relative to both sides of the center point (Figures 12b, c). Such inverse saddle contours have been reported for ethanol extraction of wheat based phenols (Liyana Pathirana, et al., 2005) but relative to temperature and solvent composition when mix time was held constant, with no further explanation of the possible mass transfer effects that induce such a configuration. However, the solid ratio (Figure 12c) showed a significant correlation with mixing time, which resulted in approximately a 0.5 mg/g increase at the mix time extremes throughout the solid:solvent range.

D.3.4.c *TF response surface plots-**Ethanol-HCl**:* According to the Pareto chart (Figure 10c) and 3D (Figure 13a,c), the negative linear coefficient for TF extraction was obviously the main influence for extracting optimal TF yields. For the solid:solvent ratio (Figure 13a,c), TF levels increased for all the ratios (10, 20,and 30%) despite the negative cross product interaction of the composition and mixing time. The solvent composition with solid ratio illustrated that the optimum amount was 70% ethanol-HCl:water (Figure 13a). The solvent composition was the only parameter that resulted in a positive linear effect (Figure 10c; Figure 13a, b). For the mixing time, the positive and negative quadratic effects were detected when there was an interaction between (mixing time vs solvent composition) and (mixing time vs solid ratio) (Figures13b, c). Finally, Figure 13b shows that the highest TF yields were obtained at low mixing time (60 min) with (~70% ethanol-HCl). Nevertheless, the maximum amount TF (1.4 mg) was obtained with a mixing time at 180 min, which had a crossproduct interaction at low solid:solvent ratios (Figure 13c).

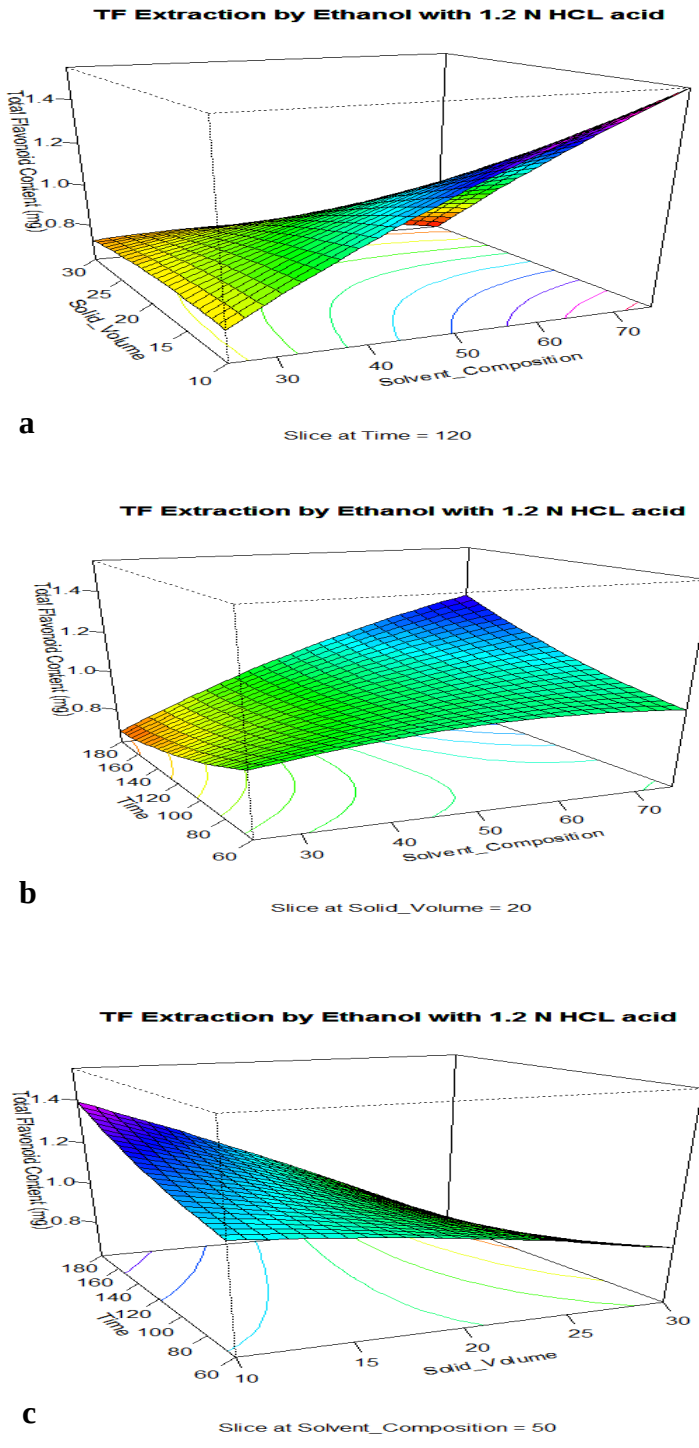


Figure 13: Response surface plots of the total flavonoids extracted with ethanol as function of time, solvent composition and solid volume: (a) time of extraction was kept constant at 120 min, (b) the solid:solvent kept constant at 20%, (c) solvent composition was kept constant at 50%.

D.3.5 *TF final optimized parameter and processing effects:* Similar to the TP model, optimized values obtained from the accepted TF model were determined (Table 22a,b,c). Considering that the highest yields were obtained with methanol consisting of 50% water, the data indicate that the flavonoids in pinto beans are more non-polar probably due to the presence of conjugates such as glycosides. Moreover, the lowest solid:solvent ratio (10%) and mixing time (60 min) have to be used for optimal yields. The lower levels extracted via the ethanol + HCl system was likely due to degradation of acid labile flavonoids. For all the systems, optimal yields are expected for a solid ratio of 10%, which was confirmed by the associated regression equations (Table 14). Considering that the optimal coded value for this factor is -1, the lowest solid:solvent ratio actually tested, even lower solid:solvent ratios may increase TF yields. Again, it should be noted that experimental data is not available as these conditions have yet to be applied to a real sample. However, these experiments are intended for future work.

Table 22: Optimized factors (in coded value) required to produce optimum TF yields for the cited system.

a.) Optimum value for methanol = 3.80 mg/g

Factor	***Optimum***
Solvent	0.486913
Solid: Liquid	-1.0
Time	1.0

b.) Optimum value for ethanol = 1.38 mg/g

Factor	***Optimum***
Solvent	0.404796
Solid:Liquid	-0.99999
Time	1.0

C.) Optimum value for ethanol + HCl = 1.91 mg/g

Factor	***Optimum***
Solvent	1.0
Solid:Liquid	-1.0
Time	1.0

D.4 Anitoxidative Capacity (AC):

D.4.1 *AC results obtained face centerd composited design (FCCC):*

The results of antioxidative capacity are shown as mean +/- standard deviation of three replicates for each extraction for solvents without HCl (Table 23) and those with HCl (Table 24). The coded and actual values used for characterizing the extraction procedures as they apply to AC are shown in Table 7). Various, but relevant AC values are also listed in Table 25 for each solvent system, i.e., the high, low, and overall range value.

The highest AC (278.42 μmole Trolox/g) was extracted from methanol + 25 HCl, but this systems also produced a low of 25.00 (μmole Trolox/g) have a significant value while the ethanol with HCl has the lowest, which resulted in a dispersion of 253.42 μmole Trolox/g. Although the remaining solvents had significantly lower AC values, they were still comparably high, with all values above 70 (μmole Trolox/g). But, the low values of 3.7 μmole Trolox/g (acetone) and 4.64 (ethanol + HCl) were obtained due only to a slight change in the process parameter.

D.4.2 *Fitting the AC models:* Multiple regression coefficients for the AC (without/with HCl) are summarized in Tables 26 and 27. The ANOVA shows that the quadratic model was adequate for the methanol without HCl, methanol + HCl, and ethanol + HCL solvent systems ($p < 0.05$, $R^2 > 75$). These solvent systems were therefore evaluated further via the lack of fit test.

Table 23: Experimental data for antioxidative capacity (in μmole Trolox/g) of pinto beans extracted under different experimental conditions and solvent systems.

Standard Order	**Methanol**	**Ethanol**	**Acetone**
1	21.57 ± 2.05	29.92 ± 3.80	3.76 ± 0.31
2	37.94 ± 4.36	11.81 ± 0.06	119.82 ± 24.12
3	55.58 ±4.29	8.92 ± 1.47	6.00 ± 0.71
4	80.69 ± 6.18	101.24 ± 58.12	78.95 ± 18.98
5	51.05 ± 2.09	135.58 ± 2.64	115.74 ±12.85
6	73.45 ± 1.39	50.45 ± 2.93	64.14 ±15.69
7	43.76 ± 3.21	20.81 ± 0.40	20.75 ± 3.80
8	71.45 ± 4.60	108.97 ± 26.05	177.02 ± 14.93
9	34.96 ±0.66	7.31 ± 0.11	173.03 ± 15.49
10	14.18 ± 1.74	13.24 ± 0.52	7.03 ± 0.65
11	77.03 ± 6.03	45.46 ± 9.65	42.21 ± 18.02
12	54.77 ± 2.73	101.22 ± 2.65	61.75 ± 8.03
13	73.04 ± 7.23	73.14 ± 3.08	20.33 ± 1.36
14	31.90 ± 0.48	25.53 ± 4.62	74.28 ± 29.57
15	20.58 ± 1.15	34.67 ± 3.78	8.36 ± 1.51
16	57.12 ± 1.22	20.97 ± 0.34	10.02 ± 0.03

* Data are shown as the mean ± standard deviation.

Table 24: E Experimental data for antioxidative capacity (in μmole Trolox/g) of pinto beans extracted different experimental conditions and solvent systems.

Standard Order	Methanol + HCl	Ethanol + HCl	Acetone + HCl
1	151.16 ± 17.36	7.85 ± 1.34	13.65 ± 1.60
2	41.13 ± 5.91	4.64 ± 0.63	22.86 ± 1.69
3	278.42 ± 9.56	18.12 ± 3.30	8.31 ± 1.66
4	67.08 ± 8.35	34.70 ± 6.06	19.26 ± 1.71
5	57.00 ± 5.42	34.75 ± 10.21	148.10 ± 8.35
6	79.26 ± 20.00	72.18 ± 12.94	12.60 ± 3.49
7	76.00 ± 1.50	59.88 ± 5.46	49.02 ± 3.40
8	70.15 ± 21. 68	36.86 ± 0.78	57.46 ± 16.58
9	36.38 ± 3.44	9.07 ± 0.35	108.10 ± 11.37
10	70.07 ± 16.31	8.94 ± 0.99	47.35 ± 4.71
11	55.62 ± 4.87	29.57 ± 1.53	121.96 ± 12.23
12	25.00 ± 3.46	12.86 ± 0.75	26.08 ± 3.52
13	58.32 ± 6.11	55.47 ± 2.37	125.26 ± 6.54
14	48.52 ± 7.10	49.09 ± 3.25	46.52 ± 2.54
15	93.07 ± 4.66	15.61 ± 1.66	38.21 ± 1.71
16	102.83 ± 4.19	35.19 ± 3.17	119.37 ± 5.33

* Data are shown as the mean ± standard deviation.

Table 25: Ranges of antioxidative capacity AC for each solvent system.

Extraction Solvent	Low and High Values of AC (μmole Trolox/g)	Range (μmole Trolox/g)
Methanol	14.18 – 80.69	66.51
Ethanol	7.31 – 135.58	128.27
Acetone	3.76 – 173.03	169.27
Methanol + HCl	25.00 – 278.42	253.42
Ethanol + HCl	4.64 – 72.18	67.54
Acetone + HCl	8.31 – 148.10	139.79

D.4.3 *Adequacy of the AC models and corresponding regression equations*. Evaluation of the ANOVA of the three solvents against the lack of fit test showed compliance of only one solvent, i.e., methanol without HCl ($p > 0.05$). Interestingly, the methanol / ethanol + HCl had R^2 value > 0.75, but failed lack of fit tests, suggesting that a higher model may be needed to describe the interactions between the analyte of interest and the process parameters used.

D.4.4 *Regression coefficients equations and Pareto charts:* The regression equation derived by the AC data using methanol as the extraction system is shown in Table 28, whereas the corresponding Pareto chart is illustrated as Figure 14. For this case all the linear coefficients for solid:solvent, time and solvent polarity significantly affect the AC but by a negative relationship. Still, the factor that primarily affected this response using methanol as the extraction solvent was the quadratic solvent composition, but again via a negative association. Combined these results demonstrate that the optimal AC values can only be obtained by applying the lower levels of the experimental values.

Table 26: Regression coefficients (coded) predicted by the quadratic polynomial model for antioxidative capacity when extracted with the cited solvent systems.

Coefficient	Methanol	Ethanol	Acetone
b_o	60.95	81.00	95.86
Linear			
b_1 (SP)	-7.33*	-9.26	-33.72**
b_2 (S:S)	-13.13*	14.54	22.38
b_3 (MT)	-8.79*	-17.45	13.64
Quadratic			
b_{11} (SP x SP)	-28.57*	-48.25*	-55.43
b_{22} (S:S x S:S)	6.94**	17.03	-4.51
b_{33} (MT x MT)	4.01	-19.46	4.93
Cross product			
b_{12} (SP x S:S)	-5.86*	-3.02	-21.60
b_{13} (SP x MT)	7.62*	19.05	-10.60
b_{23} (S:S x MT)	2.29	-19.46	16.30
R^2	87.3	77.0	71.2
p values			
Model	0.0388	0.1694	0.2794
Lack of Fit	0.0630	0.5208	0.9958

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

Table 27: Regression coefficients (coded) predicted by the quadratic polynomial model for antioxidative capacity when extracted with the cited solvent systems.

Coefficient	Methanol + HCl	Ethanol + HCl	Acetone + HCl
b_o	63.87	41.99	38.15
Linear			
b_1 (SP)	39.46*	-5.42	-13.33
b_2 (S:S)	-37.04^	-11.90**	4.80
b_3 (MT)	-20.62**	8.16	10.60
Quadratic			
b_{11} (SP x SP)	33.03	-16.26	-5.54
b_{22} (S:S x S:S)	1.25	5.78	44.82
b_{33} (MT x MT)	-12.70	-4.60	-14.46
Cross product			
b_{12} (SP x S:S)	-25.91*	-7.41	-1.73
b_{13} (SP x MT)	-30.51*	2.22	10.53
b_{23} (S:S x MT)	16.28	-2.99	3.28
R^2	91.2	76.1	32.0
p values			
Model	0.0146	0.0336	0.1862
Lack of Fit	0.0115	0.0215	0.2291

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

Table 28: Regression equations that fit the model and passed lack of fit test.

TP methanol = 60.95 -7.33*Xsp* -13.13*Xss* - 8.79*Xmt* - 28.57*XspXsp*.

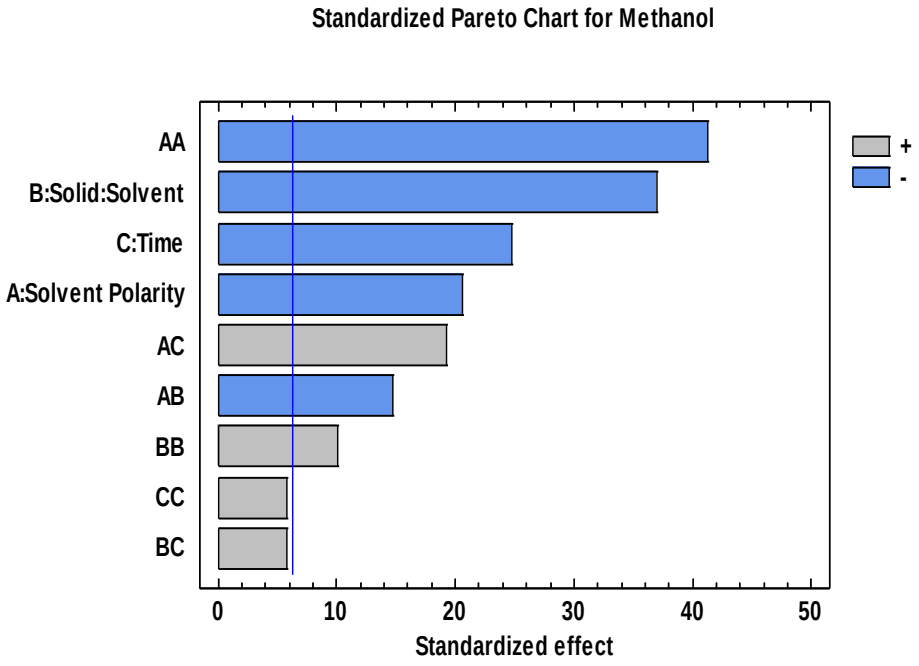


Figure 14: Pareto charts showing relative effects of regression coefficient for total antioxidant accepted models for methanol extractions.

D.4.4a. *TP Response Surface Plots-**Methanol:*** The response plots (Figure 15a, b,c), further confirm that solvent composition had the most significant impact on the extraction of high AC samples. The most revealing evidence is shown by Figure 15 c. When the composition was held at 50% methanol:water, the AC increased slightly with lower solvent:solid ratios while time had no effect. This Figure also shows the ruggedness of this extraction when 50% methanol:water is used. Basically, similar AC values will be obtained with most times and solid:solvent ratios, which is as an important factor to consider as is the high values if this process was being considered for industrial applications. However, such discussions are beyond the scope of this research, considering we are characterizing the effects of the extraction process on obtaining antioxidative rich extracts from pinto beans. Figure 15 a and b show similar contours, i.e., an inverted saddle type configuration. Both again show that solid:solvent and time have little effect but that the optimal solvent composition is 50% methanol.

D.4.5 *Final optimized AC values and processing factors*: The optimized factors predicted to produce the highest AC values are shown in Table 29.

Table 29: Optimized factors (in coded value) required to produce optimum AC yield for the cited system.

a.) Optimum value for methanol = 96.84 μmole Trolox/g

Factor	***Optimum***
Solvent	-0.159552
Solid: Liquid	-0.999895
Time	1.0

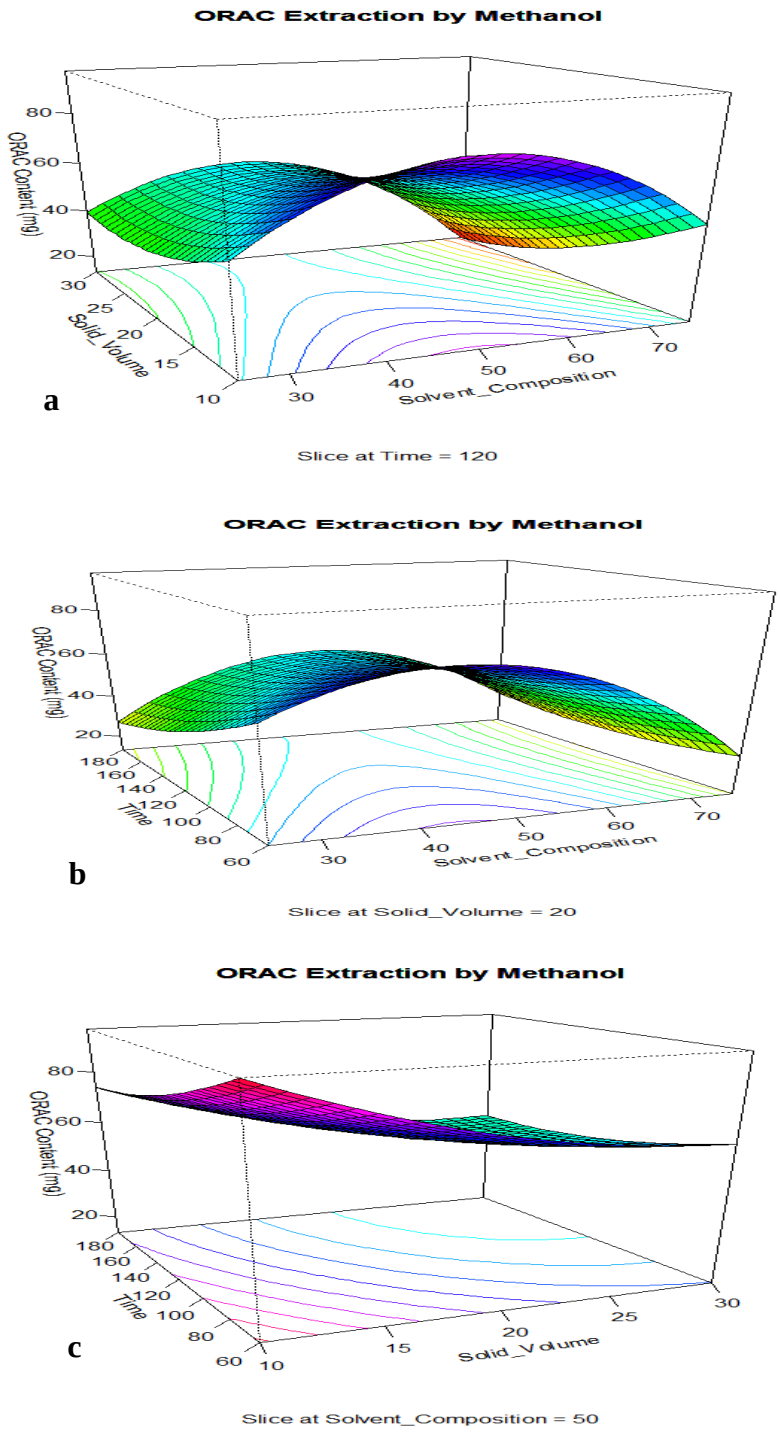
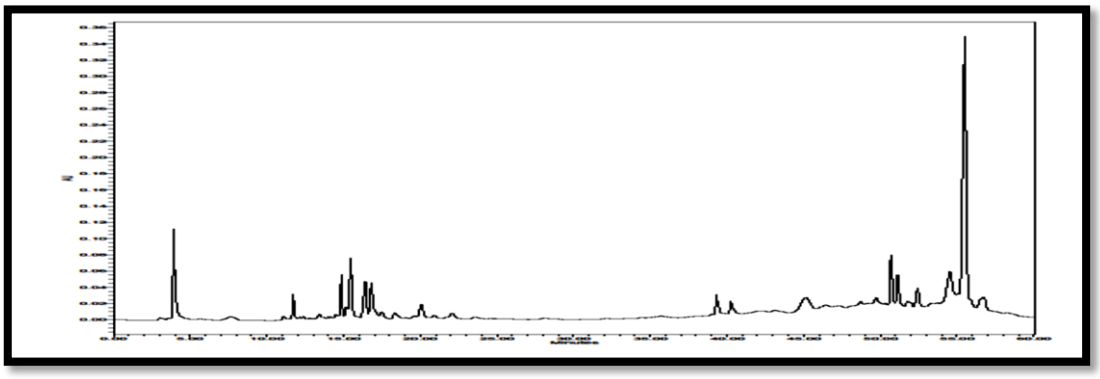


Figure 15. Response surface plots of the antioxidant capacity of samples extracted with methanol as function of time, solvent composition and solid volume: (a) the time of extraction was kept constant at 120 min, (b) the solid:solvent was kept constant at 20%, (c) the solvent composition was kept constant at 50%.

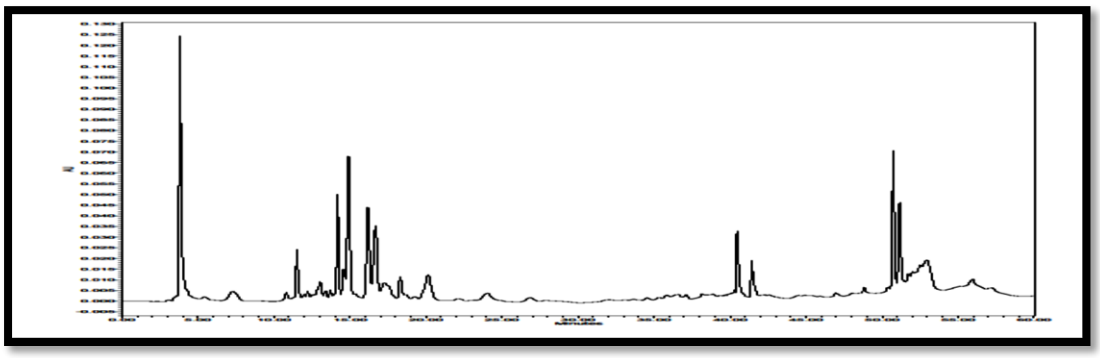
D.5. Compositional Analysis of Select Extracts:

Select extracts were analyzed via HPLC to determine qualitatively the extent of the differences (if any) in their composition profiles. The selection process was based primarily on those that exhibited high antioxidative capacities, with the addition of others that had either high TF or TPs. Figure 16 shows a sampling of chromatograms obtained from the methanol extractions. (It must be noted that the detection wavelength used was 280 nm, which is selective primarily for phenolic acids and their conjugates.)

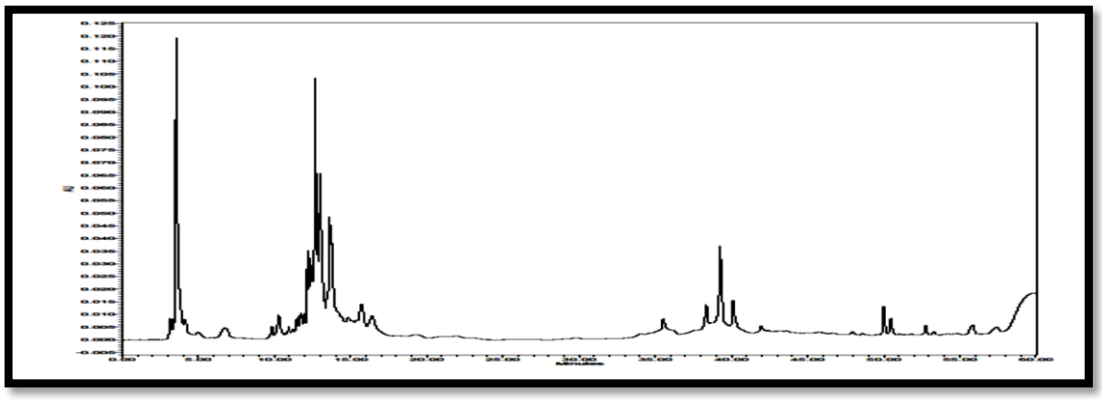
Two of these samples exhibited high antioxidative capacities relative to the other methanol extractions (Figure 16 a, c), yet had very different compositional profile, not only in relative differences in intensities but the appearance of other peaks, more specifically towards the end of the run (50-60 min) (Figure 15a). As comparable AC values were obtained for Samples 11 and 6, these peaks probably do not impact the biological response that was monitored for this project. However, it must be pointed out that only a slight difference in the extraction process resulted in fairly large differences in the composition. This in turn could affect other biological responses, positively or negatively. Figure 16c shows a chromatogram of a sample that exhibited fairly low AC. Again, a comparison of the chromatograms shows significant difference between the three, indicating that type and ratio have AC values. As TP and TF values are different between these three extracts, the results also show that further compositional profiles are needed to link the biological activity to the overall profiles, and ultimately to identify the responsible phenolic based antioxidants acting alone or in synergy in pinto beans. Characterization of the extraction parameters is a critical first step.



a.) Sample 11: Methanol without HCl, Solvent:Water – 25:275, Solid:Solvent – 10%, Mix Time – 60 min. AC: 77.03 µmole Trolox/g, TF: 1.09 mg/g, TP: 0.38 mg/g.



b.) Sample 6: Methanol without HCl, Solvent:Water – 50:50, Solid:Solvent – 10%, Mix Time – 120 min. AC: 73.45 µmole Trolox/g, TF: 0.81 mg/g, TP: 1.19 mg/g.



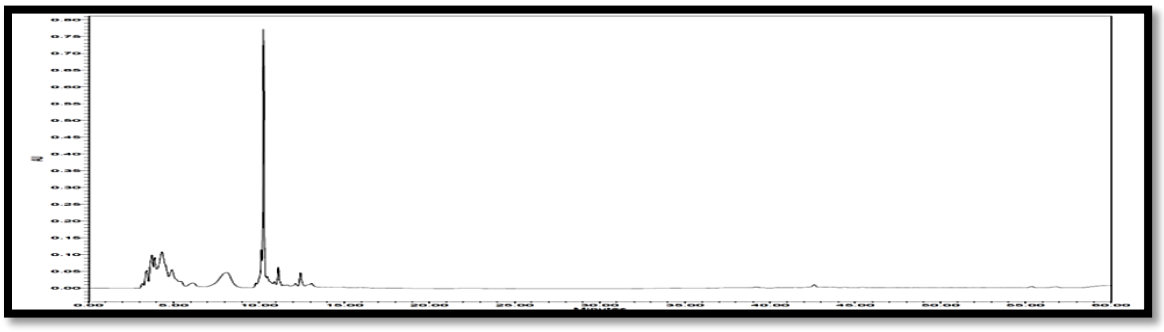
c.) Sample 14: Methanol without HCl, Solvent:Water – 25:50, Solid:Solvent – 20%, Mix Time – 60 min. AC: 31.90 µmole Trolox/g, TF: 0.59 mg/g, TP: 1.14 mg/g.

Figure 16: Reverse phase chromatograms of methanol based extract detected at 280 nm. (AC-antioxidative capacity, TF: total flavonoids, TP: total phenols).

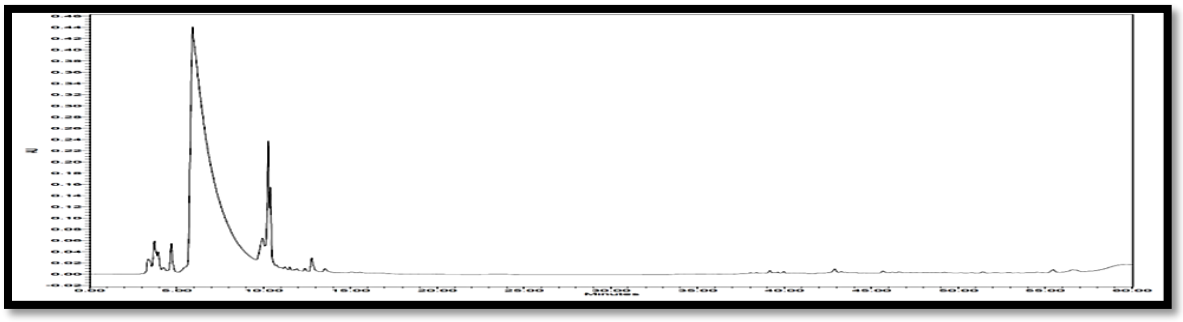
E. CONCLUSIONS.

The response surface methodology was successfully used for obtaining critical information relative to extraction of phenolic-rich extracts that deliver optimal antioxidative capacities from pinto beans. The most effective factors that resulted in overall maximum yields for TP and TF were a solvent composition of 50%, a solid ratio of 10% and a 60 min mix time, although the solvents were different. Acetone without HCL was most effective for extracting TP, while methanol without HCl yielded the highest TPs. Other solvents produced even higher values, but the data did not fit the models, which could be due to variability in the assay or pinto bean particle size, or that a higher model is needed. Still, the data suggest that HCl is not needed for these extractions, as it did not aid and often resulted in lower AC and phenolic values. Lastly, the different compositional profiles as affected by solvent, and even slight changes in process parameters, show that information on the extraction process effects specific for pinto beans needs to be collected in order to take the next step, i.e., identification of the responsible components for delivering the health promoting property.

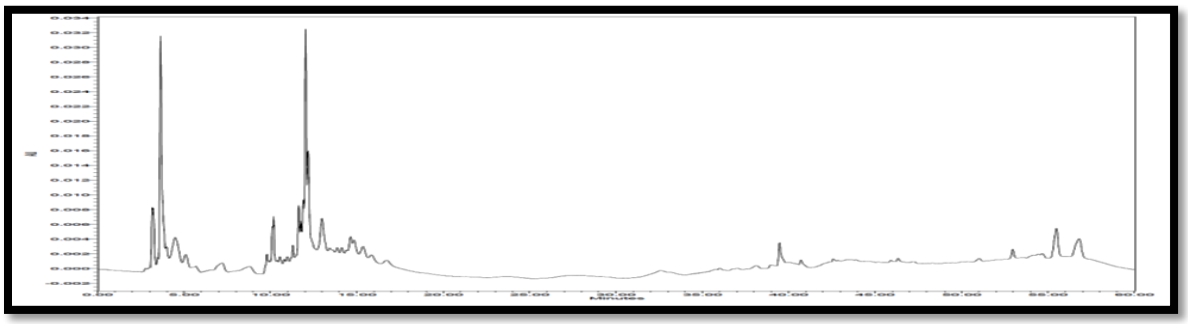
F. APPENDIX:



a) Sample 3: Methanol with HCl, Solvent:Water –75:25, Solid:Solvent – 10%, Mix Time – 60 min. AC: 278.42 μmole Trolox/g, TF:1.42 mg/g, TP:1.34 mg/g.



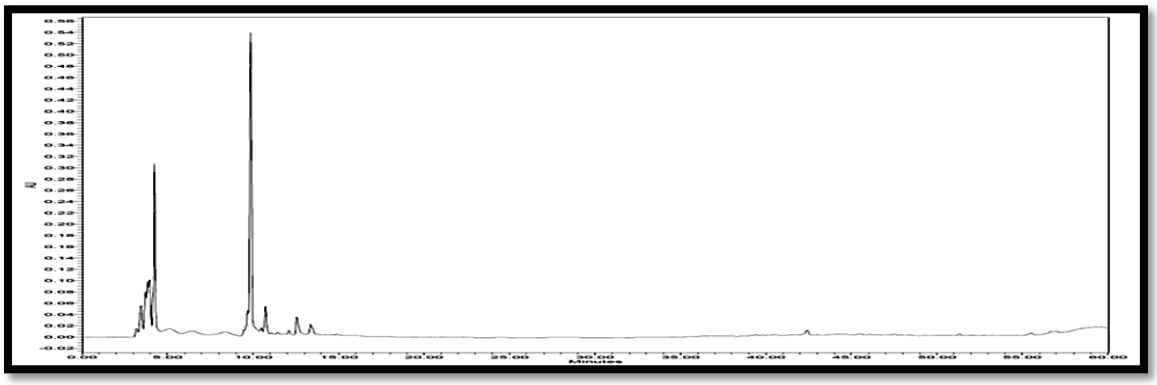
b) Sample 11: Methanol with HCl, Solvent:Water –25:75, Solid:Solvent – 10%, Mix Time – 60 min. AC: 55.62 μmole Trolox/g, TF:0.51 mg/g, TP:0.85 mg/g.



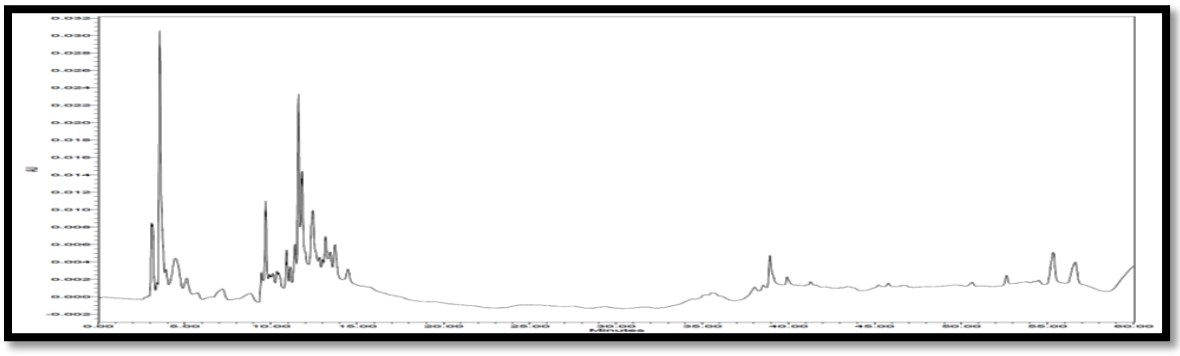
c) Sample 15: Methanol with HCl, Solvent:Water –75:25, Solid:Solvent – 30 %, Mix Time – 180 min. AC: 93.07 μmole Trolox/g, TF: 0.60 mg/g, TP: 0.19 mg/g.

Figure 17: Reverse phase chromatograms of methanol with HCl based extract detected at 280 nm. (AC-antioxidative capacity, TF: total flavonoids, TP: total phenols).

a) Sample 3: Ethanol without HCl, Solvent:Water –75:25, Solid:Solvent – 10%, Mix Time – 60 min. AC: 8.92 μmole Trolox/g, TF: 1.93 mg/g, TP: 0.53 mg/g.

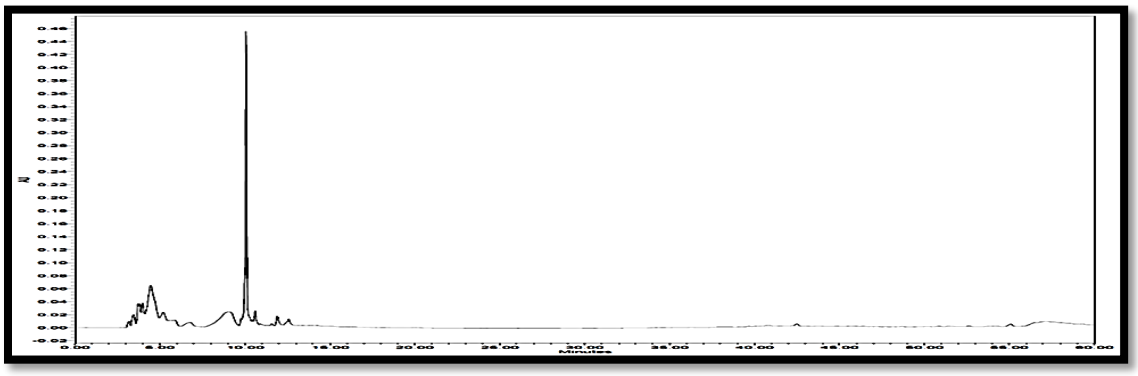


b) Sample 5: Ethanol without HCl, Solvent:Water –50:50, Solid:Solvent – 30 %, Mix Time – 120 min. AC:135.58 μmole Trolox/g, TF:0.73 mg/g, TP:1.06 mg/g.



c) Sample 15: Ethanol without HCl, Solvent:Water –75:25, Solid:Solvent – 30 %, Mix Time – 180 min. AC: 34.67 μmole Trolox/g, TF: 0.89 mg/g, TP: 0.33 mg/g.

Figure 18: Reverse phase chromatograms of ethanol without HCl based extract detected at 280 nm. (AC-antioxidative capacity, TF: total flavonoids, TP: total phenols).



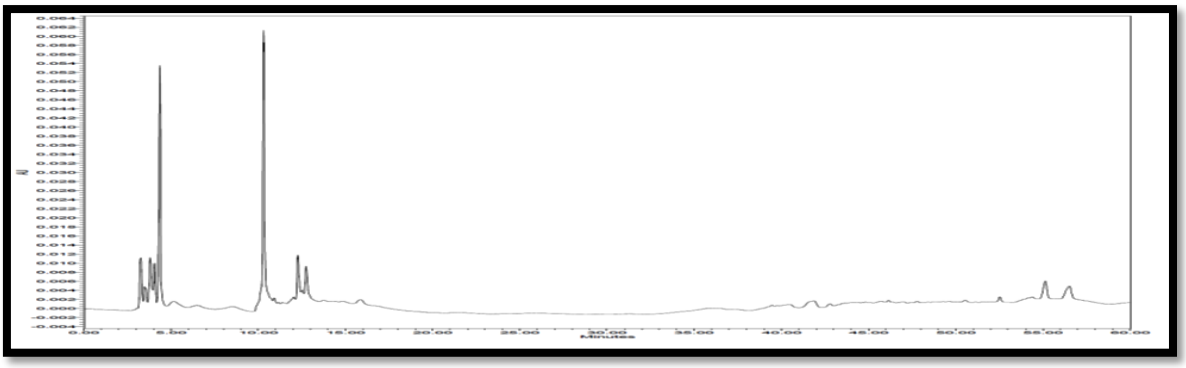
a) Sample 1: Ethanol with HCl, Solvent:Water –75:25, Solid:Solvent – 20%, Mix Time – 120 min. AC: 7.85 μmole Trolox/g, TF: 1.03 mg/g, TP: 1.37 mg/g.

b) Sample 6: Ethanol with HCl, Solvent:Water –50:50, Solid:Solvent – 10%, Mix Time – 120 min. AC: 72.18 μmole Trolox/g, TF: 1.37 mg/g, TP: 1.65 mg/g.

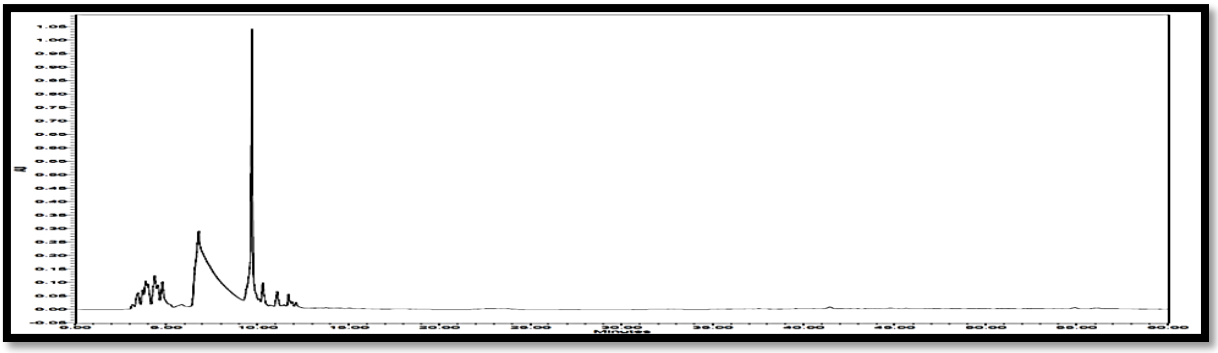
c) Sample 7: Ethanol with HCl, Solvent:Water –25:75, Solid:Solvent – 10%, Mix Time – 180 min. AC: 59.88 μmole Trolox/g, TF: 0.78 mg/g, TP: 0.83 mg/g.

Figure 19: Reverse phase chromatograms of ethanol with HCl based extract detected at 280 nm. (AC-antioxidative capacity, TF: total flavonoids, TP: total phenols).

a) Sample 5: Acetone without HCl, Solvent:Water –50:50, Solid:Solvent – 30%, Mix Time – 120 min. AC: 115.74 µmole Trolox/g, TF: 2.25 mg/g, TP: 1.93 mg/g.



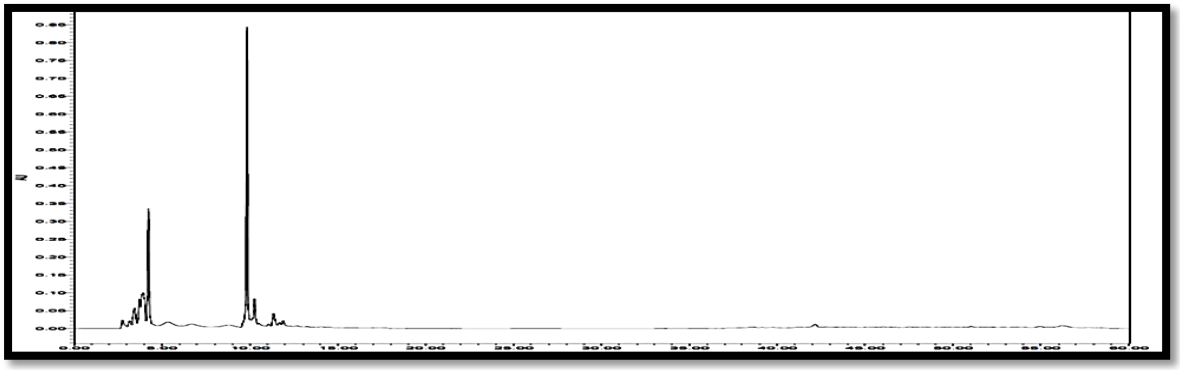
b) Sample 7: Acetone without HCl, Solvent:Water –50:50, Solid:Solvent – 30%, Mix Time – 120 min. AC: 20.75 µmole Trolox/g, TF: 3.46 mg/g, TP: 1.25mg/g.



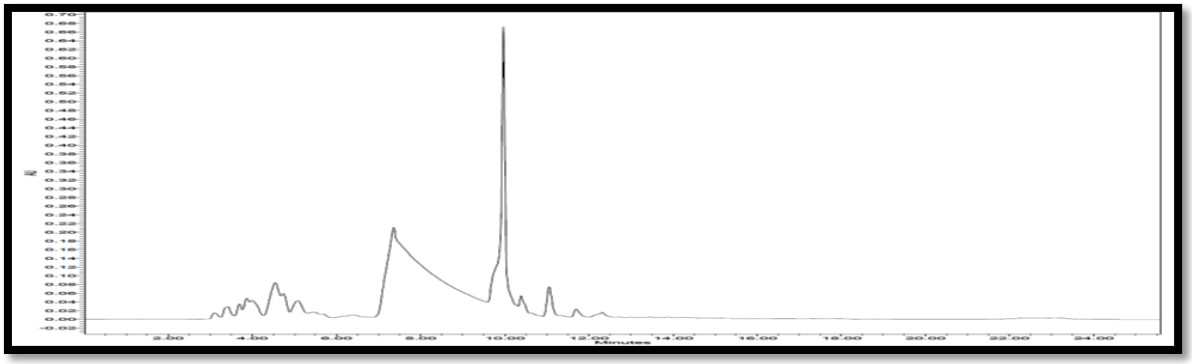
c) Sample 9: Acetone without HCl, Solvent:Water –25:75, Solid:Solvent – 30%, Mix Time – 180 min. AC: 173.03 µmole Trolox/g, TF: 1.80 mg/g, TP: 1.14 mg/g.

Figure 20: Reverse phase chromatograms of acetone without HCl based extract detected at 280 nm. (AC-antioxidative capacity, TF: total flavonoids, TP: total phenols).

a) Sample 2: Acetone with HCl, Solvent:Water –50:50, Solid:Solvent – 20 %, Mix Time – 180 min. AC: 22.86 μmole Trolox/g, TF: 0.31 mg/g, TP: 0.24 mg/g.



b) Sample 5: Acetone with HCl, Solvent:Water –50:50, Solid:Solvent – 30 %, Mix Time – 120 min. AC: 148.10 μmole Trolox/g, TF: 0.26 mg/g, TP: 0.27 mg/g.



c) Sample 10: Acetone with HCl, Solvent:Water –75:25, Solid:Solvent – 30 %, Mix Time – 60 min. AC: 47.35 μmole Trolox/g, TF: 0.20 mg/g, TP: 1.17 mg/g.

Figure 21: Reverse phase chromatograms of acetone with HCl based extract detected at 280 nm. (AC-antioxidative capacity, TF: total flavonoids, TP: total phenols

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