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COOKED COMMON BEANS (*PHASEOLUS VULGARIS* L.): PHENOLIC COMPOUND COMPOSITION AND INFLUENCE OF FRACTIONS ON COLON TUMOR DEVELOPMENT IN *OB/OB* OBESE MICE

presented by

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of the requirements for the

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By

Kathleen Grace Barrett

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ABSTRACT

COOKED COMMON BEANS (*PHASEOLUS VULGARIS* L.): PHENOLIC COMPOUND COMPOSITION AND INFLUENCE OF FRACTIONS ON COLON TUMOR DEVELOPMENT IN *OB/OB* OBESE MICE

By

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The consumption of dry beans has been correlated with the inhibition of several chronic diseases such as cardiovascular disease, type II diabetes mellitus, obesity and cancer. Dry beans contain a variety of phytochemicals (phytic acid, saponins, oligosaccharides, and phenolic compounds) that may confer these health benefits. Phenolic compounds have been shown to possess anti-cancer activity and other health promoting effects. Phenolic compounds in raw common dry beans have been studied, however, beans must be heat-treated (i.e. cooked) before consumption and there is little known about the types and amounts of phenolic compounds found in cooked beans.

Hydroxybenzoic acids and hydroxycinnamic acids were identified in the four cooked dry common beans. The hydroxybenzoic acids found were *p*-hydroxybenzoic acid, vanillic acid, and syringic acid and total concentrations ranged from 3 to 12 mg per 100 g bean flour. Protocatechuic acid was only identified in black, pinto and red beans. The hydroxycinnamic acids identified were *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid in total concentrations ranging from 11to 36 mg per 100 g bean flour. Acid hydrolysis of the beans liberated greater quantities of hydroxybenzoic acids while alkaline hydrolysis liberated greater quantities of hydroxycinnamic acids. Only black, pinto, and red beans contained the flavan-3ol (+)-catechin (0.3 to 3 mg per 100 g bean flour) and the flavonols quercetin and kaempferol (7 to 67 mg per 100 g bean flour).

Since dry beans may contain other colon cancer inhibiting compounds in addition to phenolic compounds, cooked navy beans were fractionated into an aqueous-ethanol soluble fraction (navy bean extract mix, NBEM; concentrated in saponins, oligosaccharides, and phenolic compounds) and an aqueous-ethanol insoluble fraction (navy bean residue, NBR; concentrated in fiber and protein) to narrow the search for which components inhibit the development of colon cancer. A control diet and diets containing either cooked navy beans or its fractions were fed to azoxymethane (AOM) injected mildly diabetic, obese mice (*ob/ob*; B6.V-*Lep*^{ob} /J). This mouse model has not been previously used to evaluate the effect of dietary ingredients on chemically induced colon carcinogenesis.

The *ob/ob* mouse did not have the desired sensitivity to AOM to be recommended for widespread use to test dietary ingredients for colon tumor inhibiting potential. The low tumor incidence in the control fed animals weakened the statistical power to differentiate colon cancer inhibition potential between the extract (NBEM) and the residue (NBR). Nevertheless, compounds contained in the aqueous-ethanol extract of navy beans (NBEM) reduced tumor incidence by 100% compared to the control diet. Additionally, the inhibition of chemically induced colon carcinogenesis in *ob/ob* mice fed navy bean and navy bean fractions was accompanied by a downregulation in the expression *c-fos* in the colonic mucosa. *c-Fos* is required for normal cell cycle progression and proliferation but is overexpressed in some cancers. The downregulation of *c-fos* expression in the colon of *ob/ob* mice fed the navy bean and navy bean fractions may inhibit carcinogenesis by maintaining normal colon crypt cell homeostasis.

DEDICATION

To my father Michael T. Barrett, thank you for your unconditional love and support.

I love and miss you Papa.

Love always, Toots

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I would like to thank my advisor, Dr. Maurice R. Bennink, for his guidance and continued support throughout my graduate program. I have learned so much from him and will always be in his debt. Additionally, thank you to my committee members Dr. Dale Romsos, Dr. Mike Orth, and Dr. Leslie Bourquin for their guidance, time and patience. I would like to express my gratitude to my family and extended family, especially my mom, sister and brother-in-law, for their love and support and extend my love and thankfulness to my beautiful nieces, Parker Grace and Quinn Michael Elaine, who bring a smile to my face everyday. Thank you to my colleague and friend Dr. Beth Rondini. Her assistance with my research will forever be appreciated. I would also like to thank her for lending an ear when I needed to vent and for all the great conversations. Finally and most of all, I would like to thank Cliff Carinci who has been my companion and best friend throughout this long journey. Thank you for always believing in me Cliffy, especially when I did not believe in myself. I love you.

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LIST OF ABBREVIATIONS

(+)-CE ..... (+)-Catechin Equivalent

ACF ..... Aberrant Crypt Foci

AICR ..... American Institute for Cancer Research

AMH ..... Anti-Mullerian Hormone

AOM ..... Azoxymethane

APC ..... Adenomous Polyposis Coli

APCI ..... Atomospheric Pressure Chemical Ionization

BB ..... Cooked, Dried and Ground Black Bean

BCAC ..... β-Catenin Accumulated Crypts

BMP ..... Bone Morphogenic Proteins

C ..... Carbon

Caf ..... Caffeic Acid

Cat ..... (+)-Catechin

CDK ..... Cyclin dependent Kinase

COX ..... Cyclooxygenase

CRC ..... Colorectal Cancer

CVD ..... Cardiovascular Disease

DAD ..... Photodiode Array Detector

DCC ..... Deleted in Colon Cancer

DMH 1, 2-Dimethylhydrazine

ECD ..... Electrochemical Detection

ECM ..... Extracellular Matrix

ESI ..... Electrospray Ionization

FA ..... Ferulic Acid

FAB ..... Fast Atom Bombardment

FAP ..... Familial Adenomatous Polyposis

GC ..... Gas Chromatography

GSK-3β ..... Glycogen Synthase Kinase

HNPCC ..... Hereditary Non-Polyposis Colorectal Cancer

HPLC ..... High Performance Liquid Chromatography

IGFBP ..... Insulin-like Growth Factor Binding Protein

JNK ..... Jun N-Terminal Kinase

K ..... Kaempferol

LAP ..... Latency Associated Peptide

LOH ..... Loss of Heterozygosity

LPH ..... Lactose Phlorizin Hydrolase

LTBPs ..... Latent TGF-β Binding Proteins

LTGF-β ..... Latent Transforming Growth Factor beta protein complex

m- ..... meta-

MAPKs ..... Mitogen Activated Protein Kinases

MDF ..... Mucin Depleted Foci

MMR ..... Mismatch Repair

MS ..... Mass Spectrometer

MSI ..... Microsatellite Instability

MSS ..... Microsatellite Stable

NB ..... Cooked, Dried and Ground Navy Bean

NBEM ..... Navy Bean Extract Mix

NBR ..... Navy Bean Residue

NHANES ..... National Health and Nutrition Examination Survey

NMR ..... Nuclear Magnetic Resonance

o- ..... ortho-

O ..... Oxygen

ORAC ..... Oxygen Radical Absorbing Capacity

p- ..... para-

PAI-1 ..... Plasminogen Activator Inhibitor

PB ..... Cooked, Dried and Ground Pinto Bean

PC ..... Phenolic Compounds

PC ..... Paper Chromatography

PCA ..... Protocatechuic Acid

PCA ..... *p*-Coumaric Acid

PD ..... Plasma Desorption

MALDI ..... Matrix-Assisted Laser Desorption Ionization

PHA ..... Phytohemagglutinin

PHBA ..... *p*-Hydroxybenzoic Acid

PI3K ..... Phosphoinositide Kinase-3

Q ..... Quercetin

RB ..... Cooked, Dried and Ground Red Bean

RER.....Replication Error

RP-HPLC.....Reverse Phase- High Performance Liquid Chromatography

SA.....Sinapic Acid

SCFA.....Short Chain Fatty Acids

SFE.....Supercritical Fluid Extraction

SGLT1.....Sodium Dependent Glucose Transporter 1

SI.....Small Intestine

SPE.....Solid Phase Extraction

Syr.....Syringic Acid

TGF-β.....Transforming Growth Factor Beta

TIP.....Total Identified Phenolics

TLC.....Thin Layer Chromatography

TPC.....Total Phenolic Content

USDA.....United States Department of Agriculture

UV/vis.....Ultraviolet / Visible

UV.....Ultraviolet

v-.....vic-

Van.....Vanillic Acid

WCRF.....World Cancer Research Fund

CHAPTER I. INTRODUCTION

The consumption of diets high in fruits, vegetables, grains and legumes has been associated with a reduced risk for developing chronic diseases such as cancer and cardiovascular disease (Steinmetz and Potter, 1993; Fraser, 1999; Genkinger et al., 2004; WCRF/AICR, 2007). The general recognition that plant foods are important for good health led researchers to investigate which components in plant foods impart these beneficial biological properties. Suggestions that oxidative damage to DNA, RNA and proteins leads to the initiation and progression of certain diseases spurred interest in antioxidants in foods and beverages. There is a plethora of scientific and non-scientific publications extolling the virtues of antioxidants found in various foods and beverages. Sorting fact from fiction is often difficult, particularly for consumers.

Initially, nutritionists encouraged consumption of whole grain cereal products due to the presence of more minerals, vitamins and fiber in whole grains versus products made from refined grains. Now there is an expanded push for consumption of whole grains to increase the dietary intake of non-nutrients such as phytates, sterols, and phenolic compounds. The consumption of dry common beans is typically encouraged because of the protein, fiber, and specific mineral and vitamin contributions to the diet. However dry beans are grain legumes and impart health benefits typically associated with the consumption of whole grains. Beans contain a greater quantity of potentially beneficial phenolic compounds than cereal grains (Adom and Liu, 2002; Adom et al., 2003; Heimler et al., 2005; Xu and Chang; 2007).

Numerous studies indicate that incorporating beans into the diet could aid in the prevention and/or management of chronic diseases such as diabetes, obesity, and cancer. A significant portion of the inverse relationship between bean consumption and chronic disease is related to the low glycemic index of beans and the reduced glycemic load when beans are consumed. However, it is now becoming apparent that compounds traditionally considered as anti-nutrients — phytic acid, plant sterols, phenolic compounds, enzyme inhibitors, and lectins — are beneficial, particularly in affluent Western cultures. The potential for phenolic compounds in beans to impart health benefits has not been extensively studied.

Epidemiologic studies suggest that bean consumption is inversely related to colon cancer incidence and mortality. Studies with animal models of human colon cancer consistently demonstrate that chemically induced colon cancer is reduced by more than 50% by bean containing diets (Hughes and Ganthavorn, 1997; Hangen and Bennink, 2002; Bennink and Barrett, 2004; Rondini, 2006). The component in beans responsible for the colon cancer inhibition and the mechanism of colon cancer inhibition when beans are consumed are not known. Obesity and Type 2 diabetes increase the odds of developing colon cancer (WCRF/AICR, 2007). To date, only lean animal models of colon cancer have been fed beans.

One of the most consistent findings from studies investigating mechanisms leading to colon cancer is the dysregulation of colonic crypt cytokinetics. It is well documented that mutations of the so-called "gatekeeper" gene (*APC* gene) lead to colon cancer. Dietary reduction of colon cancer proceeds through mechanisms that influence

epithelial cytokinetics prior to mutations of the *APC* gene (Lipkin, 1983; Fearon and Vogelstein, 1990).

The overall goals of this research are to:

1. Identify and quantify the principal phenolic compounds in cooked beans;
2. Determine if the phenolic content of beans is related to the development of colon cancer in an obese, diabetic animal model; and
3. Determine if there is dysregulation of gene expression in pathways that are intricately involved with colon crypt cytokinetics.

CHAPTER II. REVIEW OF LITERATURE

A. Legumes (i.e. Dry Beans)

Dry or common beans (*Phaseolus vulgaris* L.) belong to the family of plants called legumes. Legumes are recognized by their ability to "fix" nitrogen and are classified as: oil seeds such as soybeans and peanuts; and grain legumes such as common beans, lentils, chickpeas, common peas, cowpeas, lima and fava beans (Geil and Anderson, 1994). The high nutritive value of dry beans, as well as other legumes, is well established. Besides being high in protein and fiber, they contain no cholesterol and are low in fat, sodium, and calories. Additionally, they contribute iron, phosphorous, magnesium, manganese, potassium, copper, calcium, zinc, and the B vitamins; folate, thiamin, riboflavin and niacin to the diet. Due to their low cost and high protein content, they are often employed as a substitute for meat and meat products. Dry beans have long been a dietary staple in the traditional diets consumed by populations in developing countries in Asia, Africa, and in Central and South America. Even though the United States produces over 1/3 of the world's dry bean supply, the inclusion of dry beans into the typical western diet of the U.S. remains low. In fact, less than 1/3 of the U.S. adult population eats beans during any 3-day period (FASEB, 1995). Commonly consumed dry beans in the U.S. include pinto, navy, kidney, Great Northern, and lima beans. In 1995 the annual kg per person was 1.5, 0.8, 0.3, 0.2, and 0.1 for pinto, navy, kidney, Great Northern, and lima beans respectively in the U.S. (USDA, 1997). Compare this to the annual 20 kg per person consumption of just black beans in Brazil (Ribeiro et al., 2003).

Legumes, including dry beans, contain compounds often referred to as non-nutrients such as amylase inhibitors, protease inhibitors (trypsin inhibitors, trypsin and chymotrypsin inhibitors), lectins, phytates (inositol hexaphosphate), saponins, oligosaccharides (raffinose, stachyose and verbacose), and phenolic compounds (Rochfort and Panozzo, 2007). Some of these non-nutrients are referred to as anti-nutrients because they interfere with protein digestibility (i.e. trypsin inhibitors, trypsin and chymotrypsin inhibitors and some phenolic compounds), and mineral bioavailability (i.e. phytates and some phenolic compounds). Additionally, beans are known to induce discomfort, bloating and flatulence because of oligosaccharides (Champ, 2002; Rochfort and Panozzo, 2007). Many of these factors however are destroyed or inactivated with common processing methods such as heat treatment (i.e. boiling, pressure cooking or autoclaving), hydration (soaking), germination or fermentation (Champ, 2002; Ibrahim et al., 2002; Shimelis and Rakshit, 2007). Humans do not consume beans without processing. Processing improves the palatability, aroma, and acceptability of legumes, while concomitantly improving protein digestibility and mineral bioavailability (Tharanathan and Mahadevamma, 2003). Processing also induce compositional changes to dietary fiber (Kutoš et al., 2003; Costa et al., 2006).

B. Phenolic Compounds

1.1. Background

Phenolic compounds belong to an array of structurally diverse secondary plant metabolites, which are distributed throughout the plant kingdom. They are synthesized during normal plant development and in response to trauma, such as pathological infection, UV radiation or wounding (Stalikas, 2007). Although the physiological

function of some phenolic compounds are known, i.e. anthocyanins act as flower pigments and lignins as structural components of the cell wall (Harborne, 1998), the roles of other classes of phenolic compounds have yet to be fully understood. Research has suggested phenolic compounds can act as bactericides, fungicides and as deterrence factors for predators (i.e. insects and animals). They also may play a role in plant growth regulation and sexual reproduction (Ribéreau-Gayon, 1972; Harborne, 1998; Balasundram, 2006; Stalikas, 2007).

The biosynthesis, metabolism, physiological functions, and chemical structures of phenolic compounds have been studied in plant biochemistry for decades (Bravo, 1998). Food scientists have investigated their organoleptic properties in plant foods and beverages, such as color, flavor, astringency, and hardness (Krygier, 1982; Bravo, 1998; Robbins, 2003), as well as their effect on fruit maturation, role in enzymatic browning and as a food preservative (Ribéreau-Gayon, 1972; Harborne, 1998; López-Amorós et al., 2006). Often their ability to bind proteins and metal ions, leading to reduced protein digestion and mineral absorption, perpetuated the idea that phenolics also acted as anti-nutrients (Krygier, 1982; Hu et al., 2006). In contrast, scientists in various disciplines have now become interested in the potentially beneficial properties of phenolics in humans. Phenolic compounds are most recognized as antioxidants and are capable of scavenging different radicals generated in *in vitro* systems, as well as chelating metal ions that generate reactive hydroxyl radicals (Croft, 1998; Hollman, 2001). Many propose that their antioxidant properties are responsible for the protection against cardiovascular disease, as well as cancer. Additionally, plant phenolic compounds possess anti-inflammatory and antimutagenic activity (Hollman, 2001).

1.2. Classification of Phenolic Compounds

In nature phenolic compounds are found either linked to sugars as glycosides or to organic acids as esters, as such they tend to be water-soluble (Harborne, 1998). They are not found in their parent (termed aglycone) or free form in nature, with the exception of catechin (Ribéreau-Gayon, 1972; Harborne, 1998). Thus phenolic compounds can possess the same parent/aglycone but be linked to different sugar moieties or organic acids and these occur in multiple combinations (Harborne, 1998). Additionally, some phenolic compounds form polymers, as is the case with lignins and tannins (condensed and hydrolysable) (Ribéreau-Gayon, 1972). Phenolic compounds are not uniformly distributed in the plant tissues and contents can vary depending on plant species, variety, time of harvest, maturation, germination, and storage conditions (storage time and temperature) (Tsao, 2004). These factors increase the difficulty and complexity in identifying and quantifying specific compounds and classes of phenolic compounds in plant foods.

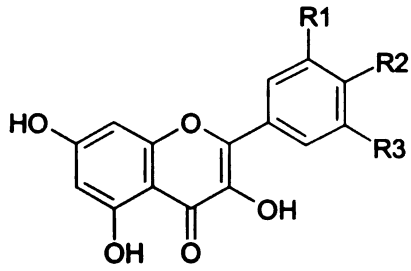
There are at least 8,000 naturally occurring phenolic compounds (Robbins, 2003; Balasundram, 2006; Stalikas, 2007), which possess a common aromatic ring bearing one or more hydroxyl substituents. This shared structure is referred to as a phenol (Harborne, 1998, Stalikas, 2007). They are further categorized into different classes and subclasses based on the number of phenol groups (Harborne, 1998; Robbins, 2003). Table 1 lists the classes of phenolic compounds found in plants. The chemical structures of flavonoids and the other common classes of phenolic compounds are shown in Figure 1. The phenylpropanoids, C_6-C_3, are structurally the most common and most important, since a large amount of phenolic compounds possess this basic carbon structure (Ribéreau-

Table 1. Classes of Phenolic Compounds[a]

Class	Carbon Structure
Simple Phenols	C_6
Hydroxybenzoic Acids and Related compounds	C_6-C_1
Acetophenones and Phenylacetic Acids	C_6-C_2
Phenylpropanoids (Hydroxycinnamic Acids, Coumarins, Chromones)	C_6-C_3
Flavones	C_6-C_3-C_6
Flavanones	C_6-C_3-C_6
Isoflavanoids	C_6-C_3-C_6
Flavonols, Dihydroflavonols, and Related Compounds	C_6-C_3-C_6
Anthocyanidins	C_6-C_3-C_6
Chalcones, Aurones and Dihydrochalcones	C_6-C_3-C_6
Biflavonyls	$(C_6$-C_3-$C_6)_2$
Lignans	$(C_6$-$C_3)_2$
Lignins	$(C_6$-$C_3)_n$
Melanins	$(C_6)_n$
Condensed Tannins (Oligomers of catechins and leucoanthocyanidins = Proanthocyanidins)	$(C_6$-C_3-$C_6)_n$
Hydrolysable Tannins	$(C_6$-$C_1)_n$
Benzophenones, Xanthones and Stilbenes	C_6-C_1-C_6, C_6-C_2-C_6
Quinones	C_6, C_{10}, and C_{14}
Betacyanins	C_{18}

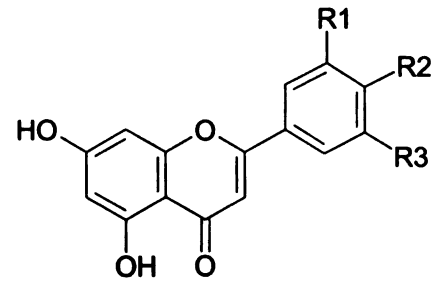
a. Table from the following references Ribéreau-Gayon, 1972 and Harborne, 1998.

A. Flavonols



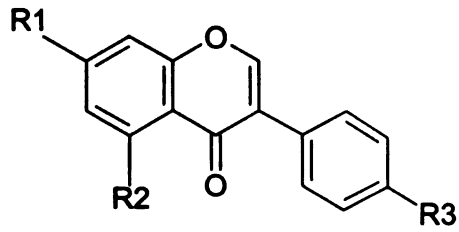
R1=R3=H, R2=OH, Kaempferol
R1=R2=OH, R3=H, Quercetin
R1=R2=R3=OH, Myricetin

B. Flavones



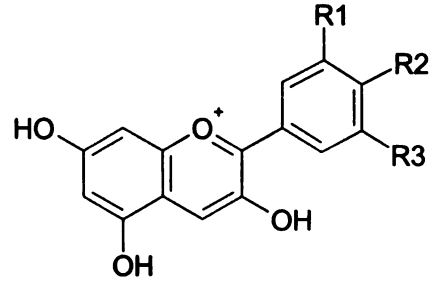
R1=R3=H, R2=OH, Apigenin
R1=R2=OH, R3=H, Luteolin

C. Isoflavones



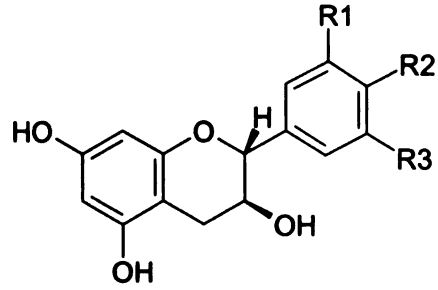
R1=R3=OH, R2=H, Daidzein
R1=R2=R3=OH, Genistein

D. Anthocyanidins



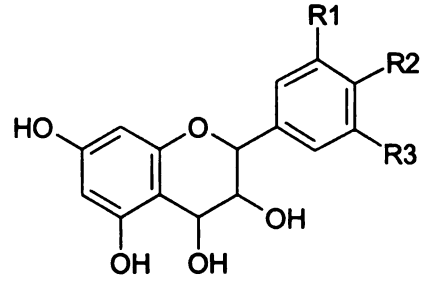
R1=R2=OH, R3=H, Cyanidin
R1=R2=R3=OH, Delphinidin
R1=OCH_3, R2=OH, R3=H, Peonidin
R1=OCH_3, R2=R3=OH, Petunidin
R1=R3=OCH_3, R2=OH, Malvidin

E. Flavan-3ols



R1=R2=OH, R3=H, (+)-Catechin

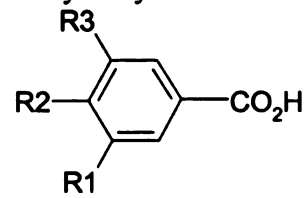
F. Leucoanthocyanidins (Flavan-3, 4diols)



R1=R2=OH, R3=H, Leucocyanidin

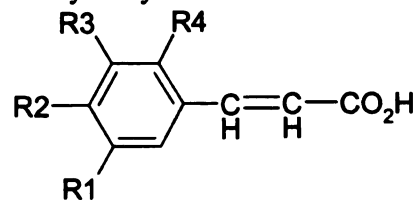
Figure 1. Structures of Phenolic Compounds (Ribéreau-Gayon, 1972)

G. Hydroxybenzoic Acids



R1=R2=R3=OH, Gallic Acid
R1=R3=H, R2=OH, *p*-Hydroxybenzoic Acid
R1=H, R2=R3=OH, Protocatechuic Acid
R1=H, R2=OH, R3=OCH_3, Vanillic Acid
R1=H, R2=OCH_3, R3=OH, Isovanillic Acid
R1=R3=OCH_3, R2=OH, Syringic Acid

H. Hydroxycinnamic Acids



R1=R3=R4=H, R2=OH, *p*-Coumaric Acid
R1=R2=R3=H, R4=OH, *o*-Coumaric Acid
R1=R4=H, R2=R3=OH, Caffeic Acid
R1=R4=H, R2=OH, R3=OCH_3, Ferulic Acid
R1=R3=OCH_3, R2=OH, R4=H, Sinapic Acid

I. Basic Carbon Skeleton of Flavonoids

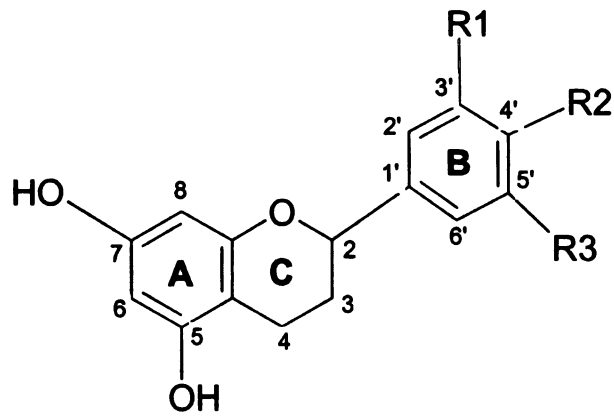


Figure 1. (cont'd)

Gayon, 1972). The flavonoids, C_6-C_3-C_6, represent the largest group of phenolic compounds (Balasundram, 2006; Stalikas, 2007).

Structurally, phenolic compounds are comprised of two phenol groups linked by a 3-carbon chain, often a heterocyclic ring linked to an oxygen molecule, except in the case of chalcones that lack the heterocyclic ring, Figure 1, I (Ribéreau-Gayon, 1972; Harborne, 1998; Balasundram, 2006; Stalikas, 2007). The flavonoids can be divided further based on the level of oxidation and saturation in the heterocyclic ring, as well as the substitution pattern (Ribéreau-Gayon, 1972). The flavonoids include flavonols, flavones, flavanones, isoflavones, anthocyanidins and the less widely distributed chalcones, dihydrochalcones, and aurones (Ribéreau-Gayon, 1972; Harborne, 1998). The degree and pattern of hydroxylation, and the alkylation and/or glycosylation of these hydroxyl groups in the subclasses of flavonoids, contributes to their structural diversity (Rice-Evans et al., 1996; Stalikas, 2007). The A ring is meta-dihydroxylated (C5 and C7) (Ribéreau-Gayon, 1972; Croft, 1998). The B ring is either monohydroxylated (C4'), ortho-dihydroxylated (C3' and C4', most common) or vic-trihydroxylated (C3', C4' and C5") (Ribéreau-Gayon, 1972; Rice-Evans et al., 1996; Croft, 1998).

Flavonols (Figure 1, A), — kaempferol, quercetin, myricetin — are the predominant flavonoids distributed in the plant kingdom. They are found as glycosides, preferentially bound to glucose, but also to galactose, rhamnose, arabinose and xylose (Rice-Evans et al., 1996, Harborne, 1998). The preferred site of glycosylation on flavonols is at the C3 in the heterocyclic C ring, and less frequently at C7 in the A ring (Rice-Evans et al., 1996).

Flavones (Figure 1, B) do not possess the C3 hydroxyl group of flavonols, but are also found as glycosides. However, unlike flavonols, they are not widely distributed in the plant kingdom and are predominantly C-glycosylated, versus O-glycosylated, at C7 in the A ring (Harborne, 1998). Two common flavones are apigenin and luteolin.

Isoflavones such as genistein and daidzein, are found as glycosides and are limited to select plant types, i.e. soybeans (Harborne, 1998). Anthocyanidins (aglycones) are highly unstable and not found in this free form (Manach et al., 2004). Anthocyanidins (Figure 1, D) are instead found as glycosides, termed anthocyanins, as well as esterified to phenolic and organic acids and sometimes in complexes with other flavonoids (Manach et al., 2004).

Flavan-3ols (catechins) and leucoanthocyanidins (flavan-3,4diols) both serve as precursors for condensed tannins (Figure 1, E and F) (Rommel and Wrolstad, 1993; Harborne, 1998; Klepacka and Fornal, 2006). Catechins, unlike most flavonoids, can be found as monomers in nature, as well as, gallic acid esters (gallocatechin). The condensed tannins, which are found universally throughout the plant kingdom, are also called proanthocyanidins. They are oligomers and polymers of catechins and leucoanthocyanidins (Ribéreau-Gayon, 1972; Harborne, 1998; Manach et al., 2004). Although they are not structurally related to anthocyanidins, proanthocyanidins will yield anthocyanidins when heated in acidic solutions (Ribéreau-Gayon, 1972; Rommel and Wrolstad, 1993; Harborne, 1998).

Hydroxybenzoic acids (Figure 1, G) and their related compounds are usually found esterified to alcohol insoluble lignins or as alcohol soluble simple glycosides. *p*-Hydroxybenzoic acid, protocatechuic acid, vanillic acid, and syringic acid are the most

common and universally widespread hydroxybenzoic acids in the plant kingdom (Harborne, 1998). Individual gallic acid subunits and dimers of gallic acid, referred to as ellagic acid, are found in a polymeric form and classified as hydrolyzable tannins (Ribéreau-Gayon, 1972; Harborne, 1998). Hydrolyzable tannins are limited to dicotyledonous plants i.e. legumes, oaks, sunflowers (Harborne, 1998).

Hydroxycinnamic acids (Figure 1, H), the most widespread phenylpropanoids, are found conjugated to organic acids such as quinic acid, tartaric acids, malic acids, and rosmarinic acid via ester bonds, esterified to hemicelluloses (i.e. arabinoxylans, xyloglucans) in the cell wall and as soluble forms in the cytoplasm (Harborne, 1998; Faulds and Williamson, 1999). They can only be found in their free form if released during food processing (Manach et al., 2004). Additionally they can provide the building blocks for lignins, be found as dimers cross-linked to hemicelluloses in the cell wall; as amides of amino compounds; esters of lipids; esters of polysaccharides, simple sugars and sugar alcohols; esters of glycosides of anthocyanins, flavonols and diterpenes; and as glycosides (Clifford, 1999; Naczk and Shahidi, 2006). Common hydroxycinnamic acids include ferulic acid, sinapic acid, caffeic acid, and *p*-coumaric acid (Harborne, 1998).

1.3. Biosynthesis of Phenolic Compounds in Plants

All phenolic compounds, except the flavonoids, originate from the shikimic acid pathway, Figure 2. Shikimic acid is a required intermediate for the formation of phenylpropanoids (Goodwin and Mercer, 1983). Furthermore this pathway is responsible for the synthesis of aromatic amino acids in plants and microorganisms (Ribéreau-Gayon, 1972; Goodwin and Mercer, 1983; Harborne, 1998).

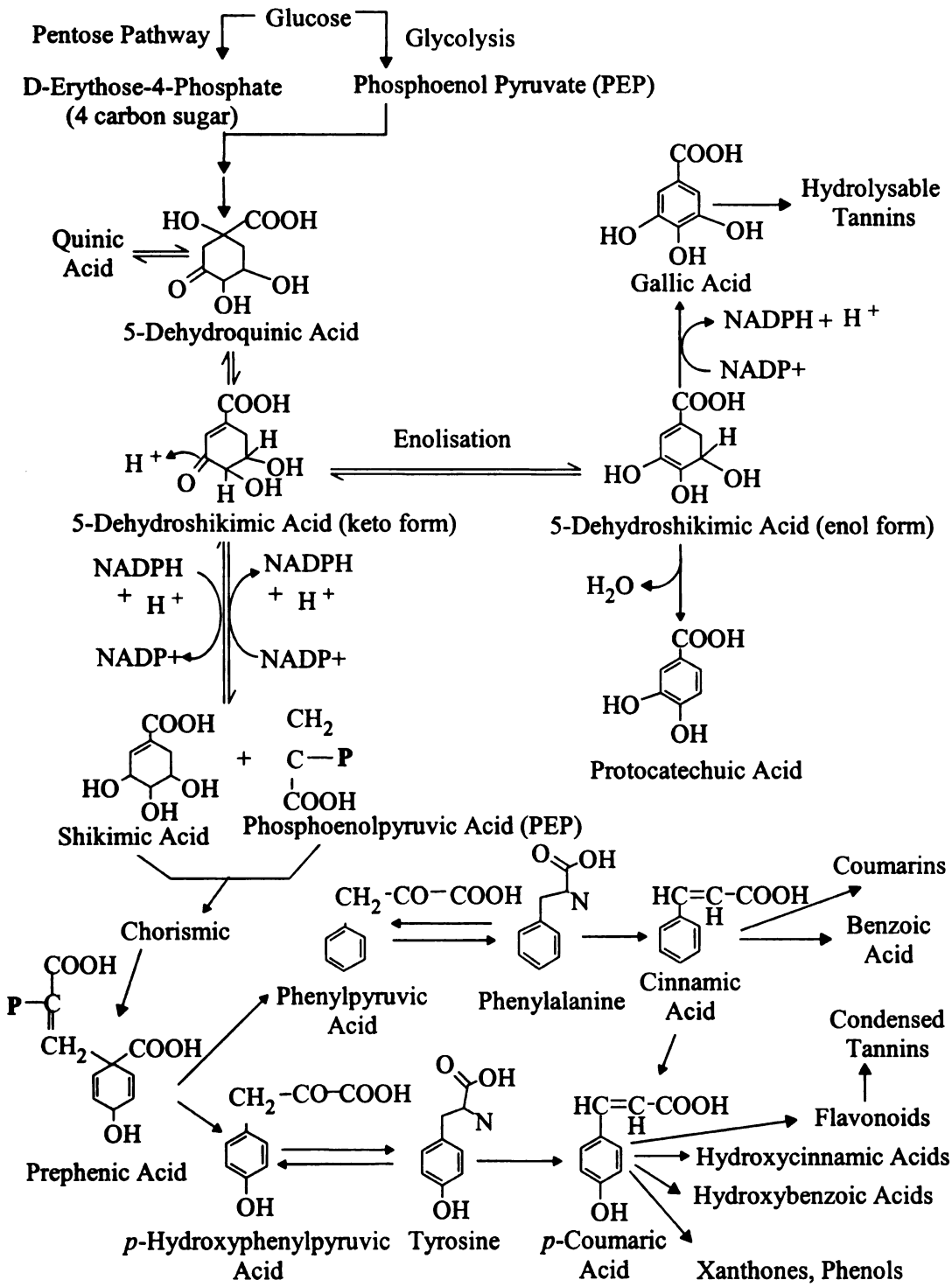


Figure 2. Shikimic Acid Pathway (Ribéreau-Gayon, 1972)

The general carbon skeleton and numbering system for flavonoids is shown in Figure 1, I. The two aromatic phenol groups in flavonoids arise from two different routes (Ribéreau-Gayon, 1972). The 3-carbon (3C) oxygenated heterocyclic ring (C ring) and one of the benzene rings (B ring, right side of the C ring) is formed via the shikimic acid pathway, Figure 2. The 2nd benzene ring (A ring, left side of the C ring) arises from the conjugation of three acetyl molecules via the acetate/malonate pathway (Balasundram et al., 2006; Goodwin and Mercer, 1983; Harborne, 1998; Ribéreau-Gayon, 1972).

1.4. Analytical Methods for Extraction, Purification, Separation and Detection

Only a small number of specific plants have been analyzed for phenolic compounds, thus the data on phenolic compounds in plants is far from complete (Robards, 2003). The plant matrix includes a complex mixture of compounds, including phenolic compounds, which all contribute to the difficulty found when attempting to extract and quantify the phenolic compounds in plant foods and beverages. Additionally, the distribution of phenolics is not uniform throughout the plant material and contents can vary depending on the plant species, variety, time of harvest, maturation, germination and storage conditions (Tsao and Deng, 2004). This results in a great challenge in "extracting a large range of compounds, each of which are structurally complex, from a small amount of material, resulting in microgram amounts" (Harborne, 1998). There are several steps for accurate analysis including: sampling and sample preparation, extraction, separation, and detection (Robbins, 2003; Luthria, 2006). Each step "introduces error, interferents and artifacts which decrease the recovery" or yield

of phenolic compounds (Stalikas, 2007). This emphasizes the importance of choosing techniques and methods with greater selectivity and sensitivity throughout the analytical process (Stalikas, 2007).

1.4.1. Sampling and Sample Preparation

As stated above, phenolic compounds are not evenly distributed throughout the plant tissue and researchers must analyze a representative sample of the entire plant, or they need to document from which part of the plant the sample was taken from, i.e. seeds, leaves, fruit, stems, peels (Luthria, 2006).

As soon as the plant tissue is damaged, compounds are susceptible to enzymatic oxidation and hydrolysis via activated endogenous enzymes e.g. esterases, glycosidases, hydrolases, decarboxylases (Harborne, 1998). Thus before and throughout the analytical process, researchers need to limit the chemical changes resulting from endogenous enzymes, and/or due to sample preparation or storage conditions that could manipulate the type and content of phenolic compounds (Robards, 2003; Tsao and Deng, 2004; Luthria, 2006). Ideally freshly collected plants are the most desirable material for phenolic analysis, but more commonly the plant material is dried by vacuum, air, or low heat. Once thoroughly dried it can be stored for long periods before analysis (Harborne, 1998). Frozen material or freeze-dried material can also be used (Robards, 2003). Other methods of sample preparation prior to extraction include milling, grinding, maceration, homogenization, centrifugation, or cooking and are dependent on the type of plant food or beverage to be analyzed. Beverages such as wines and fruit juices often require little, if any preparation, besides filtration or centrifugation followed by separation and detection (Harborne, 1998; Robbins, 2003; Stalikas, 2007).

1.4.2. Extraction

Due to the complexity of the plant food matrix and the diversity of phenolic compounds, a generalized or standardized method for extraction of all phenolic compounds, or for that matter separation and detection, is not available (Harborne, 1998; Robards, 2003). Plant tissues contain a large variety of structurally diverse compounds, including phenolic compounds, and each individual component possesses physiochemical behaviors (Stalikas, 2007). Additionally interactions between phenolic compounds and other macromolecules can take place and form insoluble complexes within the plant cells (Naczk and Shahidi, 2006). The solubility of the analytes (i.e. phenolic compounds) of interest in a particular extraction solvent and other physiochemical behaviors of these analytes will strongly influence the choice of extraction solvent (Robards, 2003; Robbins, 2003; Stalikas, 2007). Common extraction solvents include alcohols (e.g. methanol, ethanol), acetone, ethyl acetate and dimethyformamide, together with varying amounts of water, utilized alone or in combination (Harborne, 1998; Naczk and Shahidi, 2006). The amount of water added is dependent on the water content in the starting plant material (Harborne, 1998; Robards, 2003). Other measures employed to enhance the interaction between the plant material and extraction solvent include homogenization, sonication, vortexing, and increasing the solvent to sample ratio (Robbins, 2003). Extractions are often repeated two to three times and extraction time can range from 1 to 12 hours (e.g. Soxhlet extraction) (Robbins, 2003; Stalikas, 2007). Sequential extractions, via increasing or decreasing polarity of the extraction solvent, can be utilized to broaden the range of phenolic compounds extracted while limiting the amount of interfering

components such as protein. The final crude extract is often concentrated via rotary evaporation (Harborne, 1998).

Although there is not a general extraction method to extract all phenolic compounds from plant material, some methods have been optimized for specific phenolic classes. For example aqueous-methanol mixtures efficiently extract flavan-3ol monomers and condensed tannins with a low degree of polymerization, whereas aqueous-acetone mixtures maximize the extraction of oligomers and polymers of proanthocyanidins. When extracting very polar phenolic compounds i.e. hydroxybenzoic acids, hydroxycinnamic acids, and some flavonoid glycosides, increasing the percentage of water in the aqueous-alcohol solvent mixture will enhance their extraction (Robards, 2003). Adding a small amount of acetic or hydrochloric acid to aqueous-alcohol solvents enhances the extraction of anthocyanins given that they are unstable in neutral and alkaline solutions (Harborne, 1998).

Other plant components such as carbohydrates, lipid material, and/or non-phenolic compounds that interfere with the extraction and quantification of phenolic compounds are often removed prior to or after the extraction process. For example non-polar plant components i.e. waxes, chlorophyll, oils, sterols, are extracted with non-polar solvents e.g. hexane, chloroform, prior to the extraction of phenolic compounds (Harborne, 1998).

Newer extraction techniques being employed by researchers include supercritical fluid extraction (SFE) and microwave assisted extraction. These techniques attempt to reduce extraction time, heat, and oxygen exposure in order to lessen the chemical alterations to phenolic compounds (Robards, 2003; Stalikas, 2007).

Often a hydrolysis step is performed to simplify the separation and quantification of phenolic compounds, as well as to remove unwanted compounds in the plant matrix that would otherwise interfere with the analysis (Tsao and Deng, 2004; Stalikas, 2007). Since the majority of phenolic compounds are found conjugated in the plant matrix, hydrolysis is applied to release the aglycones or free forms and to simplify their characterization and quantification (Tsao and Deng, 2004; Stalikas, 2007). When attempting to identify and/or quantify phenolic compounds in their conjugated or natural state, hydrolysis would be bypassed, however this depends on the availability of authentic commercial standards and a researcher's access to specific equipment and assays.

Acid and alkaline (saponification) hydrolysis are the most common techniques applied to release the phenolic compound aglycones from their natural conjugated state. Enzymatic hydrolysis is another possibility, however it is rarely used with crude plant extracts. The enzymes: β-glucuronidase and/or sulfatase are often applied to biological samples (i.e. plasma, serum, urine, bile) to identify the parent phenolic compound of metabolites (Stalikas, 2007).

Acid hydrolysis is carried out on the solid plant food or the plant extract with 1 to 2N HCL at reflux or above reflux temperatures in aqueous or alcoholic solvents. The reaction time varies from 30 minutes to 1 hour. Acid hydrolysis is capable of cleaving glycosidic bonds (-C-O-C-) resulting in the release of sugar moieties; thus it is commonly utilized to convert flavonoid glycosides to their aglycones. Yet the resistance to acid hydrolysis for different glycosidic bonds will vary and acid hydrolysis can induce undesired changes to some phenolic compounds (Ribéreau-Gayon, 1972). The anthocyanins are more resistant to acid hydrolysis than flavonol and flavone glycosides,

with flavonoid C7-glycosides being particularly more stable compared to C3-glycosides. Ether bonds (R-O-R') and *C*-glycosides vs. *O*-glycosides are very resistant to acid hydrolysis (Ribéreau-Gayon, 1972). Additionally, acid hydrolysis cleaves esters bonds to release free phenolic acids (i.e. hydroxybenzoic and hydroxycinnamic acids). Researchers, however, have observed the loss of hydroxycinnamic acids exposed to hot acidic conditions because they undergo decarboxylation; thus they are best obtained by mild alkaline hydrolysis (Krygier et al., 1982; Gao and Mazza, 1994; Harborne, 1998). Flavanones are converted to chalcones in acid solutions, resulting in an intense color (Ribéreau-Gayon, 1972). Leucoanthocyanidins are converted to anthocyanidins and form condensation products in heated acid solutions resulting in a red colored solution. Thus a researcher must be aware that anthocyanidins in acid hydrolysates may come about from anthocyanins or leucoanthocyanidins (Ribéreau-Gayon, 1972). Catechins, naturally found as monomers, not glycosides, will also form condensation products in acid, but they are not red in color. So flavans, i.e. flavan-3ols and leucoanthocyanidins should not be analyzed following acid hydrolysis (Ribéreau-Gayon, 1972). Rommel and Wrolstad (1993) reported losses of quercetin and kaempferol aglycones following acid hydrolysis of raspberry juice extracts. The expected quantities of aglycones determined from the concentrations of quercetin and kaempferol glycosides in the unhydrolyzed raspberry juice extracts, were estimated to be much higher than the quantities observed.

Alkaline hydrolysis is carried out on the solid plant food or the aqueous plant extract with 1 to 4 M NaOH at room temperature or at low heat. To inhibit the oxidation of phenolic compounds, hydrolysis is carried out under an inert gas such as nitrogen gas. The reaction time varies from 4 hours to overnight. The alkaline hydrolysate is then

acidified with HCL before analysis (Ribéreau-Gayon, 1972; Harborne, 1998; Robbins, 2003). Alkaline hydrolysis is capable of cleaving ester bonds and removing acylated portions of flavonoids, such as acylated anthocyanins (i.e. phenolic acids acylated to sugar moiety of anthocyanins) (Gao and Mazza, 1994; Harborne, 1998; Robards, 2003; Stalikas, 2007). As stated previously, hydroxycinnamic acids are more stable in mild alkaline solutions than aqueous acidic solutions and best obtained by mild alkaline hydrolysis (Krygier et al., 1982).

Krygier et al. (1982) observed the percent loss caused by alkaline and acid hydrolysis on authentic hydroxycinnamic acids standards. They reported a 15.1% to 91.7% and 2.7% to 66.7% loss resulting from acid or alkaline treatment, respectively. The percent lost by either treatment was dependent on the compound. Acid hydrolysis resulted in almost the complete loss of caffeic acid (87%) and trans-sinapic acid (92%), whereas the losses of *o*-coumaric, *p*-coumaric, trans-isoferulic and trans-ferulic acid were 15%, 73%, 50% and 78%, respectively. The same compounds were reported to be more stable under the alkaline treatment, except for caffeic acid (67% loss).

Dabrowski and Sosulski (1984) reported the losses of authentic hydroxybenzoic and hydroxycinnamic acid standards added to rapeseed flour, extracted and exposed to alkaline conditions. They reported the losses for the hydroxybenzoic acids: vanillic, *p*-hydroxybenzoic and syringic acid did not exceed 10%. The percent loss reported for the hydroxycinnamic acids: *p*-coumaric acid, ferulic acid and sinapic acid, following alkaline treatment were 13%, 18% and 22%, respectively. As was reported by Krygier et al. (1982), caffeic acid was almost completely lost under the alkali conditions with a reported 84% loss. Both authors reported that high losses of caffeic acid under alkali or

acid treatment were the result of the high reactivity of *o*-dihydroxyphenols (Figure 1, H), which can be rapidly oxidized to quinones. Caffeic acid appears to be unstable under either hydrolysis treatment.

Common methods used to clean-up/purify crude extracts or the hydrolysates are liquid-liquid extraction or solid phase extraction (SPE). Both methods can be used to fractionate phenolic compounds as well as remove unwanted components.

The solvents used for liquid-liquid extraction depend on the desired compounds and can be based on polarity or acidity. For example when basing the liquid-liquid extraction on polarity, the solvents: ethyl acetate, ether, or the combination of the two, would be used to extract phenolic acids, flavonols and flavones. If anthocyanidins are present the solvents above would be used to separate or remove all other phenolic compounds from the anthocyanidins remaining in the more polar solution (Ribéreau-Gayon, 1972). When the liquid-liquid extraction is based on acidity, neutral compounds (e.g. flavonols) would be separated from the acidic compounds (e.g. phenolic acids) by adjusting the plant extract sample's pH to 7.0 and adding ethyl acetate. The neutral compounds would be more soluble in the ethyl acetate when the pH=7.0. Once the ethyl acetate is removed, the remaining extract would be adjusted to pH 2.0 and ethyl acetate would be used to extract the acidic compounds (Harborne, 1998).

SPE is a fast and reproducible procedure to clean-up extracts and hydrolysates and can result in fairly purified extracts (Stalikas, 2007). The most common sorbent for analyzing phenolic compounds is reverse phase C_{18} bonded to silica (Stalikas, 2007). The extracts and hydrolysates and/or solvents are slightly acidified to prevent ionization of the phenolics, which could reduce their retention (Stalikas, 2007). Similar to liquid-

liquid extraction, solvents of different polarity or pH can be used to remove unwanted components, to separate larger phenolics from smaller phenolics, or to separate different classes of phenolics from each other (Harborne, 1998; Robbins, 2003; Stalikas, 2007). For example, Skrede et al. (2000) separated anthocyanins from other phenolic compounds in concentrated blueberry juice using a C_{18} SPE cartridge. After applying the acidified aqueous sample to the activated C_{18} SPE cartridge, acidified water (0.01% HCL) was applied to elute unwanted water-soluble compounds i.e. sugars. This was followed by acidified methanol (0.01% HCL) to elute the anthocyanins and finally ethyl acetate to elute all other phenolic compounds (Skrede et al., 2000). A limitation of SPE is column saturation, so small volumes are required. Recoveries are rarely reported, yet Rommel and Wrolstad (1993) reported low and variable recovery of hydroxybenzoic acids (less than 10%), especially protocatechuic and gallic acid, but close to 100% recovery for flavonol aglycones, flavan-3ols and ellagic acid from C_{18} cartridges.

1.4.3. Separation and Detection

Thin-layer chromatography (TLC) and paper chromatography (PC) were often used to separate, characterize, and quantify phenolic compounds and other plant components (Ribereau-Gayon, 1972; Harborne, 1998; Robards, 2003). Although TLC and PC are not widely used today, they remain an important tool to screen crude plant extracts for phenolic compounds (Harborne, 1998; Robards, 2003).

High performance liquid chromatography (HPLC) is currently the predominant analytical method for the separation and characterization of phenolic compounds (Robbins, 2003; Robards, 2003). Equal in sensitivity and selectivity to HPLC, gas chromatography (GC) can be used, however it is not as popular to separate phenolic

compounds because of their low volatility and the possible chemical changes induced by the high temperatures required for GC (Robards, 2003).

Plant constituents, i.e. phenolic compounds, exhibit unique spectral characteristics, consequently a means for their detection are ultraviolet/visible (UV/Vis), photodiode array (DAD) and ultraviolet/fluorescence detectors (Harborne, 1998; Robbins, 2003; Robards, 2003; Naczk and Shahidi, 2006; Stalikas, 2007). No single wavelength is ideal for all phenolic classes because each compound displays absorption maximas at distinctly different wavelengths (Robards, 2003). Hydroxybenzoic and hydroxycinnamic acids have a maxima absorbance in the range of 200 to 290 nm, except gentisic acid (hydroxybenzoic acid) whose absorbance extends to 355 nm; hydroxycinnamic acids have a second maxima absorbance in the range of 270 to 360 nm due to the additional conjugation; flavonoids have two absorption maximas. The first is in the range of 240 to 285 nm, and a second in the range of 300 to 550 nm. The first maxima absorbance is due to the A ring, and the second is due to substitutions and the conjugation of the heterocyclic C ring. Routinely 280 nm is chosen to represent flavonoids, 254 nm for phenolic acids (hydroxybenzoic and hydroxycinnamic acids) and 520 nm for anthocyanins (Ribereau-Gayon, 1972; Harborne, 1998; Robbins, 2003; Robards, 2003; Stalikas, 2007). Analytes can be monitored after separation with 1 or more UV detectors set at different wavelengths to aid in the detection of a range of phenolic compounds, e.g. 254 nm and 280 nm; 280 nm and 320 nm. DAD detectors are capable of scanning real time UV/Vis spectra of the analytes enhancing the ability to detect a range of phenolic compounds in a complex matrix. Care must be taken in selecting mobile phases that do not absorb at the set wavelengths. Although all phenolic

compounds do not display fluorescence, when used in combination with UV detection it can distinguish between co-eluting compounds. However, fluorescence is not widely used (Stalikas, 2007).

Limitations of spectrophotometric methods of detection include poor sensitivity and the inability to distinguish between co-eluting compounds even if they absorb light at different wavelengths (Bocchi et al., 1996; Robards, 2003; Stalikas, 2007). Electrochemical detection (ECD) is becoming an increasingly popular tool for detection of phenolic compounds for its higher sensitivity and selectivity compared to UV/Vis and DAD detectors. ECD is based on the oxidation or reduction of compounds. Briefly the HPLC eluent flows over an electrode or series of electrodes set at increasely higher voltages. Electroactive compounds will either donate electrons (oxidize) or accept electrons (reduce) resulting in a current that is measured in real time. The current peak generated is dependent on the concentration of the analyte and the voltage applied. Each compound has a specific voltage at which they begin to be oxidized (Svendsen, 1993). There are two types of EC detectors: 1. flat-plate amperometric detectors which oxidize or reduce approximately 5% of the compound flowing over it; 2. porous flow-through amperometric detectors which have larger surface areas and can oxidize close to 100% of the compound flowing through them, termed coulometry (Svendsen, 1993). Serial arrays of coulometric electrodes (8 to 16 electrodes) are more sensitive and require less maintenance than amperometric arrays. As the analytes pass through the electrodes of increasing voltage, they will be oxidized 100% at their oxidation potential and no other peaks will be visible at subsequent electrodes (Svendsen, 1993). HPLC-ECD mobile

phases are acidic buffers, which suppress the dissociation of weakly acidic phenolic compounds (Svendsen, 1993; Jandera et al., 2005).

For structural identification of phenolic compounds, HPLC and GC can be coupled to mass spectrometer (MS) detectors. MS is an important tool because it can provide structural data on small (microgram) amounts of sample, can give accurate molecular weights and generate complex fragmentation patterns which can be used to characterize specific compounds (Harborne, 1998). Thus it can identify conjugated phenolic compounds. Two MS methods widely employed are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Other methods include fast atom bombardment (FAB), plasma desorption (PD) and matrix-assisted laser desorption ionization (MALDI). To further increase detection sensitivity and selectivity, MS analyzers can be coupled in series of 2 (MS-MS) or more (MS^n) (Harborne, 1998; Tsao and Deng, 2004; Stalikas, 2007).

Nuclear magnetic resonance (NMR) spectroscopy, proton-NMR and carbon 13-NMR, can be coupled to HPLC to identify novel phenolic compounds and known compounds without great isolation or separation. It is often used to identify structures of compounds that could not be distinguished by mass spectral data (Robards, 2003; Stalikas, 2007). NMR, however, can be quite complex and requires highly trained individuals to run it (Harborne, 1989).

Many researchers utilize colorimetric methods to quantify total phenolic content (TPC) in complex mixtures. The Folin-Ciocalteu method is a frequent choice. The reaction involves the oxidation of phenols by phosphomolybdic-phosphotungstic acid resulting in a reduced molybdenum-tungsten blue complex. The reaction develops over

30-90 minutes and the blue complex is detected at an absorbance between 725-765 nm. An authentic standard phenolic compound (e.g. gallic acid or (+)-catechin) generates a standard curve to quantify the total phenolics. A limitation is the presence of other phenol containing compounds and antioxidants leading to overestimations in total phenolic compound content (Singleton and Rossi, 1965).

1.5. Intake, Absorption and Metabolism of Phenolic Compounds

1.5.1. Intake

The consumption of diets high in fruits, vegetables, grains and legumes have been associated with a reduced risk for developing certain chronic diseases, i.e. cancers and cardiovascular diseases (Steinmetz and Potter, 1993; Fraser, 1999; Genkinger et al., 2004; WCRF/AICR, 2007). There is speculation that the high concentration of phenolic compounds found in plant foods and beverages may exert some of these observed health benefits. In order to investigate the proposed relationship between diets high in phenolic compounds and reduced disease risk, knowing their total content and distribution within plant based foods and beverages are crucial (Manach et al., 2004). Currently phenolic compound profiles and content for many plant foods and beverages are incomplete, making it difficult to estimate an accurate daily intake of total and/or individual phenolic compounds consumed by humans. Research has identified specific phenolic compounds or classes in widely consumed foods that have displayed the greatest biological activity in the literature (Manach et al., 2004). It must be acknowledged that the identification and quantification of phenolic compounds in plant foods are frequently based on the aglycone form following hydrolysis of the glycosidic or ester bonds (Walle, 2004; Manach et al., 2004), and not the compounds in their natural state. However as stated in the previous

section this is common practice due to the large diversity and number of phenolic compounds in nature and simplifies the identification and quantification process (Ribéreau-Gayon, 1972; Harborne, 1998). In 2007, the USDA published the most current flavonoid database that compiled data from "acceptable" studies and reported the content for five subclasses of flavonoids (flavonols, flavones, flavanones, flavan-3ols and anthocyanidins) measured in 385 food items (USDA/ARS, 2007). To date this is the only flavonoid database, yet again it only analyzes the most commonly studied flavonoids in relatively few food items.

Chun et al. (2007) utilized the USDA flavonoid database and 24 hr recall data from NHANES 1999-2002 surveys to estimate an average daily flavonoid intake of 187.7 mg/day in the U.S. If one consumes diets high in red, blue, and purple fruits, or drinks moderate amounts of red wine, ingesting over 100 mg of anthocyanins per day could occur (Manach et al., 2005). Coffee drinkers consume significant amounts of chlorogenic acid, an ester of caffeic acid, since one 200 ml cup provides approximatedly 70-200 mg of chlorogenic acid (Clifford, 1999). Flavan-3ols (catechins) and proanthocyaninidins are found in high amounts in green tea, red wine and chocolate and consumption of these products can markedly influence total phenolic intakes. There does not appear to be any estimates of typical daily intakes of hydroxybenzoic acids.

1.5.2. Absorption in stomach and small intestine

The extent and site of phenolic compound absorption is far from complete. Figure 3 presents an overview of the absorption of phenolic compounds. After ingestion, phenolic compounds enter the stomach. Small quantities of phenolic glycosides and

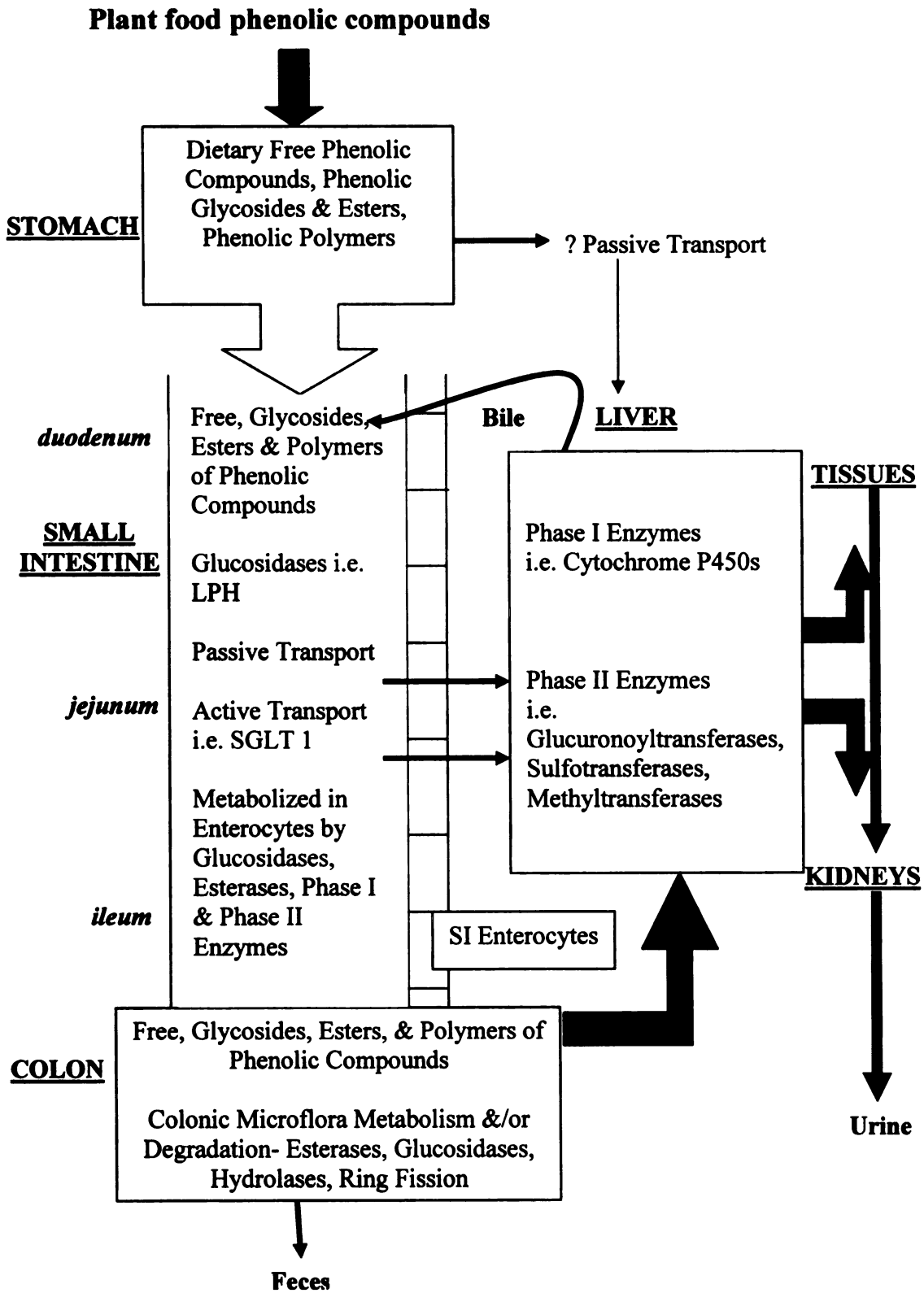


Figure 3. Absorption and Metabolism of Phenolic Compounds

phenolic esters may be freed during food storage and processing (Manach et al., 1998) making them available for absorption in the stomach. Coffee and wheat bran have been incubated with *in vitro* gastric juices to determine the effect of the acidic gastric environment on the soluble hydroxycinnamic acid esters (i.e. chlorogenic acid in coffee) and hydroxycinnamic acids esterified to the plant cell wall in wheat bran). No effect was observed on the caffeic acid derivatives in the coffee, e.g. no hydrolysis of chlorogenic acid to caffeic acid and quinic acid; and minimal amounts ferulic acid and ferulic acid esters (~1.3%) were released from wheat bran (Kroon et al., 1997; Rechner et al., 2001). There is little good evidence that phenolic compounds are absorbed in appreciable amounts from the stomach.

The majority of phenolic compounds and their derivatives would pass through the stomach and enter the small intestine (SI). Once in the SI, the glycoside derivatives could be deglycosylated by β- glucosidases in the lumen or part of the brush border. LPH (lactose phlorizin hydrolase), a β-glucosidase located on the brush border deglycosylates flavonol glucosides and diglucosides prior to absorption (Day et al., 2000; Nemeth et al., 2003). Phenolic aglycones freed prior to entering the SI or as a result from hydrolysis by glucosidases could enter the enterocyte by passive diffusion (Hollman, 2001; Petri et al., 2003). Phenolic glycosides may also enter enterocytes by the sodium-dependent glucose transporter (SGLT1) (Hollman et al., 1995; 1996; Gee et al., 1998; 2001; Hollman, 2001).

The absorption and metabolism of the flavonols has been extensively studied. Hollman et al. (1995, 1996, 1997) argue that the majority of flavonols are absorbed in the SI and maybe even in the stomach. However, research also suggests that certain flavonol

glycosides (i.e. rutin) are passed to the colon (Hollman et al., 1995; 1997; Olthof et al., 2000; Hong et al., 2004). Flavan-3ols, such as catechins and epicatechins, are found as aglycones, gallate esters or polymers (condensed tannins or proanthocyanidins) in plants. Although some of the consumed flavan-3ols — catechins and epigallocatechin — are absorbed prior to the colon (Hollman et al., 1997; Spencer et al., 2006), most flavan-3ols and proanthocyanidins pass into the colon (Hollman et al., 1997; Kahle et al., 2007). Hydroxycinnamic acids found esterified to the cell wall polymers cannot be absorbed in the SI and would also pass into the colon (Kroon et al., 1997; Rechner et al., 2001; Adam et al., 2002; Rondini et al., 2002; 2004).

1.5.3. Metabolism by microflora and absorption in the colon

Phenolic compounds not absorbed in the SI would enter the colon. The colon contains approximately 10^{12} microorganisms/cm and the microorganisms have the ability to hydrolyze and metabolize dietary compounds (Spencer et al., 2006). Colon microfloral enzymes include: β-glucosidases, β-rhamnosidases, esterases, xylanases, hydrolases and oxidases (Kroon et al., 1997; Plumb et al., 1999; Andreasen et al., 2001; 2001; Rechner et al., 2004; Jenner et al., 2005). Esterases cleave phenolic compound esters and phenolic acids esterified to plant cell walls, releasing the free phenolic compounds (aglycones) (Plumb et al., 1999; Andreasen et al., 2001; 2001). The metabolites resulting from the microfloral metabolism of phenolic compounds are phenolic acids and other aromatic acids, mainly derivatives of phenylpropionic, phenylacetic, benzoic and cinnamic acid (Gonthier et al., 2003). These microbial metabolites can then be absorbed in the colon, metabolized in the liver and excreted in the urine or excreted in the feces.

Rechner et al. (2004) specifically examined colonic metabolites formed when hydroxycinnamic acids (chlorogenic acid), flavanone glycosides (naringin) and flavonol glycosides (rutin) are incubated with a mixed culture of human colonic microflora *in vitro*. For chlorogenic acid (5-caffeoyl-quinic acid), the first metabolic product was caffeic acid resulting from the cleavage of the ester bond between caffeic acid and quinic acid, followed by the reduction of the caffeic acid double bond forming 3-(3,4-dihydroxyphenyl)-propionic acid, then dehydroxylation to form 3-(3-hydroxyphenyl)-propionic acid and finally the 2nd dehydroxylation to form 3-phenylpropionic acid (non-phenolic acid). *In vivo* the metabolites formed at each step could be absorbed from the colon. For example, caffeic acid could be absorbed intact or as any of the microbial metabolites — 3-(3,4-dihydroxyphenyl)-propionic acid, 3-(3-hydroxyphenyl)-propionic acid or 3-phenylpropionic acid (Rechner et al., 2004).

The first metabolic step for both flavanone glycosides and flavonol glycosides was deglycosylation. This was followed by ring fission in the heterocyclic C-ring (see Figure 1, I). For naringen, the C-ring ring fission was between the oxygen and C2 and between C4 on the C-ring and the A-ring. This resulted in the transient formation of phloroglucinol derived from the A-ring fragment, and either 3-(4-hydroxyphenyl)-propionic acid and 3-phenylpropionic acid (via dehydroxylation) derived from the B-ring fragment. These two metabolites can be absorbed. For example, rutin entering the colon is hydrolyzed by microbial enzymes to quercetin and rutinoside. Fission of the heterocyclic C-ring produced three different ring fission products: 1) product 1— fission between the oxygen and C2 and between C3 and C4 on the C-ring; 2) product 2 — fission between the oxygen and C2 and between C4 on the C-ring and the A-ring; 3)

product 3 — fission between oxygen and the A-ring and C2 and C3 in the C-ring. The A-ring fragments were not detected and assumed to be degraded rapidly or destroyed with the ring fission. The endproducts from the first two ring fissions include 3,4-dihydroxyphenylacetic acid or 3-(3-hydroxyphenyl)-propionic acid, respectively, both derived from the B-ring. The colonic bacteria can dehydroxylate the 3,4-dihydroxyphenylacetic acid to hydroxyphenylacetic acid which can be then be absorbed along with 3-(3-hydroxyphenyl)-propionic acid. The hydroxyphenylacetic acids are specific metabolites of flavonol glycosides arising from colonic microorganism metabolism (Rechner et al., 2004).

1.5.4. Metabolism after absorption

A phenolic compound absorbed in the SI enterocyte as a glycoside can enter the bloodstream as the intact glycoside. However they are more likely to be deglycosylated by cytoplasmic β-glucosidases. Phenolic esters absorbed from the SI are de-esterified by cytoplasmic esterases. The resulting aglycones are either released into the portal circulation or further metabolized by the intestinal cells (Andreasen et al., 2001; Hollman, 2001; Petri et al., 2003; Spencer et al., 2003). Phase I (cytochrome P450s) and phase II enzymes are found in the SI enterocytes (Spencer et al., 2006). The phase II enzymes are responsible for conjugating phenolic compounds to glucuronic acid, sulfates and methyl groups. Studies show they are conjugated to glucuronic acid via UDP-glucuronosyltransferases (Petri et al., 2003), sulfated by sulfo-transferases, and *O*-methylated by catechol-*O*-methyl transferases (COMT) (Olthof et al., 2000; Spencer et al., 2006). The phase II metabolites can be excreted from the enterocyte into the SI lumen or into the portal circulation. Phenolic compounds not undergoing phase I and II

metabolism in the enterocyte are metabolized in the liver i.e. glucuronidated, sulfated, or methylated. The liver will excrete the phenolic metabolites into the bile or into the circulation. Metabolites excreted in the bile enter the SI where they may be reabsorbed or they pass to the colon where they can be metabolized by the colonic microflora and reabsorbed or excreted in the feces (Spencer et al., 2006). Metabolites entering the circulation from the enterocyte and liver are excreted in the urine.

At normal dietary levels, absorbed phenolic compounds are unlikely to escape first-pass metabolism, thus the predominant form found in the plasma would be conjugates (i.e. glucuronates and sulfates) of the phenolic compounds (Kroon et al., 2004; Manach et al, 2004; Spencer et al., 2006). The form of the phenolic compound and dose given appear to affect where the compound is metabolized and the type of conjugates formed. When phenolic compounds are given in pharmacological doses as aglycones or in their native forms, they may saturate the phase I and phase II enzymes, resulting in higher amounts of the aglycone or native compound in circulation. For example, "the sulfate pathway is a high affinity, low capacity pathway, high doses would saturate this pathway and the compounds would be redirected to the glucuronidate pathway, which is high capacity" (Spencer et al., 2006). The metabolites formed when a pharmacological dose of a compound or a mix of compounds in a concentrated extract are given would be different then when the compounds are provided at normal dietary levels or as part of the food matrix (Kroon et al., 2004; Manach, 2004; Spencer et al., 2006). At larger doses metabolism occurs predominantly in the liver, whereas at dietary levels it occurs almost exclusively in the SI with the liver playing a secondary role (Scalbert and Williamson,

2000; Spencer et al., 2006). Moreover, the administration of single phenolic compounds may overestimate the absorption from dietary sources (Goldberg et al., 2003).

Bacterial metabolites of flavanoids — 3-(3-hydroxyphenyl)-propionic acid and 3-phenylpropionic acid —absorbed in the colon are converted to 3-hydroxyhippuric acid and hippuric acid, respectively, via β-oxidation and glycination in the liver and then excreted in the urine (Rechner et al., 2004).

1.6. Phenolic Compounds in Grain Legumes

The majority of the studies identifying and quantifying phenolic compounds in legumes has focused on the compounds found in soybeans and other oil seeds (Krygier et al., 1982). Numerous reviews examining bioactive soybean components and their health effects have been published (Messina et al., 2006; Larkin et al., 2008; Xiao, 2008). Studies identifying phenolic compounds in grain legumes, including dry common beans, are limited and were excuted to examine their organoleptic and anti-nutrient effects. Phenolic compounds (i.e. condensed tannins and flavonoids) in beans are associated with adverse tastes and colors in food products, and can reduce protein digestibility and iron bioavailability. Additionally, consumers have developed specific preferences for the size, shape and seed coat color of dry common beans and this has established the various market classes available (Beninger and Hosfield, 2003). Seed coat (i.e. hull) color varies considerably among dry common beans (*Phaseolus vulgaris* L.) and is determined by the concentration and presence of flavonol glycosides, anthocyanins and condensed tannins (i.e. proanthocyanidins). In fact various seed coat color genotypes have been identified, as well as the phenolic compounds responsible for the colors (Leterme and Muñoz, 2002; Beninger et al., 1999; 2003; 2005). Thus in many studies the seed coat is separated from

the cotyledon (meat or fruit of the bean), and only the coats are analyzed for phenolic compounds.

Investigators have identified flavonol glycosides, hydroxybenzoic acids, hydroxycinnamic acids and condensed tannins (proanthocyanidins) in the seed coats of some grain legumes (Krygier et al., 1982; Sosulski and Dabrowski, 1984; Beninger et al., 1999; 2003; 2005; Madhujith et al., 2004; Aparicio-Fernandez et al., 2005; Hu et al., 2006; Lin and Lai, 2006; Ranilla et al., 2007). Anthocyanins have only been identified in beans possessing blue and blue-violet colored seed coats (Takeoka et al., 1997; Beninger et al., 2003; Choung et al., 2003; Oomah et al., 2005; Aparicio-Fernandez et al., 2005; Macz-Pop et al., 2006). Darker seed coat colors, i.e. red, brown or black, are associated with greater total phenolic content and antioxidant activity measured by various *in vitro* assays compared to light seed coats i.e. white, gray, yellow (Madhujith et al., 2004; Oomah et al., 2005; Lin and Lai, 2006; Xu and Chang, 2007), however this is controversial (Rocha-Guzmán et al., 2007). It is dependent on the legume type, the classes of compounds being investigated and whether the whole or part of the bean is analyzed.

Several papers have characterized specific classes or phenolic compounds in different grain legumes. Beninger et al. (1999) identified quercetin, kaempferol, unknown flavonol glycosides and proanthocyanidins in raw dark kidney bean seed coats (*Phaseolus vulgaris* L. cul. Montcalm). Takeoka et al. (1997) reported the presence of anthocyanins: delphinidin-, petunidin- and malvidin-3-glycosides, in raw black beans (*Phaseolus vulgaris* L.). Aparicio-Fernandez et al. (2005) identified anthocyanins in the seed coats of raw Black Jamapa beans (*Phaseolus vulgaris* L.), as well as

proanthocyanidins and flavonol glycosides of kaempferol, quercetin and myricertin. Hu et al. (2006) investigated the phenolics in raw white, black, red and pinto bean seed coats (*Phaseolus vulgaris* L.). The flavonol kaempferol and astragalin (kaempferol-3-*O*-glucoside) were identified in the seed coats of the red and pinto beans. Kaempferol was not identified in the seed coats of black beans, but other unknown flavonols and anthocyanins were. No flavonoids were detected in the white seed coats. Choung et al. (2003) investigated the anthocyanins in seed coats of different colored raw kidney beans (*Phaseolus vulgaris* L.). They observed five different anthocyanins, whose content and composition were dependent on seed coat color. In the seed coats from Brazilian and Peruvian bean cultivars (*Phaseolus vulgaris* L.), Ranilla et al. (2007) identified condensed tannins, anthocyanins and kaempferol and quercetin glycosides. They found the cotyledons to be rich in phenolic acids such as ferulic acid, sinapic acid, chlorogenic acid and other hydroxycinnamic acids. Sosulski and Dabrowski (1984) reported the presence of hydroxycinnamic acids such as ferulic acid, *p*-coumaric acid, syringic acid in the seed coats (hulls) and flours (cotyledons) of 10 legume species. They also observed hydroxybenzoic acids, i.e. *p*-hydroxybenzoic acid, protocatechuic acid and gallic acid, in select species.

Others investigating grain legumes quantified the total phenolic content or classes of phenolic compounds e.g. total flavonoid content, total condensed tannin content. Heimler et al. (2005) investigated the phenolic compounds in 3 varieties of dry beans (*Phaselous vulgaris* L.). They reported the total phenolic content (Folin-Ciocalteu method) ranged from 117 to 440 mg gallic acid equivalents per 100g of raw bean flour and total flavonoid content ranged from 22 to 143 mg (+)-catechin equivalents per 100 g

of raw bean flour. They claimed the presence of kaempferol, quercetin and caffeic acid derivatives, but did not quantify them. Xu and Chang (2007) reported the total phenolic content (Folin-Ciocalteu method) in different grain legumes ranged from 131 to 373 mg of gallic acid equivalents per 100 g of raw bean flour and total flavonoid content 10 to 98 mg of (+)-catechin equivalents per 100 g of raw bean flour.

Sosulski and Dabrowski (1984) investigated the free and hydrolysable phenolic acids in the flours (cotyledons) and hulls (seed coats) of 10 legume species. When they attempted to hydrolyze the remaining insoluble solid residue after the removal soluble free and phenolic acid esters via alcohol-aqueous extraction, in either the flours (cotyledons) or hulls (seed coats) of raw navy beans, it failed to yield any further appreciable amount of phenolic acids. This was observed with the oil legume, rapeseed, as well. Conversely, cereals yield much higher concentrations of hydroxycinnamic acids after alkaline treatment on the insoluble solid residue (Krygier et al., 1982; Sosulski et al., 1982). Since the cell walls of dicotyledons, e.g. legumes, have much smaller amounts of phenolic acids associated with their cell walls in comparison to monocotyledons e.g. cereals and grasses (Hartley, 1987), alkaline hydrolysis of the insoluble solid residue may not be a necessary step when analyzing grain legumes.

Table 2. summarizes the quantitative data reported of specific phenolic compounds found in different whole grain legumes.

Cai et al (2003) identified seven different phenolic acids in 17 varieties of raw cowpeas (*Vigna unguiculata*) including protocatechuic acid, *p*-hydroxybenzoic acid, caffeic acid, *p*-coumaric acid, ferulic acid, 2,4-dimethoxybenzoic acid, and cinnamic

Table 2. Phenolic Compounds Quantified in Grain Legumes

Bean Class		n	Extraction, Hydrolysis and Purification	Separation and Detection	Hydroxybenzoic Acids mg / 100 g of Dry Bean Flour					
					PCA	pHBA	Van	Syr	Total HBA	
La Granja variety	*Phaseolus vulgaris* L.	2	10 g raw bean flour	80% MeOH; No Hydrolysis; Diethyl ether and ethyl acetate	RP-HPLC-PAD (210 nm-360nm)	0.03-0.04	0.03-0.04	0.09-0.1		0.15-0.18	López-Amorós et al., 2006
Cowpea (17 varieties) Free phenolics	*Vigna unguiculata*	3	500 mg raw bean flour	100% MeOH; No Hydrolysis	RP-HPLC-DAD (254 nm and 238 nm)	Trace-3.6	Trace-3.5			34.6-376.6 [b]	Cai et al., 2003
Cowpea (17 varieties) Free + liberated soluble phenolic esters	*Vigna unguiculata*	3	500 mg raw bean flour	Alkaline Hydrolysis of 100% MeOH extract[d]		9.3-92.7	Trace-3.9			9.3-96.6	
Mexican (14 varieties)	*Phaseolus vulgaris* L.	3	200 g raw bean flour	80% MeOH; Acid hydrolysis; Ethyl acetate	RP-HPLC-DAD (295nm; 257 nm)		0.6-1.4	0.5-1.7		1.1-3.1	Díaz-Batalla et al., 2006
Mexican (14 varieties)	*Phaseolus vulgaris* L.	3	200 g Autoclaved /Cooked bean flour				0.4-0.9	nd-1.2		0.4-2.1	
[c]Mexican (62 varieties)	*Phaseolus vulgaris* L.	2	Raw bean flour	80% MeOH; Acid hydrolysis; Ethyl acetate	RP-HPLC-DAD (295nm; 257 nm)		0.4-2.4	0.6-3.8	nd-0.9	1.0-7.1	Espinosa-Alonso et al., 2006
[c]Jamapa	*Phaseolus vulgaris* L.	2					1.5	1.5	1.2	4.2	
[c]Pinto	*Phaseolus vulgaris* L.	2					1.8	1.1	nd	2.9	

Table 2. (cont'd)

Bean Class		n		Extraction, Hydrolysis and Purification	Separation and Detection	Hydroxycinnamic Acids mg / 100 g of Dry Bean Flour					
						Caf	pCA	FA	SA	Total HCA	
Black (3 varieties)	*Phaseolus vulgaris* L.	3	200 mg raw bean flour	Alkaline Hydrolysis on bean flour; Ethyl acetate	RP-HPLC-DAD (270 nm and 325 nm)	1.1	7-11.6	11.7-25.5	5.7-9.0	24.4-42.5	Luthria and Pastor-Corrales, 2006
Pinto (3 varieties)	*Phaseolus vulgaris* L.	3					4.5-5.6	15.2-22.9	5.9-9.0	26.7-36.0	
Great Northern (2 varieties)	*Phaseolus vulgaris* L.	3					4.0-6.3	17.0-17.2	9.0-9.4	30.0-32.5	
Navy	*Phaseolus vulgaris* L.	3					12.4	26.6	9.2	48.3	
Dark Kidney	*Phaseolus vulgaris* L.	3					1.8	15.3	3.8	20.9	
Light Kidney	*Phaseolus vulgaris* L.	3					7.0	14.8	5.7	27.4	
Red Mexican	*Phaseolus vulgaris* L.	3					5.8	17.4	5.4	28.6	
Cranberry	*Phaseolus vulgaris* L.	3					1.7	14.0	3.5	19.1	
Pink	*Phaseolus vulgaris* L.	3					6.8	19.4	8.2	34.4	
Alubia	*Phaseolus vulgaris* L.	3					5.3	10.6	4.0	19.8	
La Granja variety	*Phaseolus vulgaris* L.	2	10 g raw bean flour	80% MeOH; No Hydrolysis; Diethyl ether and ethyl acetate	RP-HPLC-PAD (210 nm -360 nm)		nd	0.34-0.37		0.34-0.37	López-Amorós et al., 2006

Table 2. (cont'd)

Cowpea (17 varieties) Free phenolics	*Vigna unguiculata*	3	500 mg raw bean flour	100% MeOH; No Hydrolysis	RP-HPLC-DAD (254 nm and 238 nm)	Trace –1.0	Trace –4.2	Trace –6.2		34.6-376.6[b]	Cai et al., 2003
Cowpea (17 varieties) Free + liberated soluble phenolic esters	*Vigna unguiculata*	3	500 mg raw bean flour	Alkaline Hydrolysis of 100% MeOH extract[d]		0.4-2.4	1.0-5.6	0.4-12.4		1.8-20.4	
Mexican (14 varieties)	*Phaseolus vulgaris* L.	3	200 g raw bean flour	80% MeOH; Acid hydrolysis; Ethyl acetate	RP-HPLC-DAD (295nm)		0.3-0.7	1.7-3.6		2.0-4.3	Díaz-Batalla et al., 2006
Mexican (14 varieties)	*Phaseolus vulgaris* L.	3	200 g Autoclaved /Cooked bean flour				nd-0.5	1.2-2.8		1.2-3.3	
[c]Mexican (62 varieties)	*Phaseolus vulgaris* L.	2	Raw bean flour	80% MeOH; Acid hydrolysis; Ethyl acetate	RP-HPLC-DAD (295nm; 257 nm)	nd-0.9	0.1-1.0	0.9-3.8	0.3-2.0	1.3-7.7	Espinosa-Alonso et al., 2006
[c]Jamapa	*Phaseolus vulgaris* L.	2				1.8	0.3	1.8	1.0	4.9	
[c]Pinto	*Phaseolus vulgaris* L.	2				2.0	0.4	2.3	1.4	6.1	

Table 2. (cont'd)

Bean Class		n		Extraction, Hydrolysis and Purification	Separation and Detection	Flavonols mg/100 g of Dry Bean Flour			
						Q	K	Total Flav	
Mexican (14 varieties)	*Phaseolus vulgaris* L.	3	200 g raw bean flour	80% MeOH; Acid hydrolysis; Ethyl acetate	RP-HPLC-DAD (260.6nm)	0.7-2.3	1.4-20.9	2.1-23.2	Díaz-Batalla et al., 2006
Mexican (14 varieties)	*Phaseolus vulgaris* L.	3	200 g autoclaved/ Cooked bean flour			0.4-1.2	0.7-12.3	1.1-13.5	
[c]Mexican (62 varieties)	*Phaseolus vulgaris* L.	2	Raw bean flour	80% MeOH; Acid hydrolysis; Ethyl acetate	RP-HPLC-DAD (295nm; 257 nm)	0.2-5.2	0.2-8.8	0.4-14.0	Espinosa-Alonso et al., 2006
[c]Jamapa	*Phaseolus vulgaris* L.	2				2.1	1.5	3.6	
[c]Pinto	*Phaseolus vulgaris* L.	2				0.2	2.1	2.3	

a. Values are means or a range of means. HBA, hydroxybenzoic acids; PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Syr, syringic acid; HCA, hydroxycinnamic acids; Caf, caffeic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Flav, flavonols; Q, quercetin; K, kaempferol; nd, not detected. b. Represents the Total Phenolic Content determined by Folin-Ciocalteu's reagent, results expressed as protocatechuic acid equivalents. c. Values were converted to mg per 100 g of dry bean flour (dry weight basis) from mg/kg bean flour (fresh weight basis), assumed raw beans = 11% moisture. d. Aliquot of the cowpea methanol extract was hydrolyzed with alkali.

acid. The total phenolic content (TPC) determined from the Folin-Ciocalteau method was highly varied and ranged from 34.6 to 376.6 mg/100 g of bean flour. The predominant hydroxybenzoic acid present following alkaline hydrolysis was protocatechuic acid (9.3 to 92.7 mg/100 g of dry bean flour), whereas ferulic acid (0.4 to 12.4 mg/100g of dry bean flour) was the predominant hydroxycinnamic acid present after hydrolysis.

Luthria and Pastor-Corrales (2006) quantified hydroxycinnamic acids in 15 different varieties of raw dry common beans (*Phaseolus vulgaris* L.). They ranged from 19.1 to 48.4 mg per 100 g of dry bean flour following alkaline hydrolysis. Cranberry beans had the lowest content, while navy bean had the greatest, followed by black and pinto beans. *p*-Coumaric, ferulic and sinapic acid were found in all varieties analyzed, but caffeic acid was only identified in black beans. Ferulic acid was the predominant hydroxycinnamic acid identified after alkaline hydrolysis, ranging from 10.6 mg/100 g of dry bean flour in alubia beans up to 26.6 mg/100 g of dry bean flour in navy beans. López-Amorós et al. (2006) reported the phenolic compounds in raw dry beans (*Phaseolus vulgaris* L. var. La Granja). Hydroxybenzoic acids identified included protocatechuic, *p*-hydroxybenzoic and vanillic acid. Total ranged from 0.15 to 0.18 mg/100 g of dry bean flour. The only hydroxycinnamic acid identified was ferulic acid, ranging from 0.34 to 0.37 mg/100g dry bean flour. No hydrolysis was performed on the bean samples.

Díaz-Batalla et al. (2006) investigated the phenolic compounds in several varieties of Mexican common beans (*Phaseolus vulgaris* L.) raw and cooked. This was one of the few studies investigating phenolic compounds in processed beans. After acid

hydrolysis hydroxybenzoic acids identified included *p*-hydroxybenzoic, vanillic and syringic acid, totals ranging from 1.0 to 3.1 mg/100g dry bean flour for the raw beans. This was reduced to 0.4 to 2.1 mg/100 g dry bean flour if the beans were cooked, approximately 46% reduction. *p*-Coumaric and ferulic acid were the only hydroxycinnamic acids identified in the raw and cooked Mexican bean varieties. Totals ranged from 2.0 to 4.3 mg/100g dry bean flour to 1.2 to 3.3 mg/100g dry bean flour for raw and cooked beans, respectively. There was approximately a 30% reduction in hydroxycinnamic acids after processing (cooking). Ferulic acid was the predominant phenolic acid regardless of processing. Additionally they identified the flavonol aglycones, quercetin and kaempferol. Quercetin ranged from 0.7 to 2.3 mg/100g dry bean flour in raw beans and 0.4 to 1.2 mg/100g dry bean flour in autoclaved/cooked beans. Kaempferol was the predominant flavonol, ranging from 1.4 to 20.9 mg/100 g dry bean flour in raw beans and 0.7 to 12.3 mg/100 g dry bean flour in autoclaved/cooked beans. They reported the reduction from cooking the Mexican common beans ranged from 12 to 65% and 5 to 71% for quercetin and kaempferol, respectively.

Espinosa-Alonso et al. (2006) investigated the phenolic compounds in 62 different varieties and cultivars of Mexican dry common beans. They also looked at the phenolic compounds in Jamapa black beans and pinto beans. They identified hydroxybenzoic acids, hydroxycinnamic acids and flavonol aglycones following acid hydrolysis. The hydroxybenzoic acids included: *p*-hydroxybenzoic, vanillic and syringic acid. The total hydroxybenzoic acids in raw Mexican common beans ranged from 1.0-7.1 mg/100 g dry bean flour. The raw Jamapa black and pinto beans containd 4.2 mg and 2.9 mg total hydroxybenzoic acids/100 g dry bean flour, respectively. Caffeic, *p*-

coumaric, ferulic and sinapic acid were identified in all beans analyzed. The total hydroxycinnamic acid in raw Mexican common beans ranged from 1.3 to 7.7 mg/100 g dry bean flour. For raw Jamapa black bean the total hydroxycinnamic acids was 4.9 mg/100 g dry bean flour, and for pinto beans it was 6.1 mg/100 g dry bean flour. Ferulic acid was the predominant hydroxycinnamic acid in all bean samples. Quercetin and kaempferol ranged from 0.2 to 5.2 and 0.2 to 8.8 mg/100 g dry bean flour, respectively, in the raw Mexican beans. Jamapa black beans had 2.1 mg of quercetin and 1.5 mg kaempferol/100 g dry bean flour. Pinto beans had 0.2 mg of quercetin and 2.1 mg kaempferol/100 g dry bean flour. Espinosa-Alonso et al. (2006) also identified the anthocyanidins: delphinidin, petunidin, cyanidin, malvidin, pelargonidin and peonidin, in the Mexican beans possessing black, mottled gray and heterogeneous color mixture seed coats and the Jamapa black bean. A few of the varieties and/or cultivars were found to contain the isoflavones: daidzein and coumestrol. Both Díaz-Batalla et al. and Espinosa-Alonso et al., however used acid hydrolysis to quantify hydroxycinnamic acids, as stated in a previous section, acidic conditions could increase the loss of hydroxycinnmic acids and decrease the total amount of hydroxycinnamic acids.

Different processing methods will induce physical and chemical compositional changes in legumes, altering their nutrient content and these changes are dependent on the legume type and processing conditions. Legumes must be processed before consumption to inactivate anti-nutrients e.g. lectins, protease inhibitors, phytates. Processing methods include hydration (soaking), heat treatment (i.e. boiling, pressure cooking or autoclaving), germination or fermentation, or a combination of 2 or more methods (Geil and Anderson, 1994; Messina, 1999; Champ, 2002; Ibrahim et al., 2002;

Shimelis and Rakshit, 2007). Hydration or soaking legumes prior to either heat treatment, germination and/or fermentation, is a typical preliminary step. It softens the legume's texture and reduces their cooking time. Generally, the more polar compounds will be leached into the soaking water, as well as into the cooking water, which are normally discarded. Thus in studies examining the effect of soaking on grain legumes' phenolic content and antioxidant activity, a decrease has been observed (Towo et al., 2003; López-Amorós et al., 2006; Xu and Chang, 2008). It has been estimated that 30%-40% of the phenolic compounds in legumes are lost when the cooking water is discarded (Bressani and Elias, 1980).

Very few studies have investigated the effect of processing on phenolic compounds in legumes. Of those that have, the majority simply measured total phenolic content or classes of phenolic compounds. Granito et al. (2008) reported an overall decrease in the total phenolic content and antioxidant activity after cooking (boiled) or fermenting black beans (*Phaseolus vulgaris* L.). However, fermentation did not reduce antioxidant activity to the same degree as cooking. Towo et al. (2003) investigated the affect of germination, cooking or dehulling on total phenolics on several species of grain legumes. Dehulling significantly reduced the total phenolic content by 52%-67% in all legumes analyzed and condensed tannins content by 82%-93%. Cooking significantly reduced the total phenolic content and condensed tannin content by 29%-55% and 46%-69%, respectively. Germination significantly reduced the total phenolic content and condensed tannins by 25%-44% and 35%-72%, respectively. The authors concluded that dehulling and heat treatment were the most effective in reducing the total phenolic content in the analyzed grain legumes. The dramatic loss in condensed tannins following

dehulling is not surprising because they are highly concentrated in the seed coats of legumes (Beninger and Hosfield, 2003; Madhujith et al., 2004). López-Amorós et al. (2006) also examined the effect of germination on grain legumes. They concluded that germination alters the phenolic composition of legumes and their antioxidant activity, but the changes are dependent on the type of legume and the germination conditions such as length of time and the presence or absence of light (López-Amorós et al., 2006). Rocha-Guzmán et al. (2007) evaluated the effect of pressure cooking (autoclaving) on antioxidant activity in common beans. Cooking induced a large reduction in total phenolic content measured in the seed coats and cotyledons of common beans and the antioxidant activity in the cooked legumes was similar to that in raw legumes. Interestingly, when the freeze-dried material from the cooking water was included in the analysis, the antioxidant activity in cooked legumes increased. Xu and Chang (2008) investigated the effect of soaking, boiling and steaming on total phenolic content and antioxidant activity of cool season legumes i.e. green and yellow peas (*Pisum sativum* L.), chickpeas (*Cicer arietinum* L.), and lentils (*Lens culinaris*). They concluded that all processes resulted in reductions in total phenolic content ranging from 40%-68%, but the amount depended on the legume type and processing conditions. Loss due to soaking ranged from 2% to 38%. Loss in antioxidant activity depended on the *in vitro* antioxidant assay utilized, the legume type and the processing conditions. DDPH free radical scavenging activity value was significantly reduced by all processes ranging from 6%-95%. The oxygen radical absorbing capacity (ORAC) values were reduced in all legumes only after soaking or regular boiling, range of 4%-77%. Conversely, the ORAC

values significantly increased after pressure boiling (except in lentils) and pressure steaming, ranging from 5% up to 175%.

The modifications in phenolic composition via processing methods, may be a result of the following: phenolic compounds leaching into the soaking or cooking water; decreases in the amount of extractable phenolics because of the formation of complexes with macromolecules; degradation and/or formation of compounds, phenolic or otherwise; endogenous enzyme activation during germination i.e. polyphenoloxidases, hydrolases, esterases (Towo et al., 2003; López-Amorós et al., 2006; Sangronis, 2006; Shimelis and Rakshit, 2007).

With the increasing public awareness of phenolic compounds, especially as antioxidants, and the health benefits associated with fruit and vegetable consumption, identifying and quantifying phenolic compounds in all plant foods is imperative. If a plant food is processed prior to consumption, it is more nutritionally relevant and just as vital to analyze the phenolic compounds in that processed plant food. In a published review on the subject of hydroxycinnamic acids, Clifford (1999) observed that assessing the dietary intake of hydroxycinnamic acids was not possible. He stated, "With the exception of some beverages, the lack of data for the composition of commodities as consumed, i.e. the edible portion after processing, cooking, baking, etc, effectively make such an assessment impossible". Since legumes must be exposed to some sort of heat treatment to be safe to consume, it is important from a dietary standpoint to quantify the phenolic compounds in heat treated grain legumes i.e. dry common beans.

C. Colorectal Cancer (CRC)

1.1. Colorectal Cancer Statistics

Colorectal cancer (CRC) is the 3rd most commonly diagnosed cancer and the 4th most common cause of cancer death worldwide (WCRF/AICR, 2007). In the United States alone, CRC is the 4th most commonly diagnosed cancer following lung, female breast and prostate cancers, and the 2nd leading cause of cancer death (Jemal et al, 2008). It was recently noted that in the U.S. significant decreases in CRC incidence and death rates for men and women combined have been observed, 2.1% per year (from 1998 to 2003) and 2.8% per year (from 2001 to 2003), respectively (Ries et al, 2006). These declines have been attributed to increased public awareness and improvements in the methods of early detection and treatment (Jemal et al., 2007). Colorectal cancer screenings allow for the detection and removal of pre-cancerous polyps (hyperplastic polyps or adenomas) and early-stage cancers in asymptomatic individuals, preventing their progression to more invasive cancer (adenocarcinomas) and allow for detecting them at the more treatable early stages, leading to a reduction in the number of deaths from CRC (Hawk et al, 2004). In fact, experts believe as many as 50-60% of CRC deaths could be prevented if everyone age 50 or older were to be screened regularly (Selby et al., 1992). Nevertheless it is estimated that 148,810 new cases of CRC will be diagnosed this year in the U.S., resulting in 49,960 deaths (Jemal et al, 2008).

Approximately 88% to 94% of CRC cases are sporadic, occurring in individuals who do not have an inherited colon cancer syndrome (Compton, 2003; Weitz et al., 2005). An individual's risk of developing colorectal cancer increases with age. Risk rises sharply from age 40 to 50, doubles with each subsequent decade and peaks at age 70 (Compton, 2003). Other risk factors include male sex, hormone exposure, history of

colorectal polyps, history of CRC or cancers of the small bowel, endometrium, breast or ovary (Weitz et al., 2005). Lifestyle and environmental risk factors include diets rich in red and processed meats, high in animal fats and sugars, low in folate, calcium and dietary fiber; low physical activity; obesity; diabetes mellitus; smoking; high alcohol intake (especially in men); occupational hazards e.g. asbestos exposure; or radiation exposure (Weitz et al., 2005; WCRF/AICR, 2007). Approximately 20% of sporadic CRC cancers are due to hereditary factors or familial history of colorectal cancer without fulfilling the criteria for the known hereditary syndromes (Weitz et al., 2005). Chronic inflammatory bowel diseases i.e. ulcerative colitis and Crohn's disease increase the risk of developing CRC and account for 1%-2% of the CRC cases (Weitz et al., 2005).

Patients with inheritable CRC syndromes are predisposed to cancer and these syndromes account for 5%-10% of cases of CRC. Two highly studied syndromes are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). FAP is an autosomal dominant disorder, with approximately 80% of affected FAP patients possessing a germline mutation in one allele of the adenomatous polyposis coli (*APC*) gene. Although individuals with FAP are predisposed for CRC, it does not guarantee they will develop it. Studies of FAP patients and mice with similar mutations in the murine homolog of *APC* gene require the loss of the 2^{nd} allele via a somatic mutation or by chromosomal loss or deletion, the latter being more common (Kinzler and Vogelstein, 1996). The loss of the 2^{nd} allele is required for the CRC phenotype to manifest. This loss of heterozygosity (LOH) is required for the development of colon cancer, and is consistent with the Knudson "two-hit" hypothesis that requires at least two genetic alterations for the development of cancer. FAP patients will develop 10 to 1000

adenomas throughout their colons, 50% by age 15 and 95% by age 35. Although the adenomas are benign, if left untreated, CRC will develop by the age 50. The most common treatment for FAP patients is prophylactic colectomy. Additionally patients are at increased risk for developing extracolonic tumors such as duodenal carcinomas, desmoid tumors, osteomas and brain tumors (Kinzler and Vogelstein, 1996; Weitz et al., 2005; Barnetson and Dunlop, 2007).

HNPCC or Lynch syndrome is an autosomal dominant syndrome caused by a germline mutation in the DNA mismatch repair genes (MMR) i.e. *hMLH1, hMSH2.* A typical molecular characteristic of HNPCC tumors is the presence of microsatellite instability (MSI). Microsatellites are short repeats of non-coded DNA sequences distributed throughout the genome. MSI is described by small insertions or deletions in microsatellite regions in the DNA of tumors. Interestingly, MSI is found in approximately 15% of sporadic CRC not from a germline mutation in MMR genes but resulting from the epigenetic silencing of MMR genes. The onset of CRC in HNPCC patients occurs at around 40 yrs of age, and characteristically a larger proportion of tumors are located within the proximal colon (right sided) compared to sporadic cancers (left sided, distal colon). Moreover extracolonic tumors frequently develop (Weitz et al., 2005; Barnetson and Dunlope, 2007).

Other inheritable autosomal dominant syndromes include Peutz-Jegher's syndrome, Juvenile polyposis and Cowden Syndrome. These disorders are more rare compared to FAP or HNPCC and are characterized by the formation of hamartomas in the colon (Weitz et al., 2005).

1.2. Colorectal Carcinogenesis

Carcinogenesis is a multi-step process wherein a series of genetic events are necessary to drive the transformation of normal cells to malignant cancers (Hanahan and Weinberg, 2000). The process begins with an initial event where a single cell experiences a genetic mutation resulting in a selective growth advantage over surrounding cells, followed by clonal expansion of the mutated cell and the acquisition of further genetic alterations (Fearon and Vogelstein, 1990). These mutations occur in genes responsible for normal cell proliferation, differentiation, adhesion and survival, and these alterations often advance the growth of the transformed cells.

Carcinogenesis progresses slowly, taking years to fully manifest, approximately 10 to 20 years from the initiation event to malignant transformation (Lipkin, 1999; Dove-Edwin and Thomas, 2001). In "Western" populations 50% of the population by the age of 70 will likely develop colorectal tumors, and in about 10% of these individuals the benign adenoma will progress to the malignant adenocarcinoma or CRC (Kinzler and Vogelstein, 1996). CRC remains the paradigm utilized to study the molecular, biochemical, cellular, and genetic alterations occurring in the carcinogenesis process (Fearon and Vogelstein, 1990). The large intestine is made up millions of crypts, which are around 50 cells deep (see Figure 4., Lipkin, 1999; Heavey et al, 2004, Humphries and Wright, 2008). Colonic cells are under constant renewal, with the entire human colonic epithelium being replaced every 3 to 8 days (Lipkin, 1999). Stem cells located at the bottom of the crypt replicate resulting in the original stem cell and a new daughter cell. The daughter cells continue to replicate and push upward toward the top of the crypt. The cells occasionally divide as they migrate up the crypt column. There are very few

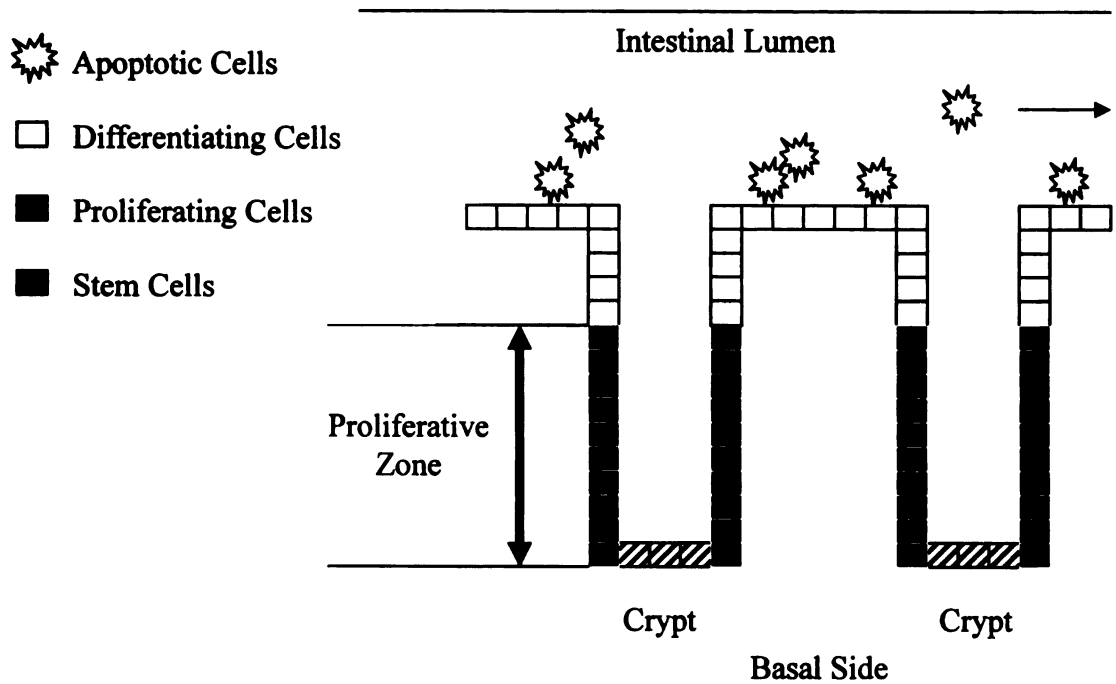


Figure 4. Normal Colonic Crypt

The large intestine is made up of millions of crypts, which are around 50 cells deep (Lipkin, 1999; Heavey et al, 2004, Humphries and Wright, 2008). Colonic cells are under constant renewal, with the entire human colonic epithelium being replaced every 3 to 8 days (Lipkin, 1999). Stem cells located at the bottom of the crypt replicate resulting in the original stem cell and a new daughter cell. The daughter cells continue to replicate and push upward toward the top of the crypt. The cells occasionally divide as they migrate up the crypt column. There are very few dividing cells in the top 1/3 of the crypt where they begin to differentiate into mature non-proliferative epithelial cells. Once at the lumenal surface the mature cells undergo apoptosis and are excreted in the feces.

dividing cells in the top 1/3 of the crypt where they begin to differentiate into mature non-proliferative epithelial cells. Once at the lumenal surface the mature cells undergo apoptosis and are excreted in the feces. The crypt design ensures that damage to any epithelial cells induced by genotoxic compounds found in the intestinal lumenal contents will not influence the integrity of the crypt (Heavey et al., 2004). Crypt cell kinetics or the molecular processes involved in maintaining the balance between new cell production and cell death are under tight regulation in highly renewing tissues like the colon (Lipkin, 1999). Disruptions in any one of these processes can contribute to the development of colorectal cancer.

Given the accessibility of the colon, animal models of colon carcinogenesis and inheritable human CRC syndromes have permitted researchers the opportunity to study the pathological, histological and molecular alterations occurring during the different stages of colorectal cancer development, or during the "adenoma-carcinoma sequence" (Figure 5). This term is used to describe the progression from normal epithelial cells to dysplastic cells to benign adenoma to malignant adenocarcinoma to metastasis, and the accompanying epigenetic and genetic alterations (Fearon and Vogelstein, 1990; Leslie et al., 2002).

Fearon and Vogelstein (1990) proposed the following: 1) colorectal tumors result from the mutational activation of oncogenes coupled to the inactivation of tumor suppressor genes, 2) at least 5 to 7 genetic alterations are required for normal cells to progress to carcinoma (malignant tumor), although less are required for benign tumorigenesis, 3) even though genetic changes commonly occur in a preferred sequence,

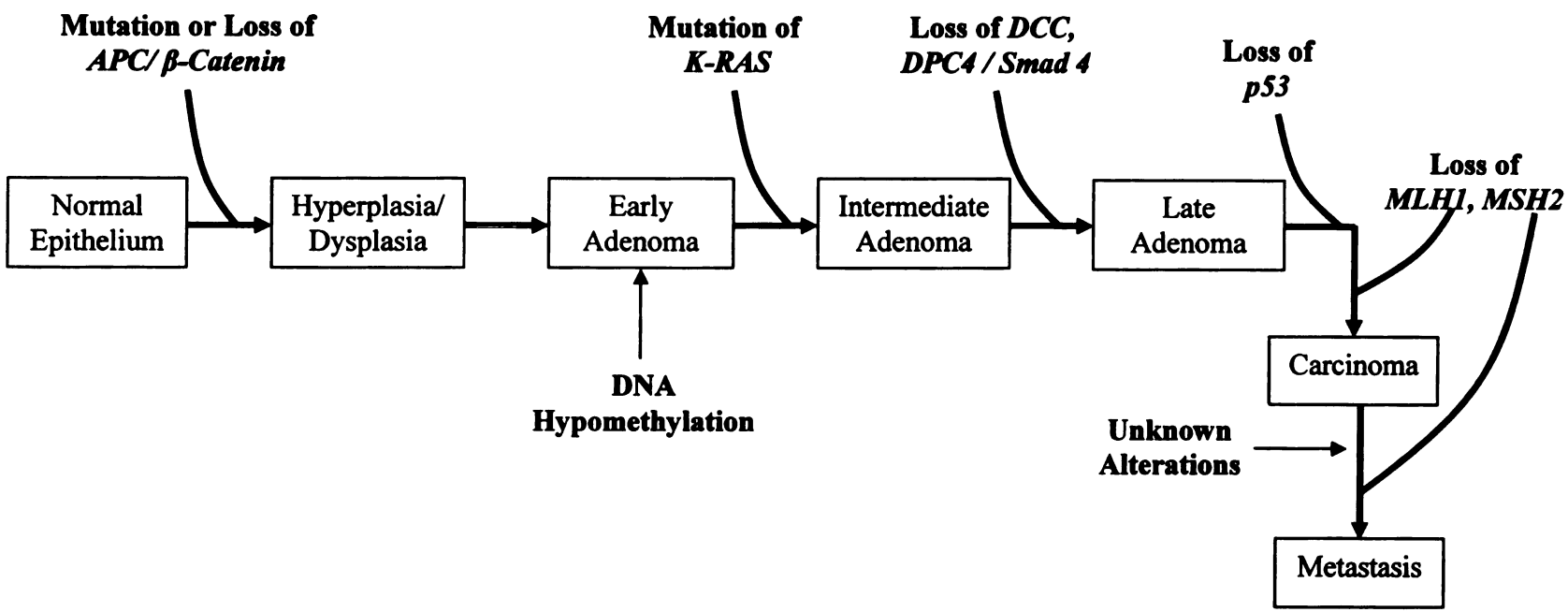


Figure 5. Adenoma-Carcinoma Sequence: A Genetic Model for Colorectal Carcinogenesis (Fearon and Vogelstein, 1990; Heavey et al., 2004)

it is the total accumulations of mutations, not their order of occurrence in respect to each other, that determines the tumor's biological properties, 4) some mutated tumor suppressor genes appear to exert a phenotypic effect even when present as a heterozygous state. Two pathways, the gateway pathway and the caretaker pathway, can be used to explain the CRC process (Figure 5). Mutations in genes that regulate cell growth such as oncogenes (e.g. K-*ras, c-myc, c-erb2, c-src*) and tumor suppressor genes (e.g. *APC, DCC, DPC4/Smad4, p53, nm32*) represent the gatekeeper pathway. Whereas the caretaker pathway involves mutations or epigenetic changes in genes that maintain genetic stability e.g. mismatch repair genes (Kinzler and Vogelstein, 1996; Leslie et al., 2002; Weitz et al., 2005). Figure 5 describes the widely accepted and well characterized, genetic alterations that occur during the adenoma-carcinoma sequence (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996). Nevertheless, the order of genetic alterations and the possibility of subsets of colorectal tumors proceeding through a different set of genetic alterations cannot be overlooked (Fearon and Vogelstein, 1990; Yamashita et al., 1995).

Animal models of colon carcinogenesis have provided a great deal of information about the pathogenesis of colon cancer and the influence of dietary and environmental factors upon its development. Unlike humans however, animals rarely develop spontaneous cancer of the colon and rectum (Femia and Caderni, 2008). Therefore colon cancer must be induced in animals using exogenous chemicals. 1, 2-Dimethylhydrazine (DMH) and its metabolite azoxymethane (AOM) are two commonly used carcinogens utilized in studies to induce colon cancer in animals (Femia and Caderni, 2008). Evidence has shown that chemically induced colon carcinogenesis follows the multi-

stage carcingoenesis process, beginning with initiation followed by promotion and progression (Reddy, 2004; Femia and Caderni, 2008).

Azoxymethane (AOM) is a potent generator of colon carcinomas of the large intestine in selective strains of rodents and an accepted model used to study the pathology of human colon carcinogenesis (Druckrey, 1973; Reddy, 2004). Many studies investigating the effect of dietary/nutritional factors and chemopreventive agents on colorectal carcinogenesis have utilized AOM (Reddy, 2004). The first tumor is often visible 15 weeks after treatment and tumors have a mean latency period of about 20 weeks (5 months). Studies using tumors as the endpoint are usually terminated 7 to 9 months after AOM treatment when tumors are developed (Reddy, 2004). AOM methylates DNA primarily at the O^6 position of guanine, resulting in O^6-methylguanine adducts. If left unrepaired, the adduct will pair with thymine, instead of cytosine, resulting in G→A transition during DNA replication (Wali et al., 1999).

In short-term colon carcinogenesis experiments, it is widely accepted to utilize aberrant crypt foci (ACF), early preneoplastic lesions, as biomarkers to screen for potential chemopreventive agents as well as carcinogens prior to long-term studies (Roncucci et al., 1991; Femia and Caderni, 2008). ACF have been observed in the intestines of rodents injected with the colon carcinogens: AOM and dimethylhydrazine (DMH, an AOM precursor). These morphological precursors can be viewed microscopically after whole colons are stained with methylene-blue. Single (aberrant crypts) or clusters (ACF) of morphologically modified colonic crypts stain darker than surrounding areas, possess altered lumenal openings (oval or slit-like), thickened epithelia and appear larger than adjacent normal crypts. They are visible by 5 weeks, but

commonly examined 12-15 weeks post-carcinogen treatment (Bird et al., 1989). Additionally ACF have been identified in the colons of humans with FAP or CRC (Roncucci et al., 1991; Pretlow et al., 1991; Yokota et al., 1997; Schoen et al., 2008). There is some debate on whether ACF correlate with tumor development and if they are true precursors of tumors (Davies and Rumsby, 1998; Mori et al., 2005; Gupta and Schoen, 2008; Schoen et al., 2008). Other preneoplastic lesions identified and suggested to that correlate better with tumor development include β-catenin accumulated crypts (BCAC) (Yamada et al., 2001) and mucin depleted foci (MDF) (Caderni et al., 2003).

Tumors remain the best endpoint to evaluate the efficacy of chemopreventive agents and dietary components against AOM-induced colon cancer. The distribution of the intestinal tumors in AOM injected rodents is similar to that exhibited in humans. Small intestinal tumors, although rare, are generally found in the duodenum, and large intestinal tumors are generally found in the distal 2/3 of the colon (corresponding to the left side or descending and sigmoid colon in humans) (Ward et al., 1973; Zedeck, 1980). Histolopathological characteristics and genetic alterations identified in sporadic colonic tumors in humans have also been identified in AOM-induced tumors in rodents (Druckrey, 1973; Ward et al., 1973; Reddy, 2004). Three types of colonic tumors identified include: adenomas, adenocarcinomas and mucinous adenocarcinomas (Ward et al., 1973; Shamsuddin, 1983).

There are numerous genetic alterations occurring during the colorectal cancer process, resulting in perturbations in signaling pathways regulating cellular homeostasis. Mutations and/or deletions in *APC*, K-*ras*, *DCC* and *p53* genes are frequently found in human colorectal cancer (Figure 5). Alterations in *APC* and K-*ras* genes often occur

during the early stages of the carcinogenesis process, whereas *DCC* and *p53* are involved during the late stages (Kinzler and Vogelstein, 1996; Takahashi and Wakabayashi, 2004). In AOM-treated rats, mutations in codon 12 of the K-*ras* oncogene have been detected at high frequencies in ACF and adenocarcinomas (Erdman et al., 1997; Takahashi and Wakabayashi, 2004). This mutation results in the continuous activation of the *K-ras* oncogene, a component in the RAS-RAF-MAPK signaling pathway involved in cell growth and survival and has been identified in 50% of colorectal carcinomas (Fearon and Vogelstein, 1990). Although mutated K-*ras* has also been detected in AOM-treated mice models, it does not occur as frequently as that in rats (Guda et al., 2004). In contrast, mutations in *APC, DCC* and *p53* are rarely detected in ACF and adenocarcinomas found in AOM-treated rodents (Erdman et al., 1997; Takahasi and Wakabayashi, 2004).

One of the earliest genetic events to occur in colorectal carcinogenesis is the loss of a functioning APC protein. Approximately 80% of affected FAP patients possess a germline mutation in the *APC* gene and somatic mutations have been observed in more than 50% to 80% of sporadic colorectal tumors (Kinzler and Vogelstein, 1996; Cassidy et al., 2007; Femia and Caderni, 2008). Yet mutations in this gene are rarely observed in AOM induced ACF, adenomas or adenocarcinomas in rodents. The APC protein is responsible for multiple cellular functions, but in carcinogenesis, its role as a tumor suppressor is of great importance. It is responsible for regulating the cellular levels and location of β-catenin, a cell adhesion protein family member and essential component of the Wnt signaling pathway, a pathway reportedly involved in the transformation of normal cells to malignant tumors (Buda and Pignatelli, 2002). The loss of a functioning APC protein leads to the accumulation of β-catenin in the cytoplasm and nucleus and

increased activation of β-catenin target genes i.e. *cyclin D* and *c-myc*, both important in cell proliferation and shown to be up-regulated in colon cancers (Takahashi and Wakabayashi, 2004). Although *APC* is not often mutated in AOM induced colon cancer, researchers have reported altered cellular location of β-catenin in AOM induced dysplastic ACF, adenomas and adenocarcinomas in rodents and mutations within the *β-catenin* gene were identified (Guda et al., 2004; Takahashi and Wakabayashi, 2004). In humans, 4 - 15% of all sporadic colorectal adenomas and carcinomas have mutations in the *β-catenin* gene, yet a functioning APC protein (Morin et al., 1997; Buda and Pignatelli, 2002). Thus mutations in the *β-catenin* gene can contribute to the nuclear accumulation of β-catenin protein and increased transcription of target genes, similar to that observed with mutant ACF protein.

High throughput cDNA microarrays have permitted researchers to analyze the expression of tens to thousands of genes in a variety of species. Using microarray technology Rondini (2006) investigated genetic alterations in AOM-induced colon tumors compared to surrounding normal appearing colonic mucosa in F344 rats. The expression of genes involved in inflammation, extracellular matrix (ECM) formation, and cell cycle regulation were upregulated in AOM-induced tumors, whereas anti-inflammory genes, tumor suppressors genes and genes encoding ion transporters and metabolic enzymes were downregulated. This is in agreement with evidence showing enhanced expression of enzymes associated with inflammation observed in human CRC and in AOM induced colon carcinogenesis. For example, COX-2 and iNOS, important inflammatory enzymes upregulated in human CRC, are reportedly over expressed in AOM induced rat colorectal cancers as well (Takahashi and Wakabayashi, 2004). In

addition to an increased expression of COX-2 in AOM induced rat colonic adenomas and adenocarcinomas, Shao et al. (1999) found an overexpression in TGF-β1 in the same colonic lesions. These authors suspected the overexpression of COX-2 observed during AOM induced colon carcnogenesis results from the overexpression of TGF-β1 (Shao et al., 1999).

The TGF-β signaling pathway controls cell division, differentiation, migration, adhesion, extracellular matrix deposition and programmed cell death (apoptosis) in many cell types (Harradine and Akhurst, 2006; Massagué et al., 2006). TGF-β and its family members can exert dual functions depending on the cell type and time of development, for example during embryogenesis TGF-β promotes tissue growth and morphogenesis, whereas in mature adult tissues it maintains homeostasis by activating cytostatic processes i.e. inhibiting cell growth and multiplication, as well as cell death processes (Massagué et al., 2006).

Transforming growth factor (TGF-β) is a member of a superfamily of cytokines that include: the nodals, activins, bone morphogenic proteins (BMP), myostatin and anti-Muellerian hormone (AMH). Three isoforms of TGF-β are expressed in mammalian cells, i.e. TGF-β1, TGF-β2 and TGF-β3. TGF-β1 is the most abundant and ubiquitiously expressed isoform. Mammalian cells secrete TGF-β into the extracellular matrix as the inactive latent protein complex, LTGF-β. The LTGF-β complex consists of the mature TGF-β protein non-covalently associated to the N-terminal TGF-β propeptide called latency-associated peptide (LAP), which is covalently bound to a latent TGF-β binding proteins (LTBPs). In order for the TGF-β ligand to associate with its membrane surface receptors it must be activated by being released from the LTGF-β complex. Although the

exact mechanism activating the TGF-β ligand is not fully understood, it involves proteolytic cleavage of LTBP and the release or a conformational change in LAP. Activation occurs in response to microenvironmental signals. Once activated the members of the TGF-β superfamily signal through one of two ligand-receptor-Smad signaling branches: 1.) TGF-βs/ activins/ nodals to Smad 2/ 3; 2.) BMPs and AMHs to Smad 1/ 5/ 8. There are two high affinity TGF-β receptors, type I and type II that are serine/threonine kinase transmembrane receptors and are ubiquitously expressed. A third low affinity TGF-β receptor, type III or betaglycan, is a membrane-anchored proteoglycan responsible for capturing the TGF-β ligand and presenting it to the TGF-β receptors type I and II. The activated TGF-β ligand binds the type II receptor which results in the recruitment of the type I receptor forming a heteromeric complex. The type II receptor phosphorylates the type I receptor and activates its kinase. The activated ligand-receptor complex attracts and phosphorylates downstream target proteins i.e. Smad proteins. Humans and mice encode for eight Smad proteins, i.e. Smad 1-5,8 and Smads 6 and 7 the inhibitor Smads. The type I receptor in the ligand-receptor complex, phosphorylates the cytoplasmic Smad proteins. These phosphorylated Smads form of heteromeric complex with Smad 4, an essential component in all Smad signaling pathways, which allows the complex to be translocated into the nucleus. The Smad complex can then combine with a variety of DNA-binding proteins or cofactors, i.e. FOX, HOX, RUNX, E2F, AP-1, CREB/ATF, Zinc-finger. It is this Smad-cofactor combination, determined by cell-specific factors, that specifies the subset of genes that will be bound, and which transcriptional co-activators or co-repressors will be recruited. Smad activation can lead to hundreds of immediate gene activation or repression

responses. Although Smad signaling is essential for most TGF-β responses, other mediators of signaling such as mitogen activated protein kinases (MAPKs)/ERK, Jun N-terminal kinase (JNK), p38, P-I3K kinase, PP2A phosphotases and Rho family members, can be activated (Derynck et al., 2001; Elliott and Blobe, 2005; Javelaud and Mauviel, 2005; Harradine and Akhurst, 2006; Massagué et al., 2006; Seoane, 2006).

TGF-β inhibits cell proliferation in specific cell types, i.e. epithelial, endothelial and hemotopoietic cells (Massagué et al., 2006; Seoane, 2006), chiefly by preventing cell progression through the G1 phase of the cell cycle. In epithelial cells in the normal colonic crypt, the expression of TGF-β increases from the bottom of the crypt to top, with the highest amounts expressed at the lumenal surface where the mature, differentiated cells are located (Shao et al., 1999). Its expression is inversely related to proliferation. TGF-β induces the transcriptional expression of *p21CIP1* and *p15Ink4b*, cyclin-dependent kinase (CDK) inhibitors, while concurrently repressing the expression of *c-myc and ID1, 2* and *3*. *c-Myc*, a proto-oncogene, promotes cell growth and proliferation and the *IDs* are nuclear factors capable of inhibiting cell differentiation. *c-Myc* is frequently mutated in many types of cancers. Furthermore the increased expression of p15 leads to the redistribution of another CDK inhibitor, p27Kip1, from the active p27-cyclin D-Cdk 4/6 complex to cyclin E-Cdk 2, inactivating Cdk 2, a late G_1/S-phase kinase. Thus in normal epithelial cells TGF-β signaling induces cell cycle arrest and clearly helps to regulate colon crypt cell kinetics (Massagué et al., 2006).

Abnormal TGF-β signaling has been associated with cancer, and plays a dual role in the tumorigenesis process by acting as a tumor suppressor during the early stages and a promoter in the late stages (Seoane, 2006). In fact, a large majority of epithelial derived

tumors (≥85% of all human cancers) no longer respond to the anti-proliferative signals induced by TGF-β, resulting in a selective growth advantage for these tumor cells (Elliott and Blobe, 2005). In CRC, resistance to the tumor suppressor effect of TGF-β results from mutations in components of the TGF-β signaling pathway, i.e. *TGF-βRII* receptor and *SMAD 4* (Markowitz et al., 1995; Zhou et al., 1998; Grady et al., 1999; Elliott and Blobe, 2005; Harradine and Akhurst, 2006). Additionally, *in vitro* studies have shown that some colon carcinoma cell lines no longer respond to the anti-growth effects of TGF-β , while other colon carcinoma cell lines remain sensitive. Sensitivity to the anti-growth effects of TGF- β was found to be positively associated to the degree of differentiation of the colon carcinoma cell lines (Hoosein et al., 1989; Hsu et al., 1994). Insensitivity to anti-growth signals represents one of the hallmarks of cancer governing the transformation of normal cells to malignant cancers (Hanahan and Weinberg, 2000).

As previously mentioned, altered TGF-β expression has been identified in AOM induced colon cancer in animal models (Shao et al., 1999). When Guda and coworkers (2001; 2003) examined the TGF-β signaling pathway in the AOM induced colon carcinogenesis in the susceptible A/J mouse, they found significantly higher expression of TGF-β1 protein and reduced expression of TGF-βRII (mRNA and protein) in colon tumors compared to normal colonic mucosa from control animals. In an attempt to clarify where the disruption in the TGF-β signaling pathway occurred the expression of several components in the pathway were analyzed. No significant changes were revealed in *TGF-β1, TGF-βRI, Smad 3* or *Smad 7* mRNA levels, however, the expression of *c-myc* mRNA was significantly upregulated, while *p15* was significantly downregulated (Guda et al., 2001; 2003). *p15* and *c-myc* are direct targets of the TGF-β. In normal cells TGF-

β would activate the transcription of *p15*, a CDK inhibitor, while simultaneously suppressing the transcription of c-*myc*, a proto-oncogene that promotes cell growth and proliferation (Massagué et al., 2006). These two opposing actions are required to block the progression of cells through the G1 phase of the cell cycle (Massagué et al., 2006). The data in this study suggest that tumor cells in AOM injected A/J mice are resistance to TGF-β growth inhibition is a consequence of reduced *TGF-βRII R* expression leading to the downregulation in *p15* expression permitting the expression of *c-myc* which promotes progression through the cell cycle and continued cell proliferation (Guda et al., 2001).

Although the mRNA levels of *TGF-β1* were not altered in AOM induced colonic adenomas in A/J mice, higher TGF-β1 protein expression was reported. Since high TGF-β1 protein levels are reported in different types of human cancer and TGF-β1is secreted in a latent inactive form, the authors wanted to determine the amount of active mature TGF-β1 protein vs. latent (i.e. inactive bound TGF-β1) in the tumors. In the tumors 50% of the TGF-β1 protein was active compared to 80% in the normal colonic mucosa. The higher amount of latent TGF-β1 protein was suspected to result from the lower plasmin activity and higher PAI-1 mRNA identified in the tumors. Plasmin is a known activator of TGF-β1, whilst PAI-1 (plasminogen activator inhibitor) regulates the production of plasmin from plasminogen and serves as a negative feedback regulator of the TGF-β signaling pathway. To confirm that a disruption in TGF-β1 signaling was occurring the expression of 21 previously identified TGF-β- specific target genes were analyzed. It was determined that in AOM induced tumors expressing high amounts of TGF-β1 protein the expression of all 21 target genes were downregulated by at least 1.5 fold compared to the normal mucosa. These target genes were involved in cell matrix interaction (i.e.

IGFBP3, fibronectin), cell-to-cell interaction (i.e. Notch2, APC, CD44) and cytoplasmic regulators and effectors (i.e. rhoB, rho GDP-dissociation inhibitor 1, P21-RAC2). The authors concluded that a defect in the activation and turnover of the TGF-β1 protein, in addition to the downregulation in TGF-βRII receptors contributes to the progression of AOM induced colon carcinogenesis in A/J mice (Guda et al., 2001; 2003).

Raju and colleagues (2002) examined alterations in TGF-β1 and TGF-β2 mRNA and protein expression in the colons of AOM injected F344 rats fed diets containing different dietary lipids. Dietary lipids differentially regulate the expression of TGF-β *in vitro* in some tissues (Biasi et al., 2007). The dietary intervention was initiated 10 weeks after the final AOM injection; thus these researchers were investigating the promotional effects of different dietary lipids on preneoplastic lesions (i.e. ACF) and whether this involved the TGF-β signaling pathway. The AOM induced tumors from all animals regardless of diet, exhibited higher levels of TGF-β1 and TGF-β2 mRNA and protein compared to the surounding normal colonic mucosa. In a minor subset of ACF, TGF-β1 and TGF-β2 protein levels were also higher than the normal mucosa. The overexpression of TGF-β1 and TGF-β2 mRNA and protein in AOM induced preneoplastic lesions and tumors suggests that during the later stages of colon carcinogenesis an overexpression of TGF-β may promote the progression of tumors to more advanced lesions (Raju et al. 2002).

1.3. Colorectal Cancer and Diet

Population-based studies have demonstrated large geographical variations in CRC incidence across the world, with lower incidences in Africa and Asia, moderate incidences in South America and high incidences in North America, Australia/New

Zealand and Western Europe (Parkin et al, 2005; WCRF/AICR, 2007). In 1975 Armstrong and Doll reported a 60-fold variation in global CRC rates among countries, whereas in 2003, Parkin and colleagues reported a 25-fold variation in CRC rates among countries. Epidemiological studies examining dietary patterns and disease risk have observed changes for risk in diseases predominantly found in higher-income, industrialized nations, beginning to increase in less developed nations as they become more urbanized or "Westernized". These countries simultaneously assimilate the dietary patterns and lifestyle practices of Western nations (Heavey et al, 2004; WCRF/AICR, 2007). The best evidence indicative of these changes in disease risk is from migrant studies. Migrant studies observing the changes in cancer rates in populations migrating from low-risk areas to high-risk areas (or vice versa) and their off-spring, have demonstrated a strong relationship between lifestyle and environmental factors, i.e. dietary patterns, cultural and social practices, physical activity, obesity, socioeconomic status, and cancer incidence (Heavey et al, 2004). This relationship is found when migrants move from rural to urban areas within their country of origin or from one country to another. Researchers have found that as migrants' dietary patterns change, disease patterns begin to reflect those of their new region within one to two generations (Willett, 2001; WCRF/AICR, 2007). "Disease rates that shift with migration are those with important environmental causes" (WCRF/AICR, 2007). Therefore migrant studies validate the causal effect of lifestyle and environmental factors in cancers of most sites, including CRC (WCRF/AICR, 2007). Although genetics accounts for 5-10% of colorectal cancer cases, the more predominant modulators in colorectal cancer development are lifestyle and environmental factors (Weitz et al, 2005; Willett, 2001).

Theoretically, an estimated 80-90% of all cancers could be prevented by altering environmental and/or lifestyle, suggesting cancer is a preventable disease (Doll, 1998; WCRF/AICR 2007).

Evidence has been shown that diet is an important lifestyle factor that can be modified and alter CRC risk. Since food components come in direct contact with the colonic epithelial cells and colorectal carcinogenesis requires a number of molecular alterations to develop, it is logical that food components can interfere, inhibit, or reverse its development (Janne and Mayer, 2000). However, distinguishing which food components or food groups specifically protect or promote the development of CRC and the molecular mechanisms by which they exert these biological activities have yet to be clearly comprehended. Until recently the suggestion of a positive association between total fat intake and the risk of developing cancers of the breast, prostate, and colon was commonly accepted, yet in the 2007 WCRF/SICR report reviewing the evidence on food, nutrition, physical activity and the prevention of cancer, a consensus panel found limited proof to suggest that high total fat intakes increase an individual's risk of developing colon, breast or prostate cancer.

When reviewing the evidence regarding CRC, the WCRF/AICR panel concluded that there was convincing evidence to support the positive association between higher intakes of red meat and processed meat, greater abdominal and total body fatness and substantial consumption of alcoholic drinks, and the risk of developing CRC, whereas physical activity was found to protect against its development. They concluded that consuming foods containing dietary fiber, garlic, milk and calcium probably protects against CRC. Studies examining legume consumption and CRC risk were not analyzed

by the panel because they were limited in number and/or they may not have met the predetermined criteria required to be included in the analysis. However the panel did report that evidence from a limited amount of studies suggested a protective effect against stomach and prostate cancer with higher legume consumption (WCRF/AICR, 2007).

1.4. Colorectal Cancer and Legumes

The consumption of legumes including dry beans has been inversely associated with the development of several chronic diseases such as cardiovascular disease (CVD), type 2 diabetes mellitus, obesity and colon cancer (Geil and Anderson, 1994; Anderson and Major, 2002; Guillon and Champ, 2002; Giovannucci, 2003; 2007; Gunter and Leitzmann, 2006). Many of the risk factors associated with obesity, type 2 diabetes, CVD and colorectal cancer overlap and nutrition plays an important role in the etiology, prevention, treatment, and management of these diseases.

1.4.1. Epidemiological Studies

Although limited, some epidemiological studies have investigated the relationship between colorectal cancer and legume consumption. Similar to studies examining legume consumption and risk of developing obesity, diabetes or CVD, the majority of studies clump legumes in with other high fiber plant foods, rather than analyzing them individually. Although it is probable that dietary fiber does protect against CRC, the relationship between fiber and cancer has not been clear. This is due to many factors including food surveys, differences in the definition of fiber, vast number of plant foods having high fiber content, and the other components exerting biological activity found in high fiber foods.

In 1981, Correa examined the role of vegetables in cancer risk and found a significant negative correlation coefficient (-0.68, P<0.05) between dry bean intake and colon cancer risk (Correa, 1981). The Seventh-day Adventists Health cohort prospective study in California, examined the relationship between intakes of specific foods or food components and colon cancer incidence. The Seventh-day Adventists consume a largely plant-based diet, lower in saturated fat and higher in fiber compared to other U.S. populations and their mortality rates from cancer, heart disease, and diabetes are lower compared to non-Adventists living within the same community. An inverse association between legume intake and colorectal cancer risk was found (>2x per week vs. <1x per week, RR=0.53 [95% CI: 0.33-0.86]; p=0.03). Additionally, the authors observed a $\geq$ 3-fold increase in colon cancer risk for individuals having a high BMI, consuming diets high in red meat, but low in legumes. The authors suggested that the consumption of legumes counteracts the colon cancer promoting effects induced by red meat (Singh and Fraser, 1998). Steinmetz and Potter (1993) reported an inverse relationship between legume intake and colon cancer risk in both men and women enrolled in a case-control study. With the observed reduction being greater in women consuming the greatest amount of legumes vs. those consuming none, approximately a 50% reduction (OR=0.53 [95% CI: 0.26-1.07]), whereas a 30% reduction was found for men (OR=0.74 [95% CI: 0.39-1.38]). When examining the association between fruit and vegetable intake and the prevalence and incidence of tumors in the distal colon and rectum in the Nurses' Health Study, Michels and colleagues (2006) reported women who regularly consumed legumes (>4 serving/week) had a lower incidence of colorectal adenomas (OR= 0.67 [95% CI: 0.51-0.90]; p=0.005). In support of the previous study, a dietary intervention study

called the Polyp Prevention Trial (PPT), researchers examined the association of fruit and vegetable intakes and the recurrence of colorectal adenomas. They found a significant inverse association between increased dry bean intake (the greatest increase in dry bean intake vs. the lowest; OR=0.35 [95% CI: 0.18-0.69]; p=0.001) over a 4 yr period and advanced adenoma recurrence (Lanza et al., 2006).

In a prospective cohort study, published by Lin et al. (2005), no significant association between fruit, vegetable and fiber intake and colorectal cancer was found in women. Yet the authors did report a significantly inverse relationship between legume fiber and colorectal cancer risk, when comparing the highest intake relative to the lowest (RR=0.60 [95% CI= 0.40-0.90], p=0.02).

1.4.2. Experimental Studies

Several researchers have investigated the antimutagenic and antigenotoxicity effect of dry beans. Using a modified version of the Ames test, Cardador-Martínez and colleagues (2002 and 2006) reported that a methanolic phenolic extract of the seed coats of raw common beans (*Phaseolus vulgaris* L., Flor de Mayo FM-38 variety) inhibited 50% of mutagenic activity induced by aflatoxin B_1 (AFB_1) *in vitro*. In addition the authors found that the phenolic extract from beans possessed antioxidant activity and was capable of scavenging free radicals; however this was not the mechanism responsible for inhibiting the mutagenic activity of AFB_1. Their data suggest that the phenolic extract from dry beans inhibits the mutagenicity induced by AFB_1 by reducing its bioavailability and the amount of the active ultimate mutagen (8,9- AFB_1 epoxide) formed by P450 enzymes, either by competing with the AFB_1 for P450 activity, directly inhibiting P450 activity or by binding the 8,9- AFB_1 epoxide (Cardador-Martínez et al., 2002; 2006). In

another study examining black beans and antimutagen activity, Azevedo et al. (2003) reported feeding diets containing 1%, 10% or 20% cooked black bean to mice injected with cyclophosphamide (CP), a well-known indirect acting mutagen, reduced the DNA damage.

Bawadi et al. (2005) investigated the anti-angiogenic activities of tannins extracted from black beans on several human cancer cell lines. The aqueous-acetone (70% acetone) soluble components in raw black beans (including tannins) inhibited the growth and angiogenesis of colon, breast, and prostate cancer cells in a dose-dependent manner yet had no effect on normal human fibroblast lung cells (Bawadi et al., 2005). Concentrations of condensed tannins (expressed as catechin equivalents) added ranged from 2 to 24 μM. The concentrations of phenolic compounds used in this study, as well as in many other cell culture studies, are unlikely to be found in the peripheral circulation *in vivo*. Human plasma concentrations of intact flavonoids have rarely exceeded 1 μM after consuming normal dietary levels of polyphenols (Scalbert and Williamson, 2000). However, there is the possibility of higher concentrations being found in the GI tract. For example, higher levels (μM to mM range) of flavonoids and simple phenolic compounds would be found in the stomach and small intestine prior to absorption after ingesting a meal containing plant foods. The colon could also be exposed to higher levels (μM range) of simple phenolic compounds; intact flavonoids; metabolites of flavonoids and other larger phenolic compounds; and microflora metabolites of phenolic compounds, all of which possess biological activity and possibly protect against colorectal cancer (Gee and Johnson, 2001; Halliwell, 2007).

It is important to note that the aqueous-methanolic and aqueous-acetone extracts from dry beans used in the previously mentioned studies were crude extracts. They most likely contained other soluble compounds that exert biological activity besides phenolic compounds, such as saponins and oligosaccharides.

Using animal models, Hughs et al. (1997) found that feeding pinto beans to AOM-injected rats significantly reduced tumor incidence compared to a casein-based control diet. Twenty-four percent of the rats fed the pinto bean diet had colonic tumors versus 50% tumor incidence in the rats fed the casein based control diet. Recently, Boateng and coworkers (2007) examined the effect of feeding dry beans: black-eyed peas, pinto beans, or soybeans, on AOM-induced colonic ACF in rats. Animals fed the bean diets weighed significantly more, and had significantly lower cecal pH than the casein control fed animals, yet all animals consumed similar amounts of food and had similar cecal weights. Compared to the casein control fed rats, the number of colonic ACF were reduced by 77%, 64% and 56% in the rats fed the black-eyed peas, pinto beans, and soybeans, respectively. The authors also found the dry bean diets significantly induced hepatic glutathione-*S*-transferase activity (GST). GST is a phase II detoxifying enzyme and its increased activity may help detoxify free radicals produced by the metabolism of carcinogens and other compounds (Boateng et al., 2007).

Our laboratory has performed several *in vivo* studies investigating the chemopreventive effect of feeding bean-based diets on AOM induced colon carcinogenesis in a rat model. Hangen and Bennink (2002) determined that feeding navy and black beans to AOM injected rats reduced colon tumor (adenoma and adenocarcinoma) incidence by 59% and 54%, respectively, compared to rats fed a control

casein-based diet. Similarly, when Rondini (2006) fed AOM injected rats defatted soybean flour or black beans, colon tumor incidence was reduced by approximately 60% with both bean based diets. Based on cDNA microarray technology, feeding beans inhibits AOM induced colon tumorigenesis by modulating the expression of genes involved in regulating colon crypt cytokinetics and in reducing inflammation (Rondini, 2006).

D. Rationale

The consumption of dry common beans is inversely associated with the development of chronic diseases, including CVD (Finley et al., 2007; Winham et al., 2007), Type 2 diabetes (Villegas et al., 2008), obesity (van Dam and Seidell, 2007) and cancers of the breast (Thompson et al., 2008; 2009) and colon (Hughes and Ganthavorn, 1997; Hangen and Bennink, 2002). Dry beans are a rich source of protein, dietary fiber, vitamins and minerals, as well as many non-nutrients such as phenolic compounds.

Phenolic compounds have been suggested to contribute to the protective effects observed when fruits, vegetables and grains, including dry beans, are consumed. They have been shown to act as antioxidants (Croft, 1998), anti-inflammatory agents (Rahman et al., 2006), and anti-cancer agents (Bode and Dong, 2004), yet the exact mechansism(s) of action has yet to be elucidated. Increased interest in the health benefits of phenolic compounds makes identifying and quantifying them in all plant foods of great importance.

Investigators have identified different types of phenolic compounds in grain legumes, including flavonol glycosides, hydroxybenzoic acids, hydroxycinnamic acids,

anthocyanins and condensed tannins (proanthocyanidins) (Krygier et al., 1982; Sosulski and Dabrowski, 1984; Beninger et al., 1999; 2003; 2005; Madhujith et al., 2004; Aparicio-Fernandez et al., 2005; Hu et al., 2006; Lin and Lai, 2006; Ranilla et al., 2007). The majority of these studies analyzed raw grain legumes, however legumes must be exposed to some sort of heat treatment to be safe to consume. Heat processing induces physical and chemical compositional changes in legumes, altering their nutrient and phenolic content. Thus from a dietary standpoint it is important to quantify the phenolic compounds in heat treated grain legumes i.e. dry common beans, in the form they are consumed by humans.

Epidemiologic studies suggest that bean consumption is inversely related to colon cancer incidence (Correa, 1981) and animal studies have found that feeding pinto beans, black beans, and navy beans inhibit chemically induced colon cancer by more than 50% (Hughes and Ganthavorn, 1997; Hangen and Bennink, 2002; Rondini, 2006). Often the anti-cancer activity of dry beans is attributed to the high dietary fiber content of beans. Although an inverse relationship between dietary fiber and CRC risk is probable the evidence supporting the relastionship has been inconsistent (WCRF/AICR, 2007). Thus narrowing which specific components in dry beans are responsible for the protection against the development of CRC are of high priority. Of equal importance is the molecular identification of the molecular mechanism by which dry beans inhibit cancer. One approach to narrowing the search for the anti-cancer components of legumes is partitioning beans into different fractions and determining the anti-cancer activity for the various fractions. Bennink and Barrett (2004) used this approach with soybeans (an oil legume). They fed heat-treated, full-fat soybean flour, soybean flour minus lipids,

defatted soybean flour minus aqueous-ethanol components and a control diet to determine which fractions protected against development of AOM-induced colon cancer in rats. Full-fat soybean flour and defatted soybean flour diets significantly reduced tumor incidence by 45% ($P < 0.05$) compared to rats fed a casein control diet. Based on this experiment and other unpublished studies it was determined that soybean fiber, sterols, fatty acids, protein and isoflavones could not be providing the anti-cancer action noted with defatted soy flour. However, removing the aqueous-alcohol soluble components completely eliminated any anti-cancer action (Bennink and Barrett, 2004). The aqueous-alcohol fraction would contain not just isoflavones, but other phenolic compounds such as hydroxycinnamic and hydroxybenzoic acids i.e. syringic acid, ferulic acid, sinapic acid and *p*-coumaric acid (Seo and Morr, 1984), as well as saponins and oligosaccharides, which are reportedly bioactive. Subsequent studies have shown soybean isoflavones do not inhibit colon cancer, which suggests further that other compounds in legumes, which are soluble in an aqueous-alcohol solvent, may be responsible for the anti-cancer activity observed in colon cancer prevention studies in rats.

Consistent with the protection observed in the previous animal study (Bennink and Barrett, 2004), *in vitro* studies have shown crude aqueous-alcohol and aqueous-acetone extracts from dry beans (*Phaseolus vulgaris* L.) possess anti-mutagenic, anti-genotoxic, and anti-angiogneic properties (Azevedo et al., 2003; Cardador-Martínez et al., 2002; 2006; Bawadi et al., 2005). These crude extracts would be concentrated in phenolic compounds as well as saponins and oligosaccharides.

Besides colorectal cancer, dry bean consumption has been inversely associated with risk of developing obesity and type 2 diabetes mellitus (Geil and Anderson, 1994; Maskarinec et al., 2000; Guillon and Champ, 2002; Venn and Mann, 2004; Villegas et al., 2008). Many of these biological perturbations resulting from obesity and diabetes, have been positively associated with colon cancer (Gunter and Leitzmann, 2006). However, few studies have evaluated the effect of chemically induced colon cancer in obese animal models, of those that have, the authors reported a higher numbers of preneoplastic lesions and tumors in the colons of obese animals compared to lean (Weber et al., 2000; Hirose et al., 2004; Ealey et al., 2008). To date an obese animal model has not been utilized to evaluate the chemopreventive affect of feeding dry beans on chemically induced colon cancer.

Colon carcinogenesis is a multi-step process requiring a number of molecular alterations to fully manifest, thus it is logical that dietary-induced changes in metabolized and undigested food components might alter colon cancer development. The mechanism by which dietary components, including dry beans, inhibit chemically induced colon cancer in animals has not been fully elucidated. Colon tumorigenesis is associated with perturbations in colon crypt homeostasis, including changes in cell proliferation, differentiation and apoptosis (Fearon and Vogelstein, 1990; Chang et al., 1997). Consistent with the idea that diet can modulate gene expression, Rondini (2006) reported the downregulation in the expression of genes involved with colon crypt cytokinetics and in anti-inflammation within the colonic mucosa of AOM-injected rats fed black beans and soybean flour diets.

The objectives of my dissertation are as follows:

1. To extract, separate, identify and quantify the principal phenolic compounds in cooked dry common beans (*Phaseolus vulgaris* L.) using high-performance liquid chromatography (HPLC)-electrochemical detection (ECD).

2. To extract a sufficient amount of phenolic compounds from cooked beans using an aqueous-alcohol solvent to allow long-term colon cancer studies to be conducted with rodents, and to identify and quantify the principal phenolic compounds in the bean extract (aqueous-alcohol soluble components) and in the residue (insoluble particulate matter remaining after extraction of alcohol-aqueous soluble compounds).

3. To evaluate whether the obese, mildly diabetic mouse (*ob/ob* mouse (B6.V-Lep^{ob} /J)) is an appropiate animal model for studying azoxymethane-induced colon cancer.

4. To feed the cooked common beans and its fractions to obese, mildly diabetic mice (*ob/ob* mice (B6.V-Lep^{ob} /J)) with chemically induced colon cancer to narrow the search for which components in dry beans are responsible for the observed reduction in the development of chemically-induced colon cancer.

5. To determine if feeding bean diets and its fractions to *ob/ob* mice reducing the development of colon tumorigenesis and if this is associated with normalizing gene expression in pathways intricately involved with colon crypt cytokinetics.

CHAPTER III. PHENOLIC COMPOUNDS IDENTIFIED AND QUANTIFIED IN COOKED DRY COMMON BEANS (*PHASEOLUS VULGARIS* L.)

A. ABSTRACT

The consumption of legumes such as dry common beans has been inversely associated with development of several chronic diseases (Thompson et al., 2008; 2009). Dry beans contain a variety of phytochemicals that may provide health benefits. Phenolic compounds in many commonly consumed fruits and vegetables, have been identified and quantified; however, there is little data regarding the phenolic compounds in cooked dry common beans (*Phaseolus vulgaris* L.).

The primary objective of this study was to extract, separate, identify, and quantify the principal phenolic compounds in cooked dry common beans using high-performance liquid chromatography (HPLC)-electrochemical detection (ECD). A secondary objective was to: a) extract a sufficient amount of phenolic compounds from cooked navy beans to allow long-term colon cancer studies to be conducted with rodents; and b) to identify and quantify the principal phenolic compounds in the navy bean extract (concentrated in phenolic compounds) and in the residue (insoluble particulate matter remaining after extraction of alcohol-aqueous soluble compounds).

Extracts of four cooked dry common beans (black, pinto, red, and navy beans) as well as in the navy bean fractions (navy bean residue and navy bean extract mix) were analyzed for phenolic compounds with and without acid and alkaline hydrolysis. Hydroxybenzoic acids and hydroxycinnamic acids were identified in the four cooked dry common beans. The hydroxybenzoic acids found were *p*-hydroxybenzoic acid, vanillic

acid, and syringic acid and total concentrations ranged from 3 to 12 mg per 100 g bean flour. Protocatechuic acid was only identified in black, pinto and red beans. The hydroxycinnamic acids identified were *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid in total concentrations ranging from 11 to 36 mg per 100 g bean flour. In general, acid hydrolysis liberated greater quantities of hydroxybenzoic acids while alkaline hydrolysis liberated greater quantities of hydroxycinnamic acids. Only black, pinto, and red beans contained the flavan-3ol (+)-catechin (0.3 to 3 mg per 100 g bean flour) and the flavonols quercetin and kaempferol (7 to 67 mg per 100 g bean flour). The large scale aqueous-ethanol extraction was more efficient in extracting hydroxycinnamic acid conjugates than hydroxybenzoic acid conjugates from cooked navy beans.

In conclusion, this is one of few studies to identify and quantify phenolic compounds in cooked dry common beans. It is important to know the types and amounts of phenolic compounds in cooked beans to further investigate their potential health benefits.

B. INTRODUCTION

The consumption of dry beans has been inversely associated with development of chronic diseases such as cardiovascular disease (CVD), type 2 diabetes mellitus, obesity, colon cancer (Hughes and Ganthavorn, 1997; Hangen and Bennink, 2002), and breast cancer (Thompson et al., 2008 and 2009). Earlier studies exploring the disease preventing components in legumes focused on dietary fiber. However, legumes, including dry beans, contain phenolic compounds that may confer some of these observed health benefits (Geil and Anderson, 1994; Messina, 1999).

Phenolic compounds belong to an array of structurally diverse secondary plant metabolites that are distributed throughout the plant kingdom (Stalikas, 2007). Phenolic compounds are not uniformly distributed in the plant tissues and tissue concentrations can vary depending on plant species, variety, time of harvest, maturation, germination, and storage conditions (i.e. time and temperature) (Tsao, 2004). There are at least 8,000 naturally occurring phenolic compounds, all of which possess a common aromatic ring bearing one or more hydroxyl substituents. This shared structure is referred to as a phenol (Harborne, 1998, Robbins, 2003; Balasundram, 2006; Stalikas, 2007).

Classes of phenolic compounds previously identified in grain legumes include hydroxybenzoic acids, hydroxycinnamic acids, flavan-3ols, flavonols, anthocyanins, and condensed tannins (Krygier et al., 1982; Sosulski and Dabrowski, 1984; Beninger et al., 1999; 2003; 2005; Madhujith et al., 2004; Aparicio-Fernandez et al., 2005; Hu et al., 2006; Lin and Lai, 2006; Ranilla et al., 2007). However, the cited publications analyzed raw, mature dry beans, but dry beans must be heat-treated before the bean is safe to consume.

Heat processing induces physical and chemical compositional changes in legumes, altering their nutrient and phenolic content. The changes are dependent on the legume type and processing conditions. Legumes are heat-treated before consumption to inactivate antinutrients such as lectins, protease inhibitors and amylase inhibitors. Processing methods include hydration (soaking), germination or fermentation, and some form of heat treatment (i.e. boiling, pressure cooking, canning, roasting, micronization, or autoclaving) (Champ, 2002; Ibrahim et al., 2002; Shimelis and Rakshit, 2007). Very few studies have investigated the phenolic compounds in legumes after processing. Of those that have, the majority only measured total phenolic content and report an overall decrease in the total phenolic content in grain legumes after cooking (Towo et al., 2003; Rocha-Guzmán et al., 2007; Granito et al., 2008; Xu and Chang, 2008).

In a published review on the subject of hydroxycinnamic acids, Clifford (1999) observed that assessing the dietary burden of hydroxycinnamic acids was not possible. He stated, "With the exception of some beverages, the lack of data for the composition of commodities as consumed, i.e. the edible portion after processing, cooking, baking, etc, effectively make such an assessment impossible". With the ever increasing public awareness of phenolic compounds as health promoting non-nutrients, especially as antioxidants, it is critical to identify and quantify phenolic compounds in plant foods that have been shown to impart health benefits when consumed.

One of the strongest health benefits derived from eating beans is the prevention of colon cancer. The cancer inhibiting component(s) of beans is(are) unknown. Since the phenolic compounds in beans could be involved in cancer inhibition, the primary objective of this study was to extract, separate, identify and quantify the principal

phenolic compounds in cooked dry common beans (*Phaseolus vulgaris* L.) using high-performance liquid chromatography (HPLC)-electrochemical detection (ECD). A secondary objective was to: a) extract a sufficient amount of phenolic compounds from cooked navy beans to allow long-term colon cancer studies to be conducted with rodents; and b) to identify and quantify the principal phenolic compounds in the navy bean extract (concentrated in phenolic compounds) and in the residue (insoluble particulate matter remaining after extraction of alcohol-aqueous soluble compounds).

C. MATERIALS AND METHODS

Chemicals

Methanol, 2N Folin-Ciocalteu phenol reagent, ellagic acid, gallic acid, isovanillic acid, salicylic acid, gentisic acid, chlorogenic acid, (-)-epicatechin, luteolin, apigenin, esculetin, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, isovanillic acid, syringic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, *o*-coumaric acid, (+)-catechin, quercetin, and kaempferol were purchased from Sigma Chemicals (St. Louis, MO). Acetonitrile — HPLC grade, acetic acid and hydrochloric acid were obtained from EMD Chemicals Inc. (Gibbstown, NJ). Sodium monobasic phosphate-monohydrate, sodium dibasic phosphate-anhydrous, phosphoric acid, sodium hydroxide were from J.T. Baker (Phillipsburg, NJ).

Navy, black, pinto and red beans were kindly provided by the Michigan Bean Commission and were stored at 4°C in dry storage prior to cooking.

Preparation of Whole Bean Flours

Five kg of raw, dry, common beans — black, pinto, red and navy beans — were soaked overnight at 4°C in sufficient distilled water to keep the beans covered with water. The soaked beans and soak water were added to large steam kettles (Model N 30 SP, Groen M.F.G. Co., Chicago, IL) and cooked for one hour at 98°C. Immediately after cooking, the beans and cooking water were dried in stainless steel trays in a convection oven at 75°C ± 5°C overnight (Machine # K 12395, Proctor and Schwartz, Inc., Philadelphia, PA). Once dried, the beans were ground to pass through a screen with 1.6 mm holes using a Fitzmill (Model D Comminuting Machine, The W.J. Fitzpatrick Co., Chicago, IL).

Preparation of Navy Beans and Fractions

Navy Beans (675 kg raw dry bean weight) were soaked and cooked as described above. Approximately 65% of the cooked navy beans were immediately ground with the Fitzmill fitted with a screen with 5 mm holes. Ethanol (95%) was added to the cooked ground navy beans and was mixed periodically for 18 – 24 hr to extract phenolic compounds. The final concentration of ethanol was approximately 60%. The liquid fraction (navy bean extract) was separated from the insoluble particulate matter (navy bean residue) by screen filters. The residue was placed into burlap bags and pressed at 20 kg/cm^2 (Bar N.A., Inc., Seymour, IL) to remove additional liquid in the wet residue. The liquid collected after pressing was combined with the filtrate. The liquid extract was left undisturbed for 12 – 24 hr to allow particulate matter passing through the screen filter to settle out. The liquid fraction was decanted and the solid sediment was combined with the residue. The navy bean residue (NBR) was dried and ground as described above.

The aqueous-alcohol liquid navy bean extract (approximately 3,200 L) was concentrated (approximately 130 L) using a Marriot Walker Falling Film Evaporator (Marriot Walker, Birmingham, MI). The concentrated liquid extract contained 27 g of dry material per 100 ml of liquid concentrate. Since the dry extracted material was very hygroscopic, the concentrated liquid bean extract was mixed with cellulose, casein, and cornstarch (27%:15%:19%:39%, wt:wt:wt:wt, on a dry basis, respectively) to facilitate drying and storage stability. The wet mix was oven dried overnight at 75°C ± 5°C. The final dried product (navy bean extract mix, NBEM) was then ground using a Fitzmill equipped with a screen with 1.6 mm holes.

For each navy bean fraction — whole beans, bean extract mix (NBEM) and navy bean residue (NBR) — a portion representing the entire ground fraction was immediately removed and stored at 4°C for subsequent analyses.

Extraction of Phenolic Compounds

Two g of each flour were extracted with 30 ml of 50% methanol containing 1% acetic acid (vol:vol), sonicated for 30 minutes, centrifuged (3,000 x g for 10 min) and the supernatant was decanted. The sediment remaining after centrifugation was extracted three more times using 70% and 90% methanol containing 1% acetic acid followed by 100% methanol. Supernatants from each cycle of the extraction process were combined. An additional 35 ml of 100% methanol was added to the supernatants to obtain a final concentration of approximately 80% methanol. The combined extracts were stored at 4°C overnight to precipitate macromolecules and then centrifuged at 3,000 x g for 20 min. The supernatants were evaporated to dryness by rotary evaporation, using vacuum

and low heat (≤ 45 °C). The residue after evaporation was dissolved in 15 ml of 80% methanol. Two extractions were performed on different days for each bean sample.

Total Phenolic Content Analysis

Total phenolic content (TPC) was determined using a modified version of the Folin-Ciocalteu method according to Singleton and Rossi (1965) and similar to Mosca et al. (2000). Briefly, 200 µl of bean extract and 50 µl of Folin-Ciocalteu reagent were combined in a test tube and vortexed. After 3 to 8 minutes, 100 µl of 35% sodium carbonate (wt:vol) was added and tubes were vortexed. Samples were brought to a final volume of 2.25 ml with distilled water (2.15 ml) and vortexed again. Samples were incubated in the dark at room temperature for 90 minutes to allow the color reaction to come to completion. Absorption was determined at 725 nm on a spectrophotometer. Bean extracts were diluted with 80% methanol as necessary to fall within the standard curve. Each sample was measured in triplicate on two different days. Calibration curves were prepared daily by diluting the (+)-catechin stock solution (0.503 mg/ml, diluted in 80% methanol) to a final concentration range of 2 - 34 µM (0.67-10.07 µg/ml). Total phenolic content (TPC) was expressed as mg of (+)-catechin equivalents ((+)-CE) per 100 g bean flour or bean fraction.

Hydrolysis of Conjugated Phenolic Compounds

Three aliquots (1 ml) of each bean extraction were evaporated under nitrogen gas at 50 °C. Before evaporation, 200 µl of the internal standard *o*-coumaric acid (10.2 µg) was added to each aliquot. One ml of distilled water was added to one of the dried aliquots and served as the control/non-hydrolyzed sample. Then the sample was acidified to a pH of 2.0 with 5 N HCL.

To identify and quantify the principal phenolic compounds present in the bean extractions, the second and third dried aliquots were hydrolyzed using alkali or acid to convert conjugated phenolic compounds to their free form (parent or aglycone form). Alkali hydrolysis cleaves ester bonds, whereas acid hydrolysis cleaves glycosidic bonds.

Alkali hydrolysis was performed as follows (Cai et al., 2003). One ml of 5N NaOH was added to the dried extracts and vortexed. The mixtures were continually flushed with nitrogen gas, heated for 4 hours at 50 °C and vortexed periodically. After hydrolysis, the alkali treated bean extracts were acidified to a pH of 2.0 with 5 N HCL.

Acid hydrolysis was performed as follows (Harborne, 1998). Five N HCL (0.800 ml) and 1.2 ml of distilled water were added to the dried extracts, (final concentration of HCL was 2N). Tubes were flushed with nitrogen gas, capped and placed in boiling water for 30 min. After acid hydrolysis, samples were placed in ice water until cool.

Sample Clean-up and Concentration

Solid-phase extraction (SPE) was used to purify and concentrate the free and hydrolyzed phenolic compounds. The control/non-hydrolyzed, alkali and acid hydrolyzates were applied to preconditioned (washed with 15 ml of 80% methanol and 15 ml of distilled water) SPE columns (Alltech Extract-Clean C18 column, (500 mg bed volume), Alltech, Deerfield, IL). After loading the extract onto the SPE column, the column was washed with 15 ml of distilled water to remove the unadsorbed, unwanted water soluble compounds such as sugars. To elute the free and hydrolyzed phenolic compounds, 5 ml of 80% methanol was applied to the columns 1 ml at a time. Pressure was applied to the top of the columns to obtain a drop-wise flow rate. The eluates were

collected and evaporated to dryness under nitrogen at 50°C. After drying they were re-dissolved in 1.5 ml of 80% methanol.

The hydrolysis and purification processes were performed on duplicate bean aliquots on different days. Duplicate control/non-hydrolyzed bean aliquots were passed through SPE columns on different days.

Preparation of Standard Solutions and Calibration Curves

Preliminary experiments revealed that the following phenolics were not present in the four bean market classes of beans used in this research: ellagic acid, gallic acid, salicylic acid, gentisic acid, (-)-epicatechin, luteolin, apigenin, and esculetin. Therefore, these phenolics were not included in standards. Stock standard solutions of protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, isovanillic acid, syringic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, *o*-coumaric acid (internal standard), and (+)-catechin were prepared by dissolving 20 mg in 20 ml of 80% methanol [1 mg/ml]. Chlorogenic acid, quercetin, and kaempferol stock solutions were prepared by dissolving 11 mg in 10 ml [1.1 mg/ml], 40 mg in 30 ml [1.3 mg/ml] and 5 mg in 10 ml [0.5 mg/ml] -in 80% methanol, respectively. Each standard was injected three times to establish retention times and oxidation profile. Then phenolic compounds that did not have similar retention times were combined to form four standard mixes. The mixed standards were diluted from 1:5 to 1:2500 with 80% methanol to establish calibration curves. Standard mix 1 contained: protocatechuic acid, (+)-catechin, vanillic acid, and ferulic acid; Standard mix 2 contained: *p*-hydroxybenzoic acid, chlorogenic acid, syringic acid, and sinapic acid; Standard mix 3 contained: isovanillic acid, *p*-coumaric

acid, and kaempferol; Standard mix 4 contained: caffeic acid, quercetin, and *o*-coumaric acid (internal standard).

Estimation of Phenolic Compound Loss During Sample Clean-up and Hydrolysis

To estimate loss of phenolic compounds due to sample preparation, a 1:10 dilution of the standard mixes were subjected to hydrolysis with alkali and with acid and SPE clean-up as described for the bean extracts.

High-Performance Liquid Chromatography-Electrochemical Detection

Separation and quantification of the phenolic compounds was achieved by using high-performance liquid chromatography (HPLC) equipped with an electrochemical detector (ECD). The HPLC-ECD equipment included a Dionex GS50 gradient pump (Dionex, Corp., Sunnydale, CA), a Spectra System autosampler injector (AS1000, Thermo Separation Products) and an 8-channel 5600A ESA Coularry® Electrochemical Detector (ESA Inc., Chelmsford, MA). The detector channels were set at –200 mV and from 200 to 800 mV, in increments of 100 mV. As a compound passes through the coulometric array detector cells, it is oxidized in a stepwise fashion until complete electrochemical conversion occurs resulting in a peak at a particular set current (Aaby et al., 2004). If the compound has more than one oxidizable moiety, than another peak at the current at which that moiety is oxidized will also be displayed. The ESA Coularray® software was used to analyze the chromatograms.

Separation was performed using a reversed phase Luna C18 Column, (250 x 4.60mm, 5µm particle size, Phenonenex, Torrance, CA). A guard column packed with C18 silica (20 µm) was placed before the separation column. Column temperature was maintained at 37°C. The mobile phases were as follows: solvent A - 2% Acetonitrile in

30mM phosphate buffer (pH 2.3); solvent B - 70% Acetonitrile in 30mM phosphate buffer (pH 2.3); and solvent C - 90% Acetonitrile and 10% water. The gradient applied was: 0-30 min, 0%-26% B; 30-65 min, 26%-65% B; 65-70 min, 0%-100% C; 70-75 min, 0%-100% A. The column was equilibrated with 100% A for 15 min before the next injection was made. The flow rate was 1ml/min and the injection volume was 20 µl.

Each reagent used to make up the mobile phases was measured individually using graduated cylinders, before being combined and mixed. The water used to prepare mobile phases was reverse osmosis water passed through a Millipore Milli-Q purification system (Millipore Corp., New Bedford, MA) and then "polished" by passing through a Millipore C18 SPE column (Millipore Corp., New Bedford, MA). Mobile phases A and B were filtered through a Millipore nylon membrane filter (47 mm) using vacuum (Millipore Corp., New Bedford, MA). The mobile phases were degassed by sonication for 5 min. All standards and bean extracts were filtered through a 0.45µm nylon filter (Alltech, Deerfield, IL) prior to HPLC-ECD analysis.

Quantification of Phenolic Compounds

Amounts of phenolic compound standards injected into the HPLC were plotted versus peak height and linear regression equations ($Y = aX + b$) were computed to establish standard curves. The regression equations were then used to quantify the phenolic compounds in bean extracts and in the standard mixes that had been treated to estimate losses during sample clean-up and hydrolysis. Phenolic compounds in bean samples were identified by retentions times, oxidation profile, and co-elution of spiked standards. All data were corrected for losses that occurred during sample clean-up and hydrolysis steps and are expressed as mg per 100 g of bean flour or bean fraction.

D. RESULTS

Separation, Identification, and Quantification of Phenolic Compounds in Dry Common Beans by HPLC-ECD

Chromatograms showing the four phenolic acid standard mixtures are displayed in Figures 6–9. Varying concentrations of these 4 standard mixes were injected into the HPLC to generate the standard curves used to quantify the various phenolic compounds in beans.

The phenolic compounds in plant materials are present conjugated to a variety of moieties at one or more sites on the aromatic rings. Thus, several compounds could be the source of one aglycone. To make the identification and quantification of plant phenolics even more difficult, only the most common phenolic glycosides or phenolic esters are available commercially for preparing standards. Therefore, to simplify identification of the various phenolic compounds, researchers frequently hydrolyze plant extracts so that only phenolic aglycones need to be identified. Representative chromatograms showing the effect of alkaline and acid hydrolysis on cooked dry common beans and navy bean fractions are shown in Figures 10a – 15c in Appendix A. Table 3, 5, and 7 in Appendix B list the concentrations of unconjugated phenolic compounds; unconjugated phenolic compounds plus phenolic compounds liberated by alkaline hydrolysis; and unconjugated phenolic compounds plus phenolic compounds liberated by acid hydrolysis, respectively, identified in cooked dry common beans. Tables 4, 6, and 8 in Appendix B list the concentrations of unconjugated phenolic compounds; unconjugated phenolic compounds plus phenolic compounds liberated by alkaline hydrolysis; and unconjugated phenolic compounds plus phenolic compounds

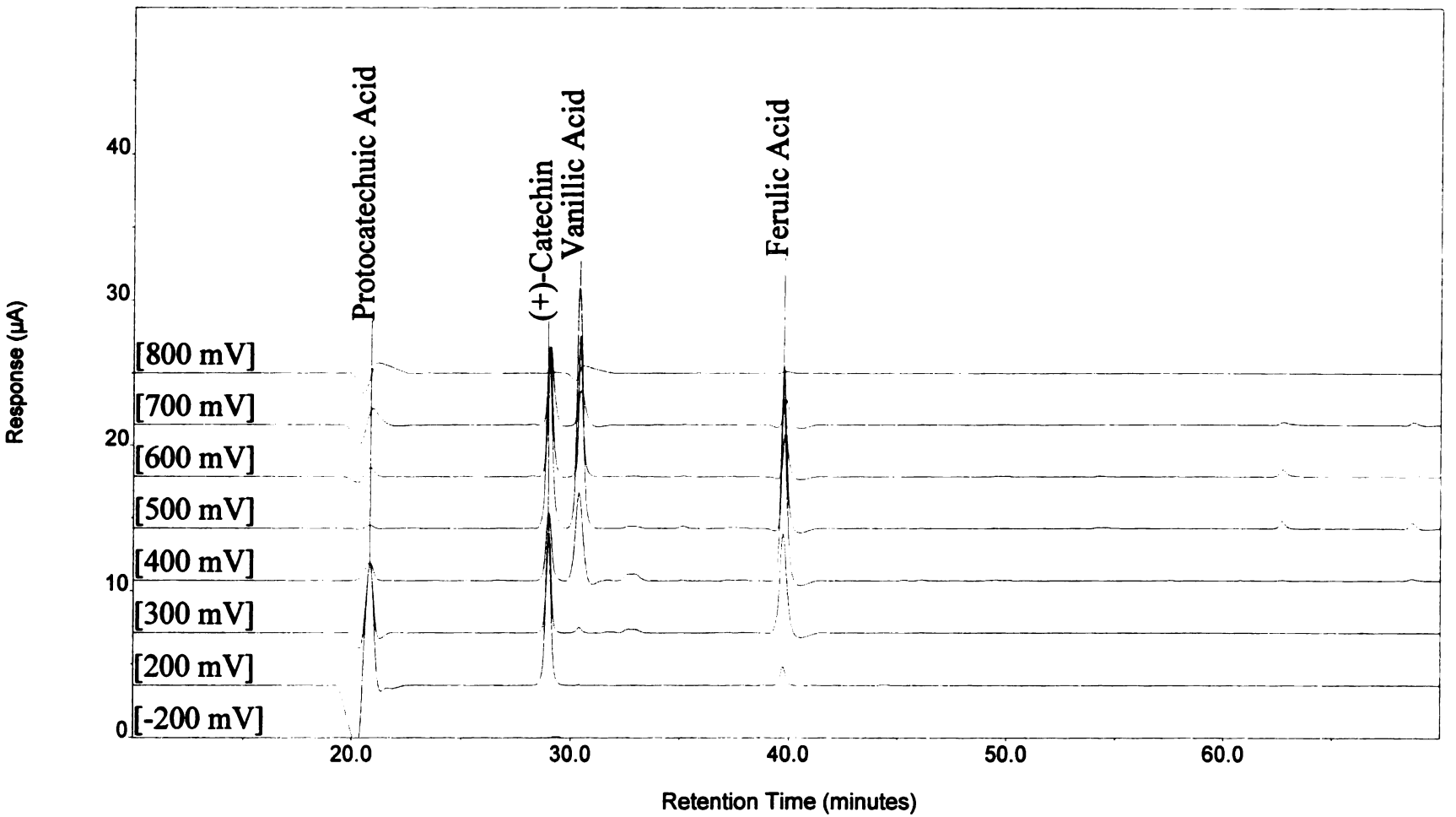


Figure 6. Chromatogram of Standard Mix 1: Protocatechuic acid, (+)-Catechin,Vanillic acid, Ferulic acid

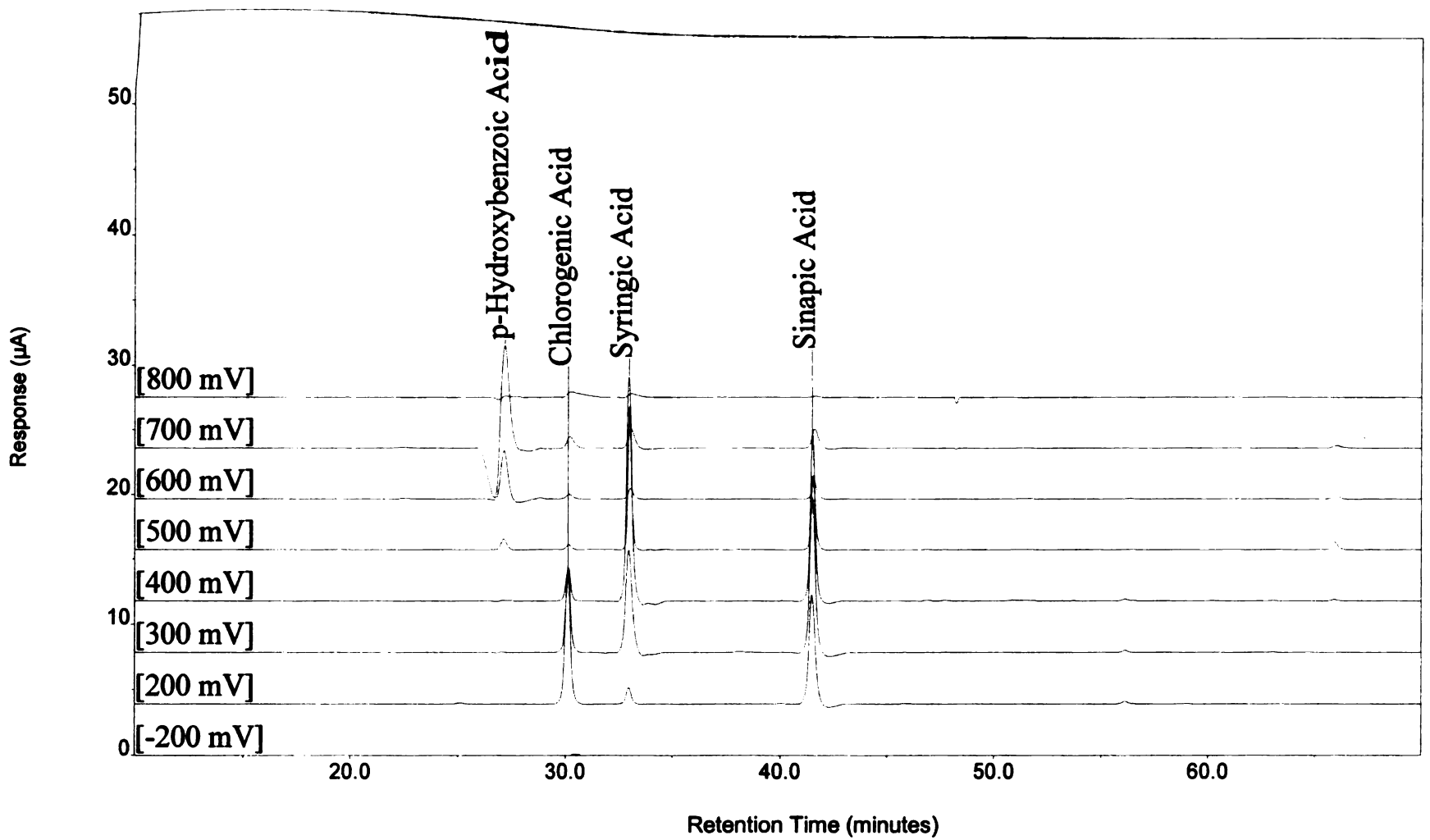


Figure 7. Chromatogram of Standard Mix 2: *p*-Hydroxybenzoic acid, Chlorogenic acid, Syringic acid, Sinapic acid

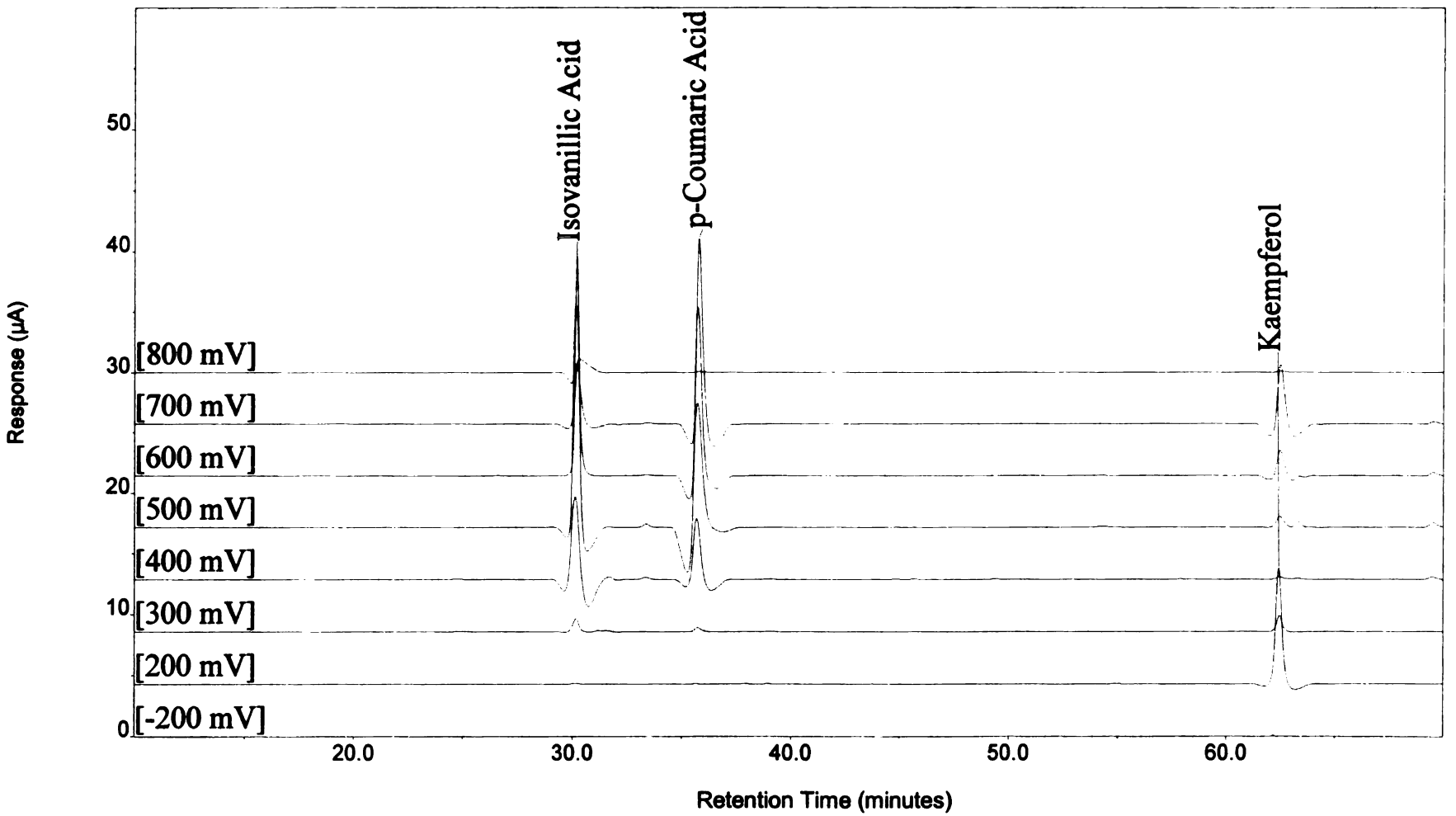


Figure 8. Chromatogram of Standard Mix 3: Isovanillic acid, *p*-Coumaric acid, Kaempferol

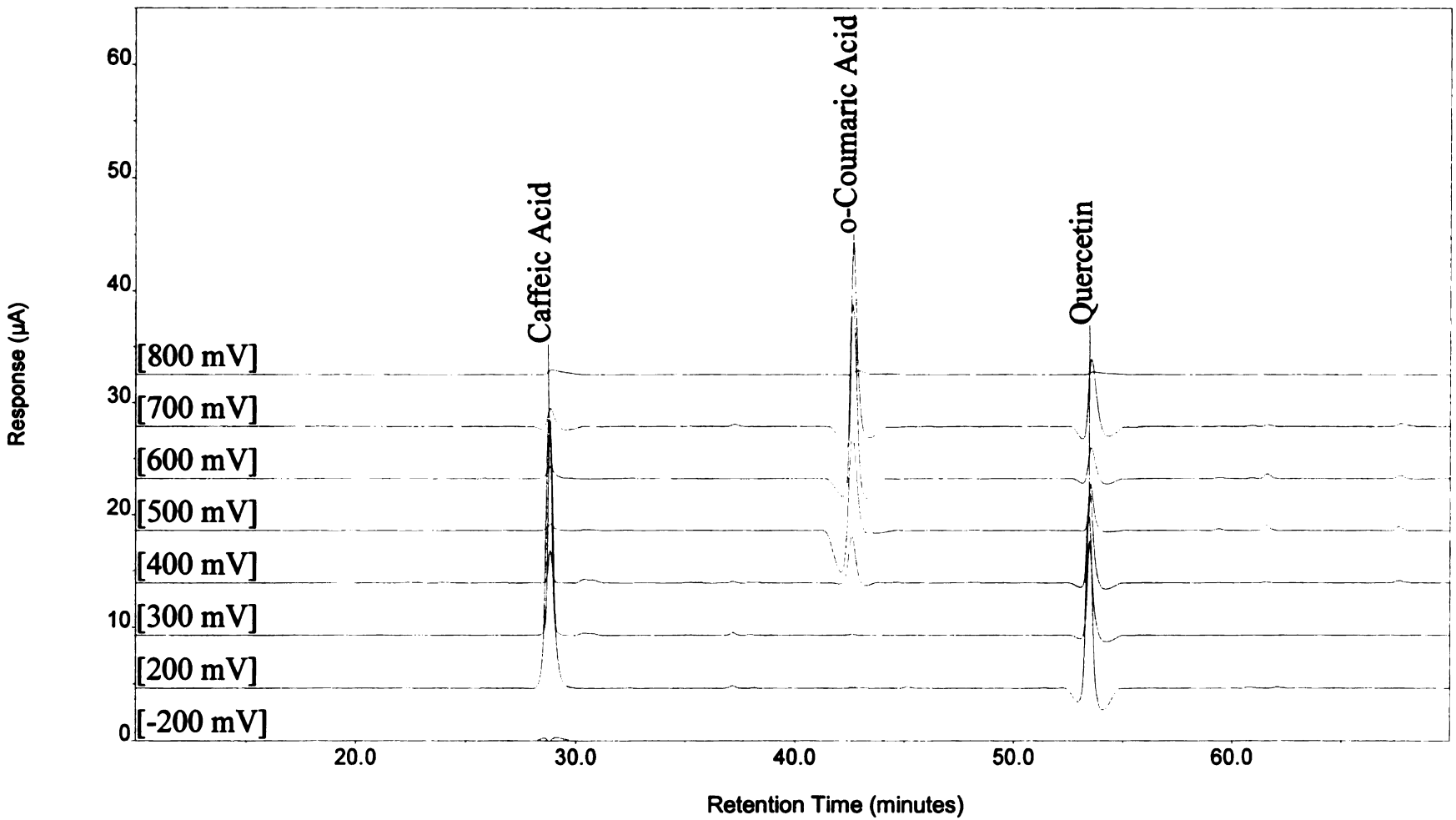


Figure 9. Chromatogram of Standard Mix 4: Caffeic acid, *o*-Coumaric acid (Internal Standard), Quercetin

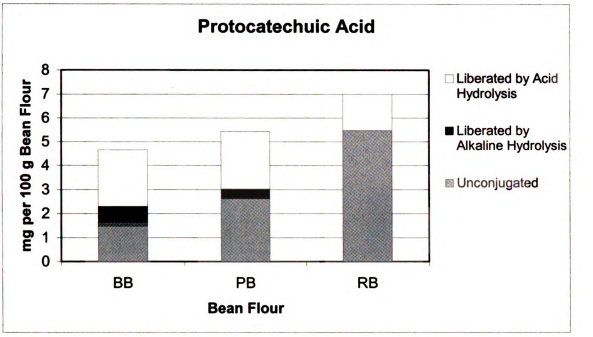
liberated by acid hydrolysis, respectively, identified in the navy bean fractions, NBR and NBEM.

The hydroxybenzoic acids identified in extracts from the cooked dry common beans were: protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, and syringic acid. The relative amounts of these acids present as aglycones and after hydrolyses are shown in Figures 16a-d. Protocatechuic acid was not found in navy beans, but it was the major hydroxybenzoic acid in black, pinto and red beans. Isovanillic acid was not detected in any of the bean extracts.

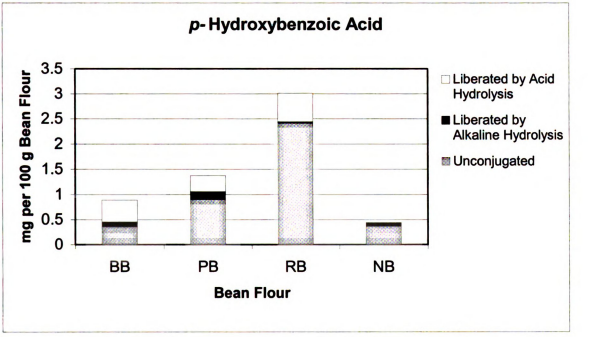
Four hydroxycinnamic acids were identified in the cooked dry common beans — caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid — and the relative amounts that were present as unconjugated aglycones and the amounts liberated by hydrolysis are shown in Figures 17a–d. Ferulic acid was the major hydroxycinnamic acid found in each of the whole bean extracts. Chlorogenic acid, a quinic acid ester of caffeic acid, was not identified in any of the bean extracts.

The flavan-3ol — (+)-catechin — and the flavonols — quercetin and kaempferol — were not present in navy bean, but were identified in black, pinto and red beans (Figures 18 - 19b).

Figures 20 a—c and 21 a—d show the hydroxybenzoic acids and hydroxycinnamic acids, respectively, identified in the navy bean fractions, NBEM and NBR. The same phenolic acids identified in the whole, cooked navy beans were also found in its fractions. The NBEM (concentrated in phenolic compounds) contained 1.2 to 3.6 fold more hydroxybenzoic acids than the NBR fraction, and 3.2 to 6.4 fold more hydroxycinnamic acids.

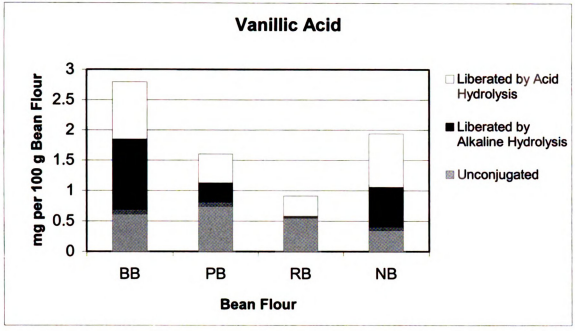


a.

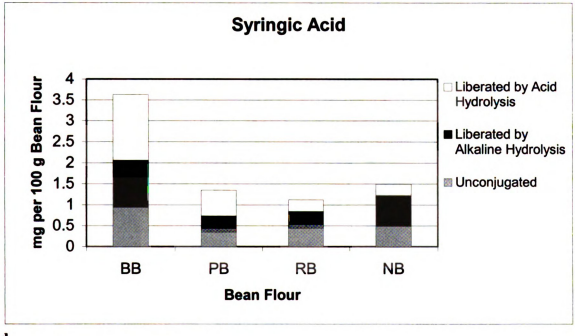


b.

Figure 16a—d. Hydroxybenzoic acids in Bean Flours*
*BB, Black beans; PB, Pinto beans; RB, Red beans; NB, Navy bean.

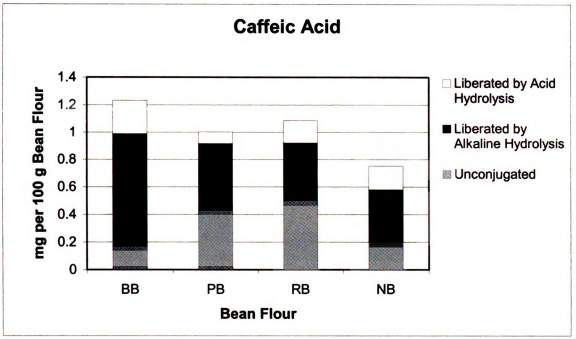


c.

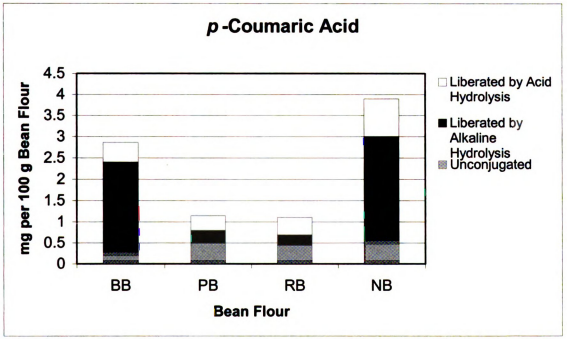


d.

Figure 16a—d. (cont'd)
*BB, Black beans; PB, Pinto beans; RB, Red beans; NB, Navy bean.

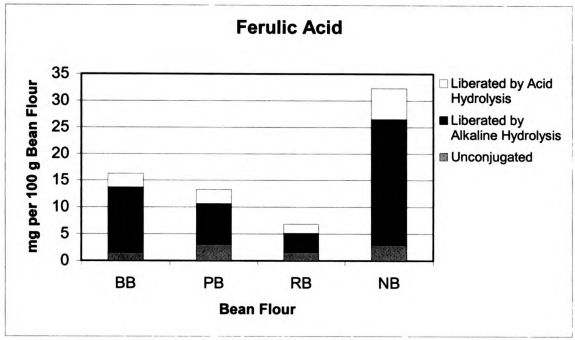


a.

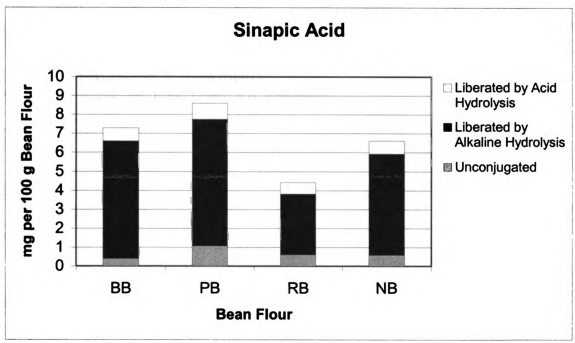


b.

Figure 17a—d. Hydroxycinnamic acids in Bean Flours*
*BB, Black beans; PB, Pinto beans; RB, Red beans; NB, Navy bean.



c.



d.

Figure 17a—d. (cont'd)
*BB, Black beans; PB, Pinto beans; RB, Red beans; NB, Navy bean.

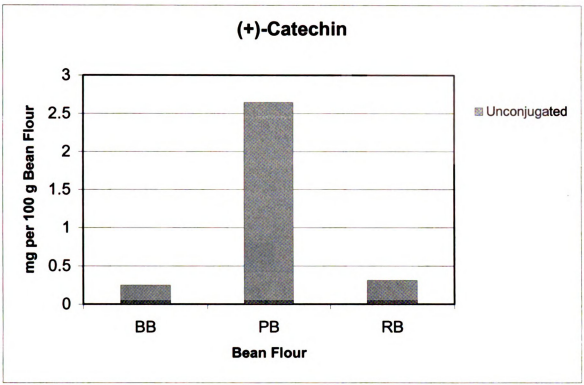
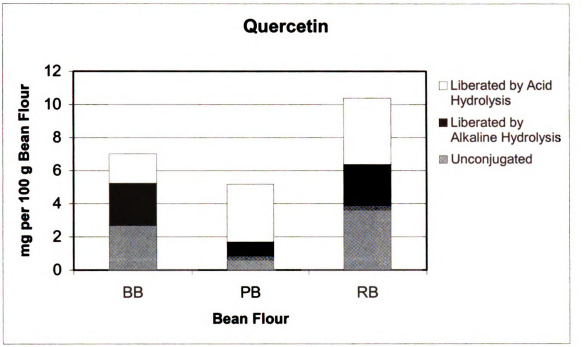
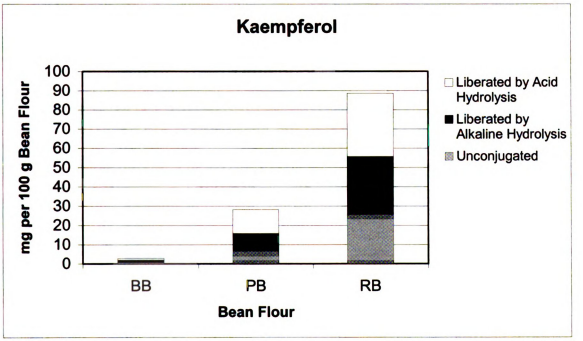


Figure 18. Flavan-3ols in Bean Flours*
*BB, Black beans; PB, Pinto beans; RB, Red beans.

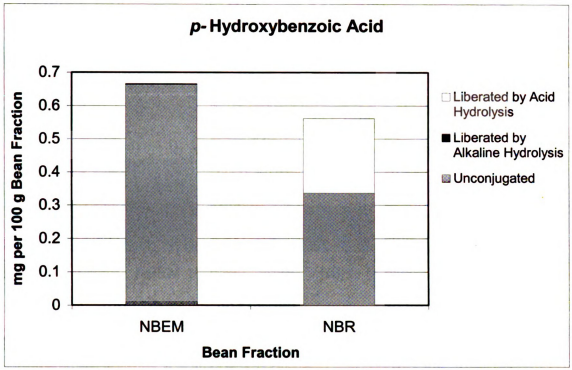


a.

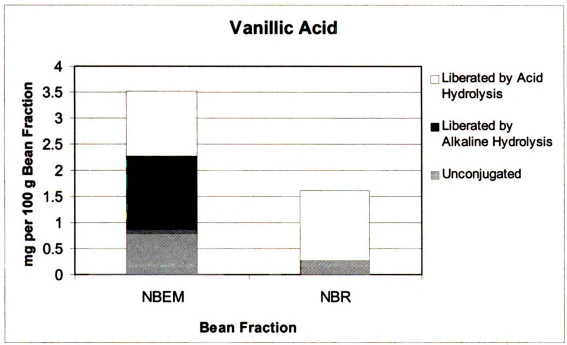


b.

Figure 19a—b. Flavonols in Bean Flours*
*BB, Black beans; PB, Pinto beans; RB, Red beans.

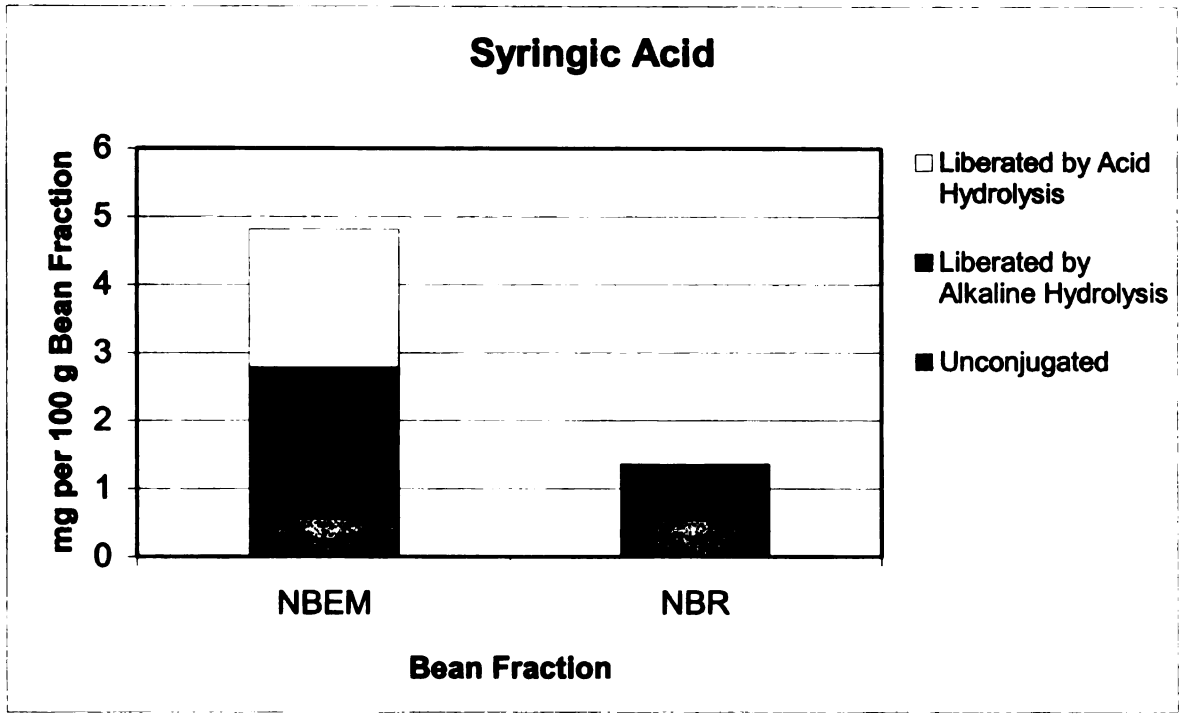


a.



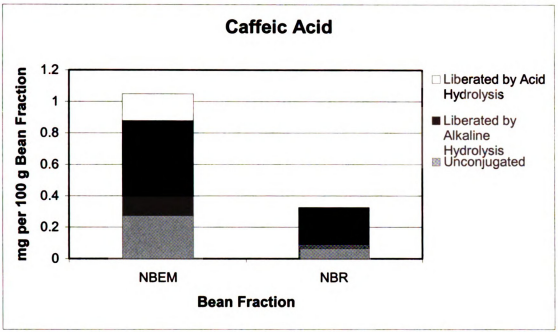
b.

Figure 20a—c. Hydroxybenzoic acids in Navy Bean Fractions*
*NBEM, Navy bean extract mix; NBR, Navy bean residue.

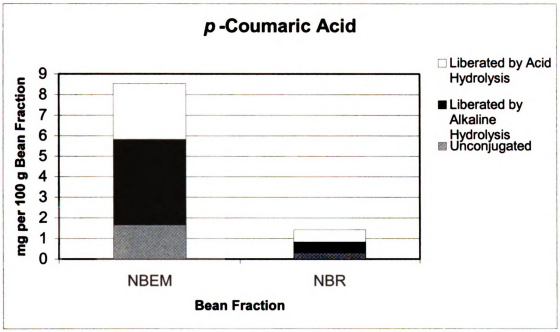


c.

Figure 20a—c. (cont'd)
*NBEM, Navy bean extract mix; NBR, Navy bean residue.

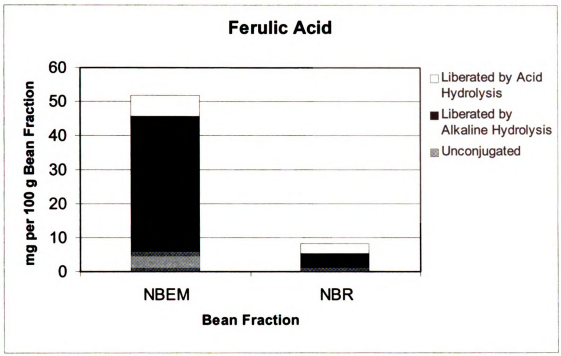


a.

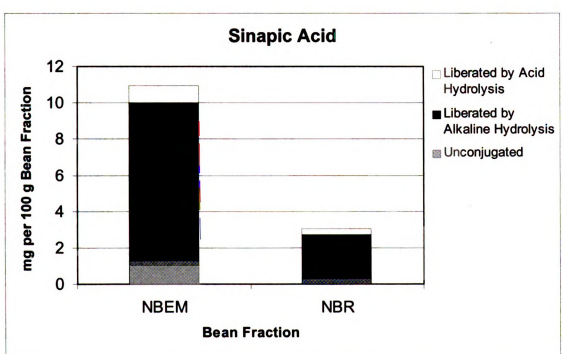


b.

Figure 21a—d. Hydroxycinnamic acids in Navy Bean Fractions*
*NBEM, Navy bean extract mix; NBR, Navy bean residue.



c.



d.

Figure 21a—d. (cont'd)
*NBEM, Navy bean extract mix; NBR, Navy bean residue.

Tables 9a—b show the method (no hydrolysis, alkali hydrolysis or acid hydrolysis) that resulted in the maximum amount of phenolic aglycones in cooked beans and navy bean fractions, respectively. Of the 11 phenolics identified in cooked beans, only (+)-catechin was present entirely in the free form and not conjugated. In general, alkaline hydrolysis resulted in the greatest concentration of hydroxycinnamic acids. For some phenolics in certain beans, acid or base hydrolysis led to similar estimates (e.g., syringic for red beans and *p*-coumaric acid for pinto beans).

The data in Tables 9a—b is displayed as a bar graph in Figures 22 and 23, to visualize the relative amounts of the identified phenolics in cooked beans and navy bean fractions, respectively. In Figure 22 the first four bars (left to right) for black bean, pinto bean, and red bean and the first three bars for navy bean show the relative amounts of the hydroxybenzoic acids. In Figure 23, the first three bars for the navy bean fractions, NBEM and NBR, show the relative amounts of the hydroxybenzoic acids. The amounts of *p*-hydroxybenzoic, vanillic, and syringic acids are relatively low and similar in all four bean classes and in the navy bean fractions. Protocatechuic acid was the major hydroxybenzoic acid in the black, pinto and red bean, but was not detected in the navy bean or its fractions.

The hydroxycinnamic acids are shown in Figure 22, bars 5-8 from the left for black bean, pinto bean and red bean and the last four bars for the navy bean, and in Figure 23, bars 4-7 for the navy bean fractions. Ferulic and sinapic acids were present in much greater quantities than caffeic and *p*-coumaric acids. Ferulic acid was the major hydroxycinnamic acid in navy bean, the navy bean fractions and black bean. Red bean had the least amount of ferulic acid. Although (+)-catechin was detected in black, pinto,

Table 9a—b. Phenolic Compounds Identified by HPLC-ECD[a] in Whole Bean Flours and Navy Bean Fractions

a.

	Bean Flour (mg / 100 g of Bean Flour)			
PC	**BB**	**PB**	**RB**	**NB**
PCA	4	5	7	ND
PHBA	0.8	1.2	3	0.4
Van	1.9	1.3	0.9	1.3
Syr	2.5	1.1	0.8	1.2
Caf	1	0.9	0.9	0.6
PCA	2.4	0.8	0.9	3
FA	13.7	10.6	5.1	26.5
SA	6.6	7.8	3.8	5.9
Cat	0.3	2.7	0.3	ND
Q	5.2	4	4	ND
K	2	10.2	38.6	ND

b.

	Navy Bean Fractions (mg / 100 g Bean Fraction)	
PC	**NBEM**	**NBR**
PCA	ND	ND
pHBA	0.7	0.6
Van	2.3	1.6
Syr	2.8	1.4
Caf	0.9	0.3
pCA	5.8	0.9
FA	45.5	5.4
SA	10.0	2.7

a. All values are dry basis. PC, Phenolic Compound; BB, black bean; PB, pinto bean; RB, red bean; NB, navy bean; NBEM, navy bean extract mix; NBR, navy bean residue; PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Cat, (+)-catechin; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Q, quercetin; K, kaempferol; ND, not detected.

No Hydrolysis

Unconjugated PC plus PC liberated by Alkaline Hydrolysis

Unconjugated PC plus PC liberated by Acid Hydrolysis

PC liberated by Alkaline or Acid Hydrolyses were similar

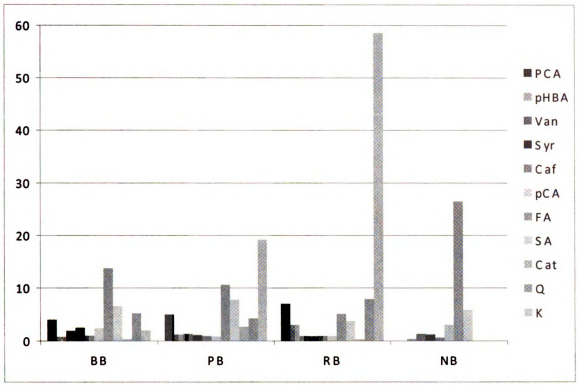


Figure 22. Phenolic Compounds Identified in Whole Bean Flours by HPLC-ECD (mg/100 g of Bean Flour)[a]

a. BB, black bean; PB, pinto bean; RB, red bean; NB, navy bean; PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Cat, (+)-catechin; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Q, quercetin; K, kaempferol.

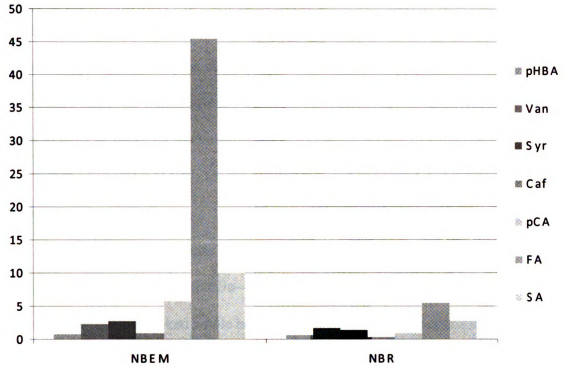


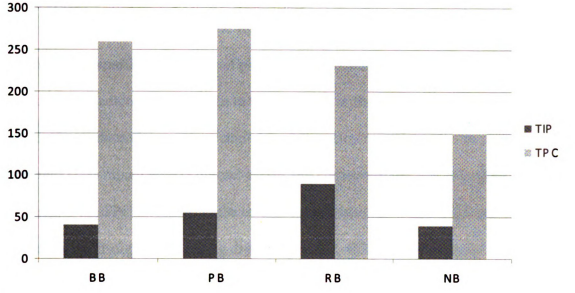
Figure 23. Phenolic Compounds Identified in Navy Bean Fractions by HPLC-ECD (mg/100 g of Bean Fraction)[a]

a. NBEM, navy bean extract mix; NBR, navy bean residue; pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid.

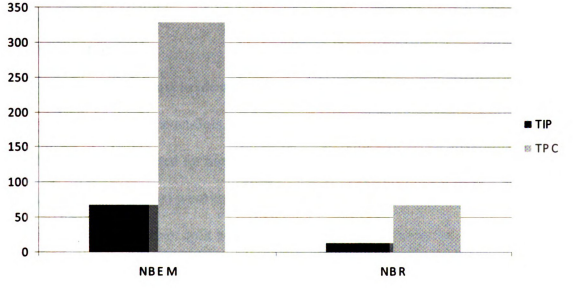
and red bean, (Figure 22, bar 9 from the left) it is present in relatively small amounts compared to kaempferol, quercetin, ferulic acid, and sinapic acid. Kaempferol (Figure 22, bar 11 from the left) was the major phenolic in red beans and pinto beans. In red beans, kaempferol was 8 times greater than any other phenolic. Kaempferol was not present in navy beans or its fractions. Quercetin (Figure 22, bar 10 from the left) content was 4 to 8 mg per 100 g of flour for the black, pinto and red beans, but it was absent in navy beans or its fractions.

Total Phenolic Content of Beans and Bean Fractions

The values shown in Table 9a—b were summed and the total identified phenolics (TIP) are shown in the cooked whole bean flours and navy bean fractions in Figure 24a—b. Figure 24a—b also shows the total phenolic content (TPC) of beans and navy bean fractions as estimated by the Folin-Ciocalteu method. TPC ranged from 149 to 275 mg of (+)-catechin equivalents ((+)-CE) per 100 g of bean flour or bean fraction and the values are listed in Appendix B, Table 3 and Table 4. There is a large difference between TIP and TPC. It is acknowledged that not all peaks observed by HPLC-ECD were identified and quantified. Figures 13a – 15c (navy beans, NBEM, NBR) show only 2 unidentified peaks, but there were more unidentified peaks (up to 13) for the other beans. Based on peak heights of unidentified peaks, it was estimated that unidentified peaks accounted for approximately 5% (navy beans), 18% (red beans), 20% (black beans), and 30% (pinto beans) of the phenolic compounds in the bean extracts. Thus, there remains a very large gap between the phenolics identified by HPLC-ECD and TPC and this difference is most likely due to the non-specific nature of the Folin-Ciocalteu method.



a.



b.

Figure 24a—b. Total Identified Phenolics[a] and Total Phenolic Content[b]

a. TIP, total identified phenolics—mg of unconjugated phenolics plus phenolics liberated by alkaline or acid hydrolysis (which ever was greatest) per 100 g of Bean Flour or Bean Fraction quantified by HPLC-ECD. b. TPC, total phenolic content—mg of (+)-catechin equivalents per 100 g Bean Flour or Bean Fraction as determined by Folin-Ciocalteu method.

Cooked navy beans were separated into two fractions, the aqueous-ethanol soluble components (NBEM) and the aqueous-ethanol insoluble components (NBR) in order to extract a sufficient amount of phenolic compounds from cooked navy beans to allow long-term colon cancer studies to be conducted with rodents. Prior to drying the hygroscopic condensed aqueous-ethanol navy bean extract (concentrated in phenolic compounds) was mixed with cellulose, casein and cornstarch, the TPC was determined. Compared to the TPC measured in the cooked navy bean (1487 mg (+)-CE per kg dry bean flour vs. 671 mg (+)-CE per kg dry bean residue, 45% of total phenolic compounds was extracted from the cooked navy beans using ~60% ethanol, and 10% was lost or could not be accounted for after the extraction process.

D. DISCUSSION

This study is the first to identify and quantify the major hydroxybenzoic acids, hydroxycinnamic acids, flavan-3ols, and flavonols in four market classes of dry common beans that had been prepared for human consumption. In previous studies raw legumes or just the seed coats (hulls) were analyzed; however, people do not consume raw beans or hulls. People eat whole or split beans that have undergone some form of heat treatment. Therefore, one goal of this research was to identify and quantify the phenolic compounds in beans that a person would consume.

Bean processing usually includes hydration (soaking) and a heat treatment (i.e. boiling, pressure cooking, canning, roasting, micronization or autoclaving). Some cultures germinate or ferment beans prior to consumption, but a heat treatment is imposed at some point prior to consumption. The various processing methods induce physical and chemical compositional changes that result in the loss of phenolic compounds (Champ,

2002; Ibrahim et al., 2002; Shimelis and Rakshit, 2007). For example, Díaz-Batalla et al. (2006) found a 42% to 48% reduction in both quercetin and kaempferol in autoclaved (cooked) beans compared to raw beans. Others have reported a decrease in TPC by 29% to 68% during preparation of beans for consumption (Towo et al., 2003; Xu and Chang, 2008; Granito et al., 2008). These studies underscore the importance of identifying and quantifying phenolics in foods in forms that are normally consumed.

With the exception of (+)-catechin, phenolic compounds are not found in their free, aglycone form in nature, but bound as glycosides and esters (Ribereau-Gayon, 1972; Harborne, 1998). When free soluble aglycones are found in plant foods, they are in all probability released during storage, processing, and extraction process (Harborne, 1998). As soon as plant tissue is disrupted it becomes susceptible to enzymatic oxidation and hydrolysis via endogenous enzymes such as esterases, glycosidases, and decarboxylases (Harborne, 1998; Cheynier, 2005). Although researchers attempt to limit oxidation and decarboxylation of phenolic compounds to avoid alterations in chemical structures, inevitably some will occur.

When identifying and quantifying phenolic compounds in plant foods a hydrolysis step is commonly performed to simplify identification and quantification. Hydrolysis also removes unwanted compounds in the complex plant matrix that would otherwise interfere with the analysis (Tsao and Deng, 2004; Stalikas, 2007). Acid and alkaline (saponification) hydrolysis are the most common techniques applied to release the phenolic compound aglycones from their natural conjugated state (Stalikas, 2007). Each step during the extraction process introduces errors and artifacts that will reduce the yield

of total phenolic compounds (Stalikas, 2007). These losses are rarely reported and the data are not corrected for these losses.

In the present study, the percentage of phenolic compounds lost during the clean up step (solid phase extraction, SPE C_{18} cartridges) of the extraction process, and due to alkaline or acid hydrolysis was assessed by applying the same conditions to pure standards. Losses varied greatly and were dependent on the hydrolysis treatment used and the phenolic compound, similar to previous studies. Krygier et al. (1982) observed a 3% to 67% and 15% to 92% loss in hydroxycinnamic acids resulting from alkali or acid treatment, respectively. Dabrowski and Sosulski (1984) reported the losses of hydroxybenzoic acids and hydroxycinnamic acids in rapeseed flour exposed to alkaline conditions. Losses in hydroxybenzoic acids did not exceed 10% and losses in hydroxycinnamic acids ranged from 13%-22%. Both authors reported high losses of caffeic acid under alkali or acid treatment and this was the result of the high reactivity of *o*-dihydroxyphenols, which can be rapidly oxidized to quinones (Ribereau-Gayon, 1972). In the present study, 10% to 90% of phenolic compounds were lost due to sample clean up and hydrolysis by base or acids. The data in the present study were adjusted for these losses.

The classes of phenolic compounds previously identified in the grain legumes were flavonols, hydroxybenzoic acids, hydroxycinnamic acids, condensed tannins (proanthocyanidins), and for a few bean classes, anthocyanins (Krygier et al., 1982; Sosulski and Dabrowski, 1984; Beninger et al., 1999; 2003; 2005; Madhujith et al., 2004; Aparicio-Fernandez et al., 2005; Hu et al., 2006; Lin and Lai, 2006; Ranilla et al., 2007).

Protocatechuic acid was the predominant hydroxybenzoic acid identified in the black, pinto and red beans, but navy beans did not contain protocatechuic acid. Previous studies have not reported the presence of protocatechuic acid in beans, although Cai et al (2003) reported that protocatechuic acid was in cowpea (*Vigna unguiculata*). Díaz-Batalla et al (2006) quantified hydroxybenzoic acids in several varieties of raw wild and weedy Mexican common beans (*Phaseolus vulgaris* L.) and Espinosa-Alonso and co-workers (2006) quantified hydroxybenzoic acids in black Jamapa beans and pinto beans. Both studies reported the presence of *p*-hydroxybenzoic acid, vanillic acid and syringic acid in beans. Espinosa-Alonso et al (2006) did not detect syringic acid in pinto beans. Syringic acid was found in pinto beans when analyzed by HPLC-ECD, but it was found in the lowest concentrations. The two groups analyzing hydroxybenzoic acids in beans reported totals ranging from 1.0 to 7.1 mg/100 g bean flour. Total hydroxybenzoic acids determined in this study were slightly higher (2.9 – 11.7), probably due to differences in methods, corrections for losses, and identification of more acids.

The hydroxycinnamic acids identified in cooked dry common beans were caffeic, *p*-coumaric, ferulic and sinapic acids. Total amounts of hydroxycinnamic acids in beans were 11 (red beans), 20 (pinto beans), 24 (black beans) and 36 (navy beans) mg/100 g bean flour. Navy beans had the largest concentration hydroxycinnamic acids. The predominant hydroxycinnamic acid was ferulic acid followed by sinapic, *p*-coumaric and caffeic acids. Ferulic acid was present in 1.3 – 4.5 times greater than the amount of sinapic acid. Four other studies have reported data for hydroxycinnamic acids in dry common beans. The other studies either used raw samples or they did not hydrolyze with base, which is required to free the majority of the hydroxycinnamic acids.

Espinosa-Alonso et al. (2006) and Luthria and Pastor-Corrales (2006) identified and quantified the same four hydroxycinnamic acids as found in this study. Luthria and Pastor-Corrales (2006) analyzed 15 varieties of raw dry common beans (*Phaseolus Vulgaris* L.) for hydroxycinnamic acid content and reported totals ranging from 19.1 to 48.3 mg/100 g bean flour. Since cooking results in a 23%-40% reduction in the total amounts of hydroxycinnamic acids (Díaz-Batalla et al., 2006), the values found in this study and those reported by Luthria and Pastor-Corrales (2006) are comparable. Luthria and Pastor-Corrales (2006) identified caffeic acid only in black beans, whereas Espinosa-Alonso et al. (2006) reported the presence of caffeic acid in all bean varieties as in this study. Díaz-Batalla et al (2006) quantified hydroxycinnamic acids in several varieties of raw wild and weedy Mexican common beans (*Phaseolus vulgaris* L.) after acid hydrolysis and reported values for ferulic and sinapic acids. Both Díaz-Batalla et al (2006) and Espinosa-Alonso et al. (2006) used acid hydrolysis in their measurements of the hydroxycinnamic acids and so the values they reported are much lower than reported in this study or by Luthria and Pastor-Corrales (2006).

Cai et al. (2003) quantified hydroxycinnamic acids in raw cowpeas. Following alkaline hydrolysis, they identified caffeic, *p*-coumaric, ferulic and cinnamic acids with totals ranging from 1.8 to 20.4 mg /100 g bean flour. Ferulic acid (0.4 to 12.4 mg /100 g bean flour) was the predominant hydroxycinnamic acid present in cowpeas, similar to what has been reported for dry common beans. However, the amounts of hydroxycinnamic acids present in cowpeas appear to be less than what is in dry beans.

Previously (+)-catechin has been identified in the seed coats (hulls) of light brown Brazilian beans (*Phaseolus vulgaris* L.) (Ranilla et al., 2007). Table 9a shows that (+)-

catechin was in black, pinto and red beans samples, but not in navy beans. Pinto had the greatest amount, 2.7 mg /100 g bean flour, followed by red and black (0.3 mg /100 g bean flour). (+)-Catechin was not detectable following hydrolysis with alkaline or acid. This was not unexpected, because (+)-catechins form condensation products in acid (Ribereau-Gayon, 1972), and are not usually quantified after alkaline hydrolysis.

Flavonols and their glycosides have been identified in common beans previously (Beninger et al., 1999, 2003, 2005; Aparicio-Fernandez et al., 2005; Hu et al., 2006; Díaz-Batalla et al., 2006; Espinosa-Alonso et al., 2006; Ranilla et al., 2007). All reported the presence of quercetin and kaempferol and their glycosides, but one study reported the presence of myricetin glycosides in the seed coat of black Jamapa beans (*Phaseolus vulgaris* L.) (Aparicio-Fernandez et al., 2005).

In the current study, quercetin and kaempferol were identified in cooked black, pinto and red beans, but not in navy beans. Total flavonols were 7 (black beans), 24 (pinto beans) and 67 (red beans) mg/100 g bean flour. Red beans had much more total flavonols than pinto and black beans. Díaz-Batalla et al. (2006) and Espinosa-Alonso et al. (2006) reported total flavonol concentrations of 0.4 to 23.2 mg/100 g bean flour in several varieties of raw wild and weedy Mexican common beans (*Phaseolus vulgaris* L.). Espinosa-Alonso et al. (2006) and Ranilla et al. (2007) reported that pinto beans had more kaempferol than quercetin, whereas the black bean had more quercetin than kaempferol. The results shown in Table 9a are consistent with these reports; black beans had a more quercetin than kaempferol whereas pinto and red beans had more kaempferol than quercetin.

Díaz-Batalla et al (2006) additionally quantified flavonols in autoclaved (cooked) beans and found a 42% to 48% reduction in both quercetin and kaempferol (1.1 to 13.5 mg of total flavonols per 100 g bean flour (cooked beans) compared to 2.1 to 23.2 mg/100 g bean flour (raw beans)). Despite analyzing cooked beans, which would be expected to have reduced total flavonols, the flavonol concentrations shown in Table 9a (7 – 67) are much greater than previously reported. The greater concentrations most likely resulted from improved methodology and the analysis of red beans which had high concentrations of kaempferol.

Pinto beans had the greatest TPC, followed by black, red and navy bean. Previous studies reporting the total phenolic content (TPC) in raw grain legumes using the Folin-Ciocalteu method, observed ranges of 35 to 440 mg per 100 g of raw bean flour (Cai et al., 2003; Heimler et al., 2005; Xu and Chang, 2007). In this study the TPC (Figure 24a; Appendix B, Table 3) in the cooked dry common bean extracts ranged from 149 to 275 mg per 100 g bean flour. The values reported here for the cooked beans are consistent with those in the literature, even though cooked beans were analyzed.

Treating the cooked dry common bean extracts with acid resulted in greater amounts of hydroxybenzoic acids being liberated compared to treatment with alkali. This suggests that the majority of hydroxybenzoic acids in the cooked common beans analyzed in this study are simple glycosides. Treatment with alkali liberated larger quantities of hydroxycinnamic acids than acid hydrolysis, suggesting that hydroxycinnamic acids in black, pinto, red and navy beans are predominantly found as esters. Hydroxycinnamic acids, especially caffeic acid, have been shown to be unstable

under acidic conditions, thus they are best obtained under mild alkali hydrolysis (Krygier et al., 1982; Gao and Mazza, 1994; Harborne, 1998).

Cooked navy beans were extracted with aqueous-ethanol (~60% ethanol) to separate navy beans into two fractions for future animal studies: 1. navy bean extract (NBEM)—aqueous-ethanol soluble components (concentrated in phenolic compounds); 2. navy bean residue (NBR)—aqueous-ethanol insoluble solid components (i.e. dietary fiber and protein). The aqueous-ethanol solvent extracted 45% of the total phenolic compounds quantified in cooked navy beans.

The hydroxybenzoic acids were fairly similar in the NBR and navy beans suggesting that the aqueous-ethanol solvent extracted only small amounts of the hydroxybenzoic acids (Figure 20a—c). Although NBEM had approximately 2 times as much total hydroxybenzoic acids, the NBEM is a concentrate and does not reflect efficient extraction of the hydroxybenzoic acids.

The aqueous-ethanol was more efficient in extracting hydroxycinnamic acids (Figure 21a—d) than it was in extracting hydroxybenzoic acids. Fifty to 74% of the hydroxycinnamic acids were extracted and identified by HPLC-ECD. Since ferulic acid was the predominant acid in navy beans and since ferulic acid was efficiently extracted (74%), the extraction of total hydroxycinnamic acids was 74% and the extraction of all phenolics was 67%. The NBEM had approximately 1.7 times more total hydroxycinnamic acids and total identified phenolics than navy beans.

In conclusion, all four cooked dry common beans (*Phaseolus vulgaris* L.), black, pinto, red and navy beans contained hydroxybenzoic and hydroxycinnamic acids. The hydroxycinnamic acids included *p*-coumaric, caffeic, ferulic and sinapic acids. Ferulic

acid was the predominant hydroxycinnamic acid. The hydroxybenzoic acids — *p*-hydroxybenzoic, vanillic and syringic acids — were present in all four beans whereas protocatechuic acid was not in navy beans. Black, pinto and red beans contained, (+)-catechin, quercetin and kaempferol, but navy beans did not contain these phenolics. Additionally, a large scale aqueous-ethanol extraction of phenolic compounds in cooked navy beans was performed. Aqueous-ethanol was more efficient in extracting hydroxycinnamic acid conjugates than hydroxybenzoic acid conjugates in cooked navy beans.

CHAPTER IV. INHIBITORY EFFECTS OF COOKED NAVY BEAN (*PHASEOLUS VULGARIS* L.) ON AZOXYMETHANE (AOM)-INDUCED COLON CANCER IN *OB/OB* OBESE MICE

A. ABSTRACT

Epidemiological and experimental studies have observed an inverse relationship between dry bean consumption and colorectal cancer (CRC). Additionally, dry bean consumption has been inversely associated with risk of developing obesity and type 2 diabetes mellitus, both are independent risk factors for CRC.

The overall goals of this study were to 1) evaluate whether obese, mildly diabetic mice (*ob/ob* mice (B6.V-*Lep*^{ob} /J)) are good animals for studying azoxymethane (AOM)-induced colon cancer and, 2) to narrow the search for which component in dry beans inhibits the development of chemically-induced colon cancer, by feeding a) navy beans (*Phaseolus vulgaris* L.); b) an aqueous-ethanol extract of navy beans (NBEM) and c) the residue remaining after aqueous-ethanol extraction (NBR).

The *ob/ob* mice fed the control diet had a low tumor incidence, only 10% adenoma incidence and 10% adenocarcinoma incidence, which seriously compromised the ability to distinguish dietary induced differences in colon cancer inhibition. Although increasing the AOM dosage would have increased tumor incidence, higher doses of AOM would have increased mortality in this animal model because AOM is a hepatotoxin and *ob/ob* mice have impaired hepatic repair and regenerative responses to injury.

Feeding the aqueous-ethanol extract of navy beans rich in phenolic compounds (NBEM) completely inhibited tumor development (tumors include adenomas and adenocarcinomas, tumor incidence = 0%). Although tumor incidence was inhibited by 69% in mice fed the aqueous-ethanol insoluble components of navy beans (NBR) compared to the control diet fed mice this was not statistically significant. The low tumor incidence in the control fed animals weakened the statistical power in this study making it impossible to partition the cancer inhibiting activities of navy beans clearly.

In conclusion, the *ob/ob* mouse did not have the desired sensitivity to the colon carcinogenic properties of AOM to be recommended for widespread use to test dietary ingredients for colon tumor inhibiting potential. Nevertheless, the compounds contained in the aqueous-ethanol extract of navy beans (NBEM) reduced tumor incidence by 100% compared to the control diet.

B. INTRODUCTION

Colorectal cancer (CRC) is the 3rd most commonly diagnosed cancer and the 4th most common cause of cancer death worldwide (WCRF/AICR, 2007). In the United States alone, CRC is the 4th most commonly diagnosed cancer following lung, female breast, and prostate cancers, and the 2nd leading cause of cancer death (Jemal et al., 2008). While some individuals are predisposed to developing CRC because of inheritable factors, environmental and lifestyle factors such as — being a non-smoker, being physically active, maintaining a healthy body weight, and consuming a healthy diet — can greatly reduce an individual's risk (WCRF/AICR, 2007). Approximately 70% of colorectal cancers may be preventable by a healthful diet and other lifestyle choices (Platz et al., 2000). Thus, diet is an important factor that can be modified and alter CRC risk.

Evidence demonstrating the important role environmental and/or lifestyle factors play in modulating CRC risk was first observed in population-studies. Large geographical variations in CRC incidence across the world were reported, with lower incidences in Africa and Asia, moderate incidences in South America and high incidences in North America, Australia/New Zealand and Western Europe (Parkin et al, 2005; WCRF/AICR, 2007). Dry beans have long been a dietary staple in the traditional diets consumed by the populations living in the aforementioned low risk regions. Moreover, epidemiological studies have observed an inverse relationship between dry bean consumption and CRC risk (Correa, 1981; Steinmetz and Potter, 1993; Michels et al., 2006). In the Polyp Prevention Trial (PPT, a prospective trial-based cohort), the association between the consumption of fruits, vegetables, dry beans and other vegetable

groups and the recurrence of colorectal adenomas in humans was examined. Researchers reported a significant 65% reduction in the recurrence of advanced colorectal adenomas in individuals who increased their dry bean intake (highest quartile) compared to those with the least change in intake (lowest quartile) over a 4 yr period ($p = 0.001$). Four-day food records revealed the five most highly consumed dry beans were baked beans (navy beans), kidney beans, pinto beans, and lima beans (Lanza et al., 2006).

Besides colorectal cancer, dry bean consumption has been inversely associated with risk of developing obesity and type 2 diabetes mellitus (Geil and Anderson, 1994; Maskarinec et al., 2000; Guillon and Champ, 2002; Venn and Mann, 2004; Villegas et al., 2008). Characteristics of obesity include impaired glucose tolerance, hyperinsulinemia, dyslipidemia, high blood pressure, elevated circulating proinflammatory cytokines (i.e. TNF-α, IL-6) (Gunter and Leitzmann, 2006). Many of these metabolic perturbations ensuing from obesity, have been positively associated with colon cancer (Gunter and Leitzmann, 2006). Hyperinsulinemia (high circulating levels of insulin) results from increased synthesis and secretion of insulin from the pancreatic β-cells. Hyperinsulinemia is a compensatory response that occurs because higher blood levels of insulin are required to maintain glucose homeostasis. As insulin resistance persists and worsens, the pancreatic β-cells lose the ability to secrete sufficient insulin to maintain glucose homeostasis and that leads to hyperglycemia and to the development of type 2 diabetes mellitus (Sandhu et al., 2002). Both obesity and type 2 diabetes mellitus are independent risk factors for the development of CRC (Coughlin et al., 2004; Brun et al., 2007; WCRF/AICR, 2007).

Aberrant crypt foci (ACF) are preneoplastic lesions commonly used as an early biomarker in short term colon carcinogenesis studies (Bird, 1995). Additionally, they have been identified in the colon of humans at high risk for CRC such as those with inheritable syndromes such as familial adenomatous polyposis (FAP) or a history of carcinomas or adenomas (Glebov et al., 2006; Femia and Caderni, 2008). Several studies utilizing chemically-induced colon cancer in genetically obese animal models have found an increased number of ACF compared to their lean, wild type littermates. Increases in ACF have been reported in genetically obese, diabetic mouse models (*db/db* mice and *ob/ob* mice) injected with the colon carcinogen azoxymethane (AOM) compared to their lean counterparts (Hirose et al., 2004; Ealey et al., 2008). Similarly, in obese rats (Zucker, *fa/fa*) injected with AOM, the number of colon tumors and aberrant crypts were greater compared to their lean littermates (Weber et al., 2000).

Several studies have evaluated the potential for dry beans and other legumes to reduce chemically-induced colon carcinogenesis in lean rodents. Tumor incidence was reduced ~52 to 60% when cooked dry common beans (*Phaseolus vulgaris* L.) were fed to AOM-injected rats (Hughes and Ganthavorn, 1997; Hangen and Bennink, 2002; Rondini, 2006). In a recent study, a group fed pinto beans, black-eyed peas and soybeans to AOM-injected rats and found a 64%, 77% and 56% reduction in ACF, respectively (Boateng et al., 2008). There does not appear to be any study that has evaluated the potential for dry beans to reduce colon carcinogenesis in an obese animal model.

Dry beans are a great source of dietary fiber, containing 8% to 28% on a dry weight basis (Guillon and Champ, 2002; USDA/ARS, 2009), and it is probable that the dietary fiber is protective against CRC, however the evidence has not always been

consistent (Fuchs et al., 1999; Schatzkin et al., 2000; McCullough et al., 2003; WCRF/AICR, 2007). Dry beans contain numerous bioactive constituents that may slow the development of colon cancer. Potentially bioactive non-nutrients found in dry beans include phytic acid, saponins, oligosaccharides, and phenolic compounds (Geil and Anderson, 1994; Messina, 1999; Guillon and Champ, 2002). Phenolic compounds have been shown to possess anti-cancer activity and numerous studies have been published examining the mechanism of action (Kwon et al., 2007; Stevenson and Hurst, 2007). In Chapter III, phenolic compounds were identified and quantified in four dry, cooked common beans — black, pinto, red and navy beans. All four beans contain hydroxybenzoic acids and hydroxycinnamic acids. Navy beans were found to contain approximately 2 to 3 times more total hydroxycinnamic acids than the black, pinto and red beans. Several phenolic compounds identified in black, pinto and red beans were not found in navy beans, including protocatechuic acid (hydroxybenzoic acid), (+)-catechin (flavan-3ol), quercetin (flavonol), and kaempferol (flavonol). Additionally anthocyanins have been identified in beans possessing blue and blue-violet colored seed coats, i.e. black beans (Beninger et al., 2003). Yet despite these differences in phenolic compound composition pinto beans, black beans and navy beans all inhibited chemically induced colon cancer in a rat model by > 50% (Hughs et al., 1997; Hangen and Bennink, 2002; Rondini, 2006).

Distinguishing which components found in dry beans protect against the development of CRC and the molecular mechanism by which they confer protection has not been delineated. Partitioning beans into different fractions and determining the anti-cancer activity for the various fractions is one approach to narrowing the search for the

anti-cancer components of legumes. Bennink and Barrett (2004) used this approach with soybeans (an oil legume). They fed heat-treated, full-fat soybean flour, soybean flour minus lipids, defatted soybean flour minus aqueous-ethanol components and a control diet to determine which fractions protected against development of AOM-induced colon cancer. Full-fat soybean flour and defatted soybean flour diets significantly reduced tumor incidence by 45% ($P < 0.05$) compared to rats fed a casein control diet. Based on this experiment and other unpublished studies it was determined that soybean fiber, sterols, fatty acids, protein and isoflavones could not be providing the anti-cancer action noted with defatted soy flour. However, removing the aqueous-alcohol soluble components completely eliminated any anti-cancer action (Beninnk and Barrett, 2004). The aqueous-alcohol fraction would contain not just isoflavones, but other phenolic compounds such as syringic acid, ferulic acid, sinapic acid and *p*-coumaric acid (Seo and Morr, 1984), as well as saponins and oligosaccharides. Subsequent studies showed that the isoflavones do not inhibit colon cancer.

In the previous study (Chapter III) the aqueous-alcohol soluble components (NBEM) in navy beans were separated from the aqueous-alcohol insoluble components (NBR) in order to extract a sufficient amount of phenolic compounds from cooked navy beans to allow long-term colon cancer studies to be conducted with rodents. Forty-five percent of the total phenolic compounds in cooked navy beans were extracted by an aqueous-ethanol solvent (~60% ethanol) and identified in the NBEM. The aqueous-ethanol solvent was more efficient in extracting the hydroxycinnamic acid conjugates than the hydroxybenzoic acid conjugates from cooked navy beans.

Although the bioactive constituents in dry beans responsible for inhibiting the development of colon cancer have not been fully elucidated, phenolic compounds (i.e. flavonoids, phenolic acids) have been identified in the human fecal water in concentrations ranging from 0.56 μM and 627.9 μM, with the majority being phenolic acids. These levels are comparable to those found to exert anti-cancer activity in *in vitro* and cell culture studies (Jener et al., 2005). Phenolic compounds in dry beans not absorbed in the small intestine will enter the colon. Some will remain in the solid phase of the human feces (bound to solid food components) while others will be part of the aqueous phase or fecal water. The biochemical composition of fecal water is directly influenced by the diet and varies greatly between individuals (Rafter et al., 1987; Nordling et al., 2003; Record et al., 2003). The fecal water is able to interact more with the colonic epithelial cells in comparison to the solid material, and can influence cellular, molecular and genetic changes associated with the etiology of colon cancer (Nordling et al., 2003; Karlsson et al., 2005). Thus, phenolic compounds in dry beans may be responsible for some of the anti-cancer activity observed in previously studies.

Since navy beans have previously been shown to inhibit chemically induced colon cancer, are the type of bean used to make baked beans, and the dominant type of dry bean consumed by non-Hispanic patients enrolled in the Polyp Prevention Trial, they were chosen to conduct the current study.

The overall goals of this study were to evaluate whether obese, mildly diabetic mice (*ob/ob* mice (B6.V-*Lep*^{ob} /J)) are good animals for studying azoxymethane-induced colon cancer and to narrow the search for which component in dry beans inhibits the development of chemically-induced colon cancer.

The specific objectives of this study were to determine: 1) if greater than 50% adenoma and greater than 20% adenocarcinoma incidence could be induced when a control diet was consumed and 2) if feeding a) navy beans (*Phaseolus vulgaris* L.); b) an aqueous-ethanol extract of navy beans and c) the residue remaining after aqueous-ethanol extraction would reduce adenoma and adenocarcinoma incidences.

C. MATERIALS AND METHODS

Chemicals

Azoxymethane (AOM) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Ethanol, Paraplast® Tissue embedding media (paraffin pellets), Protocol Eosin Y (1% Alcohol), Hematoxylin Stain Solution Gill 3 Formulation (pH 2-4) were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). 37% Formaldehyde and Xylene were from J.T. Baker (Phillipsburg, NJ, U.S.A.).

Experimental animals

Male *ob/ob* mice (B6.V-*Lep*^{ob} /J), n=160, 4 to 5 weeks in age were obtained from Jackson Laboratory in Bar Harbor, ME. They were housed 4 mice per cage in a temperature (24°C ± 2°C) and humidity (40%-60%) controlled room with a 12 h light/dark cycle. Animals were allowed free access to food and water. The animals were checked daily for health status and weighed weekly for 9 weeks, then monthly. This study was approved and conducted under the guidelines of the All University Committee on Animal Use and Care at Michigan State University.

Diet Preparation

Preparation of the navy bean flour and the fractions derived from it is described in Chapter III, C. Material and Methods. The beans were kindly provided by the Michigan Bean Commission and were stored at 4°C prior to cooking.

Table 10 lists the composition of the four diets fed to the mice; 1) control diet — modified AIN-93G diet; 2) NB — cooked navy bean diet, 3) NBR — navy bean residue diet; and 4) NBEM—navy bean extract mix diet. All four diets were formulated to provide 17.1% protein, 16.7% fat, 14.4% fiber (wt:wt) and 4 kcal/g. The vitamin mix was added according to the American Institute of Nutrition standard AIN-93G for laboratory rodents (Reeves et al., 1993). The mineral mix did not contain calcium, but all other mineral concentrations were as described for the AIN-93G mineral mix. Calcium was added at a lower concentration than the AIN-93G since the high concentration of calcium in the standard AIN-93G mineral mix may inhibit colon cancer. All diets were supplemented with 0.3 g of methionine per 100 g of diet and 0.005 g of tryptophan per 100 g of diet was added to the NB and NBR to meet amino acid requirements for growing rodents (Reeves et al., 1993).

Navy beans contain approximately 19.5% dietary fiber. In order to minimize dietary fiber effects, all diets were modified to match that found in the navy bean and navy bean residue diets or 14.4% dietary fiber (wt:wt), compared to the 4% found in AIN-93G diet (Reeves et al., 1993).

The diets were formulated to provide 36% of the energy from fat, which simulates the average American diet, but the 16.7% fat content is much higher than 7% fat in the

Table 10. Composition of Diets Fed to *ob/ob* Mice[a]

Ingredients	Diets (g/100g)			
	Control	NB	NBR	NBEM
Beans		74.0		
Residue			74.0	
Concentrate				9.0
Casein	18.0			18.0
Methionine	0.3	0.3	0.3	0.3
Tryptophan		0.005	0.005	
Corn oil	1.1			1.1
Lard	12.9	12.9	12.9	12.9
Soybean oil	2.6	2.6	2.6	2.6
Cornstarch	44.4	4.0	4.0	35.4
Fiber (cellulose)	14.4			14.4
Sucrose	1.0	1.0	1.0	1.0
Calcium Carbonate	0.3	0.3	0.3	0.3
Mineral mix	3.6	3.6	3.6	3.6
Vitamin mix	1.0	1.0	1.0	1.0
Choline bitartrate	0.3	0.3	0.3	0.3
BHT	0.0014	0.0014	0.0014	0.0014
Total	100.0	100.0	100.0	100.0
TPC[b]	*0*	*110.6*	*49.4*	*109.8*

a. Control is a modified AIN 93 G (Reeves et al., 1993); NB = whole navy bean diet; NBR = navy bean residue diet; NBEM = navy bean extract mix diet. b. TPC, total phenolic content—mg of (+)-catechin equivalents per 100 g of diet as determined by Folin-Ciocalteu method.

standard AIN-93G diet. The fatty acid profile also simulated the typical American diet (Ernst et al., 1997).

The NBEM contained the same amount of total phenolics as the NB diet, 110.6 and 109.8 mg (+)-catechin equivalents per 100 g of diet, respectively. The NBR diet contained 49.4 mg (+)-catechin equivalents of phenolic compounds per 100 g of diet. The NB and NBEM were formulated to be equivalent in total phenolic acid content, and contained 2.2 times more phenolic compounds than the NBR diet.

Initiation of Colon Cancer

Male *ob/ob* mice were received when they were 4 to 5 weeks of age. The mice were given two subcutaneous injections of 7 mg of AOM dissolved in saline per kg of body weight one week apart to initiate the cancer process. The control diet (Table 10) was fed from arrival to 1 week following the second injection. The animals (9 to 10 weeks of age) were then randomized by weight to 1 of the 4 dietary treatment groups (n=40 mice per dietary treatment). The study was terminated 27 to 29 weeks after the second injection of AOM.

Necropsy and Histology

Animals were sacrificed using carbon dioxide inhalation and exsanguination. Colons were removed, cut longitudinally, rinsed with lukewarm tap water and flattened on filter paper and visually inspected for lesions. The colons were then fixed in 10% neutral buffered formalin (NBF, pH 7.4) for 5-6 hours. Other internal organs were also examined grossly for lesions and fixed in 10% NBF if an organ appeared abnormal.

Mucosa (epithelial cells) was collected from the mid colon region of 12-15 animals per dietary treatment by gently scraping the normal appearing tissue with the edge of a glass slide. The mucosa was immediately snap frozen on dry ice and then stored at -80°C until analyzed.

After fixation in NBF, the colons were re-examined by two researchers, independently, for lesions. Locations of visible tumors and any slightly raised and opaque areas were recorded, dissected, and placed in pathology cassettes. The remaining colon tissue was transected into 4 segments: proximal colon, mid-colon 1 (2 cm), mid-colon 2 (2 cm) and distal colon and placed cassettes. Any organ tissue suspected of being a tumor was dissected and place in cassettes. All cassettes were dehydrated and infiltrated with paraffin using standard histological procedures. Tissue sections (4 micro thickness) from all lesions and colon segments were processed using standard histological procedures and subsequently stained with hematoxylin and eosin.

A pathologist examined all tissues, and lesions were categorized as focal hyperplasia, dysplasia, adenoma and adenocarcinoma based on standard histological grading criteria (Krutovskikh and Turusov, 1994).

Statistical Analysis

Two-way ANOVA with repeated measures was used to analyze the growth data and *post-hoc* analysis was done by the Fisher's LSD method. The Chi square test was used to determine statistical differences in lesion incidence. A probability of $P < 0.05$ was considered statistically significant.

D. RESULTS

Growth

The mean weights of the *ob/ob* mice fed the various dietary treatments are shown in Figure 25. After feeding the assigned dietary treatments for 3 or 4 weeks, *ob/ob* mice fed the NB and NBR weighed more ($P<0.05$) than mice fed the control diet. Mice fed the NBEM for 15 weeks weighed less ($P<0.05$) than mice fed the NB diet.

Lesion Incidence

Lesions include focal hyperplasia, dysplasia, adenomas and adenocarcinomas (Table 11). All lesions were located in the mid- or distal colon. In mice fed the control diet, the lesion incidence was 40% (16/40). There was no difference ($P>0.05$) in lesion incidence between mice fed the control diet and mice fed the NB diet. Mice fed the NBR diet had fewer lesions than mice fed the control diet ($P<0.05$), but the number of lesions was similar for mice fed the NB diet and the NBR diet ($P>0.05$). Also, the number of lesions was similar for mice fed the NBR diet and the NBEM diet ($P>0.05$). Mice fed the NBEM diet had the fewest lesions. The lesion incidence for mice fed the NBEM was less than mice fed the control or the NB diets ($P<0.05$).

Tumor Incidence

Tumors included adenoma and adenocarcinoma (Table 12). In mice fed the control diet, the tumor incidence was 18% (7/40) and the number of tumors per tumor bearing mice was 1.3 per mouse. Mice fed the whole navy bean (NB) diet had a tumor incidence of 15% (5/33) and the tumor incidences for the mice fed the control and NB diets were similar ($P>0.05$). For mice fed the NBR diet, the tumor incidence was 5%

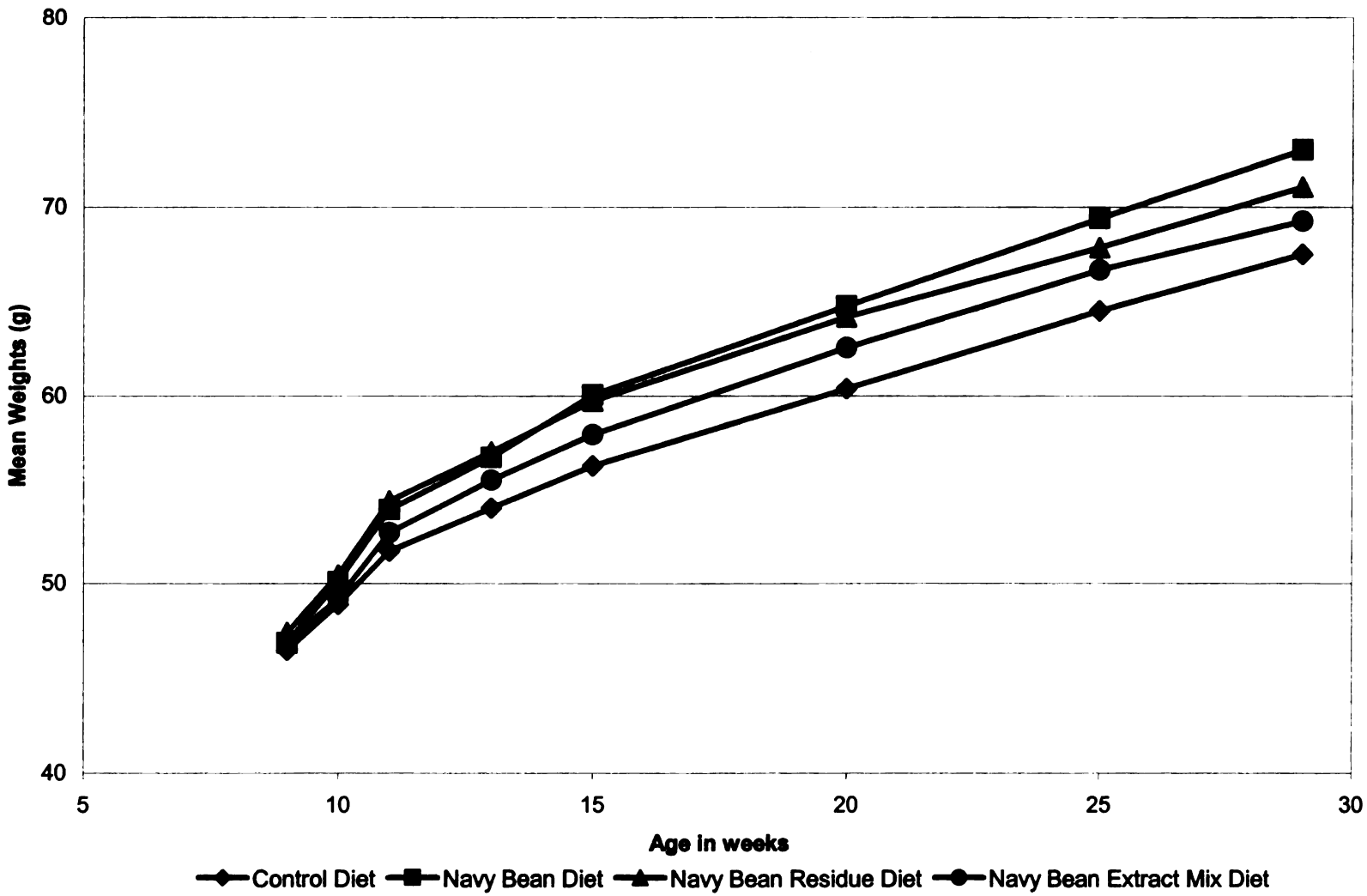


Figure 25. Growth of Age-Matched ob/ob Mice Fed Control or Navy Bean Diets

Table 11. Lesion Incidence[a] in Colons of *ob/ob* Mice Fed Control or Navy Bean Diets[b,c]

Diets	# Lesions / Lesion bearing mouse	% Incidence[d]	% Inhibition
Control	1.6	40 (16/40)[a]	
NB	1.0	21 (7/33)[ab]	
NBR	1.0	14 (5/37)[bc]	66% vs. control
NBEM	1.0	5 (2/39)[c]	87% vs. control & 76% vs. NB

a. Lesions include: focal hyperplasia, dysplasia, adenomas and adenocarcinomas. b. Control = Control diet; NB = Navy bean diet, NBR = Navy bean residue diet, NBEM = Navy bean extract mix. c. Values in a column are significantly different if they don't share the same superscript ($P < 0.05$). d. In parenthesis is the number of animals with lesions per number of mice alive at the end of the experiment.

Table 12. Tumor Incidence[a] in Colons of *ob/ob* Mice Fed Control or Navy Bean Diet[b,c]

Diets	# Tumors / Tumor bearing mouse	% Incidence[d]	% Inhibition
Control	1.3	18 (7/40)[a]	
NB	1.0	15 (5/33)[a]	13% vs. control
NBR	1.0	5 (2/37)[a,b]	69% vs. control
NBEM	0.0	0 (0/39)[b]	100% vs. control & 100% vs. NB

a. Tumors include: adenomas and adenocarcinomas. b. Control = Control diet; NB = Navy bean diet, NBR = Navy bean residue diet, NBEM = Navy bean extract mix. c. Values in a column are significantly different if they don't share the same superscript ($P < 0.05$). d. In parenthesis is the number of animals with tumors per number of mice alive at the end of the experiment.

(2/32) and similar to the control and NB diets ($P>0.05$). No adenomas or adenocarcinomas were present in the colons of mice fed the NBEM diet and mice fed the NBEM diet had a significantly ($P<0.05$) reduced tumor incidence compared to the control and NB diets.

E. DISCUSSION

The goal of producing an adenoma incidence ≥ 50% and an adenocarcinoma incidence ≥ 20% in obese, mildly diabetic *ob/ob* mice (B6.V-*Lep*^{ob}/J mice) fed a control diet was not achieved. The mice fed the control diet had only 10% adenoma incidence and 10% adenocarcinoma incidence. The low tumor incidence seriously compromises the ability to distinguish dietary induced differences. Increasing the AOM dosage would increase tumor incidence, but the trade-off would be increased mortality. A second approach would be to use earlier end-points such as ACF, β-catenin accumulating crypts (BCAC), or mucin depleted foci (MDF) that occur prior to dysplastic and hyperplastic lesions and tumors.

End-points that occur prior to the appearance of tumors have been used in short-term chemically induced colon carcinogenesis experiments. One widely accepted biomarker often used to screen for potential chemopreventive agents as well as carcinogens prior to long-term studies is ACF (Roncucci et al., 1991; Femia and Caderni, 2008). ACF, early preneoplastic lesions, have been observed in the colon of rodents injected with colon carcinogens as well as in the colons of humans with hereditary forms (i.e. familial adenomatous polyposis) and spontaneous CRC (Bird, 1989; Roncucci et al., 1991; Pretlow et al., 1991; Yokota et al., 1997; Schoen et al., 2008). More than 98% of

ACF never progress to macroscopically visible lesions and the correlation between ACF and colorectal tumors in chemically induced animal models and humans have been inconsistent (Davies and Rumsby, 1998; Wijnands et al., 2004; Mori et al., 2005; Gupta and Schoen, 2008; Schoen et al., 2008). These two criticisms seriously weaken the predictive value of ACF for the development of colorectal tumors. However, papers using ACF as the end-point for assessing chemoprevention continue to be published. Other preneoplastic lesions have been identified and suggested to correlate better with tumor development include BCAC (Yamada et al., 2001) and MDF (Caderni et al., 2003). BCAC are technically very difficult to detect and only a few labs in the world have successfully utilized this model. The MDF end-point has not been fully evaluated regarding the potential for predicting tumor incidence.

When evaluating the efficacy of chemopreventive agents and/or dietary components to prevent colon cancer, the occurrence of adenocarcinomas in the colon is a good end point. In the AOM model of colon carcinogenesis, it appears that most adenomas progress to adenocarcinomas with time. Additional considerations are that the distribution of the intestinal tumors in AOM injected rodents has been found to be similar to that exhibited in humans, with large intestinal tumors generally found in the distal colon (corresponding to the left side or descending and sigmoid colon in humans) (Ward et al., 1973; Zedeck, 1980). Also, histopathological characteristics and genetic alterations identified in sporadic colon tumors in humans have also been identified in AOM-induced tumors in rodents (Druckrey, 1973; Ward et al., 1973; Reddy, 2004). Therefore, the best available end-point is adenocarcinoma or adenoma plus adenocarcinoma.

Prior to the current study, our lab administered six weekly injections of AOM (15 mg per kg body weight; based on the weights of age matched lean C57BL/6J mice) to male and female *ob/ob* mice. This was equivalent to AOM doses ranging from 5.5-6.0 mg and 6.7-8.6 mg per kg of body weight in female and male *ob/ob* mice, respectively. This AOM dosing regimen was based on previous research (Guo et al., 2004; Hirose et al., 2004). The mice were fed the same 4 dietary treatments used in the current study throughout AOM administration until the end of the experiment. Unexpectedly, 80% of the male mice fed the control diet died before the experiment ended, the first dying approximately 4 days after the 3rd AOM injection. When the experiment was terminated, the livers from the remaining males fed the control diet appeared a great deal smaller than those from mice in the other dietary groups. Although the female mice fed the control diet survived until the end of the experiment, when sacrificed roughly 65% had abnormal appearing livers. They contained nodules throughout, a common characteristic of cirrhosis. It was concluded that the male *ob/ob* mice fed the control diet died from AOM induced liver failure. The females most likely survived the toxic effects of AOM because they received a smaller dose.

The mouse model used in the previous and current experiments (*ob/ob* mice) exhibit impaired hepatic repair and regenerative responses to injury (Yang et al., 2001; Leclercq et al., 2003) and increased sensitivity to endotoxin hepatic injury (Yang et al., 1997). In order to avoid the hepatotoxic effects of AOM observed in the preliminary study, the number of AOM injections was reduced to two (7 mg per kg of body weight; based on the average weight of *ob/ob* mice in the study). However beginning sixteen weeks after the 2nd AOM injection until the end of the study (27 to 29 weeks after the 2nd

AOM injection), 9 animals spontaneously died. The hepatotoxic effects of AOM have been demonstrated, for example a single high dose (ranging from 30-200 mg AOM per kg of body weight) induced fulminant hepatic liver failure (FHF) in C57BL/6J mice, the background strain of *ob/ob* mice (B6.V-*Lep*^{ob} /J mice), in a dose-dependent manner (Matkowsky et al., 1999). It is probable that o*b/ob* mice are more susceptible to the hepatotoxic effects of AOM even when administered at subtoxic doses than C57BL/6J mice and the consequences of AOM hepatotoxicity manifest before colon tumors can fully develop.

The statistical power in this study was less than optimal because of the hepatotoxic effects of AOM in *ob/ob* mice and because of the low adenoma and adenocarcinoma incidence in control fed *ob/ob* mice. For example, if there was one less lesion in the NB group, then feeding NB would have been considered to have reduced lesion incidence ($P<0.05$). In addition, the NBR would have to have zero adenomas + adenocarncinomas to have significantly less tumors than the controls (i.e., just one adenoma or adenocarcinoma in the NBR group made it so that the NBR was not significantly different from the mice fed the control diet ($P>0.05$)). These points underscore the importance in having a higher tumor incidence in experiments designed to show a cancer inhibiting effect of diets.

In the current study, the animals thrived on all four dietary treatments: control diet, navy bean diet or navy bean fraction diets (NBR and NBEM). However, the *ob/ob* mice fed the control diet gained less weight than those fed the whole NB diet and NBR diets. The *ob/ob* mice fed the NBEM diet also gained less weight than mice fed the NB diet or the NBR diets. Investigators have previously shown energy restriction is

associated with increased inhibition of chemically induced colon carcinogenesis. Kumar et al. (1990) reported feeding rats a 20% and 30% calorie restricted diet (resulting in 23% and 38% reduction in total weight gain respectively) significantly inhibited AOM-induced colon tumor incidence in rats by 34% and 39%, respectively. Whereas feeding a 10% calorie restricted diet (16% reduction in total weight gain) did not significantly reduce tumor incidence (Kumar et al., 1990). It is unlikely that the differences in weight influenced tumor incidence in the present study for the following reasons. The *ob/ob* mice fed the control diet gained 20% and 11% less weight than those fed the whole NB diet and NBR diets, yet the control mice had the highest lesion (40% vs. 21% or 14%) and tumor incidence (18% vs. 15% or 5%). Additionally, the *ob/ob* mice fed the NBEM diet gained 15% and 5% less weight than those fed the whole NB diet and NBR diet, respectively. However, these animals had the lowest lesion and tumor incidence. In this study if a reduction in weight gain affected lesion and tumor incidence, the lesion and tumor incidences in the control diet fed group and the NBEM diet fed group would have been similar. Additionally *ob/ob* mice are overtly obese by 4 to 5 weeks of age compared to their lean littermates, thus they were grossly obese before the beginning of the study and all weighed 2 to 3 times more than an average lean mouse at the end of the study. Thus any differences found in weight are negligible and had no consequence on tumor outcome.

The second objective of this study was to determine if feeding diets containing a) navy beans (*Phaseolus vulgaris* L.); b) an aqueous-ethanol extract of navy beans and c) the residue remaining after aqueous-ethanol extraction would reduce adenoma and adenocarcinoma incidences in AOM injected *ob/ob* mice. Due to the limitations of the

ob/ob mouse model discussed above, it could not be ascertained if feeding navy beans inhibited lesion and tumor incidence as reported previously in a rat model (Hangen and Bennink, 2002).

All three navy bean diets were designed to contain the same amount of dietary fiber to eliminate its protective affect on chemically induced colon cancer. The NB and NBEM were formulated to be equal in total phenolic content, 110.6 and 109.8 mg (+)-catechin equivalents per 100 g of diet, respectively (Table 10). Both diets contained approximately twice the amount found in the NBR diet. In Chapter III the ~60% ethanol extraction of the aqueous-alcohol soluble components in navy beans was more efficient at extracting hydroxycinnamic acids conjugates than hydroxybenzoic acids conjugates. Using the data from Chapter III, the NB and NBEM diets were found to contain similar amounts of hydroxycinnamic acids, whereas all three navy bean diets had similar amounts of hydroxybenzoic acids. No flavan-3ols or flavonols were identified in navy beans or its fractions. Besides being rich in phenolic acids, the aqueous-alcohol fraction of cooked navy beans (NBEM) would contain oligosaccharides, saponins and phytic acids which were not quantified in this or the previous study. These non-nutrients have been shown to possess anti-cancer activity (Jenab and Thompson, 2000; Francis et al., 2002; Guillon and Champ, 2002). The NB diet would contain these non-nutrients as well. We expected the tumor incidence to be reduced by at least 50% in animals fed both the NB diet and NBEM diet, similar to that observed previously in a rat model fed a navy bean diet (Hangen and Bennink, 2002). Tumor incidence was expected to be reduced in animals fed the NBR diet, but to a lesser extent compared to the other two navy bean diets.

Feeding the aqueous-ethanol extract of navy beans (NBEM) reduced tumor incidence (tumors include adenomas and adenocarcinomas) by 100% compared to animals fed the control diet and the navy bean diet ($P<0.05$). Interestingly tumor incidence was inhibited by 69% (not statistically significant) in mice fed the aqueous-ethanol insoluble components of navy beans (NBR) compared to the control diet fed mice The finding that feeding NBR and NBEM, were more protective against AOM-induced colon carcinogenesis was unexpected. When bioactive molecules in beans are part of the whole food matrix, the bioactivity may work together or synergistically, whereas others may work against one another or antagonistically (Jacobs and Steffen, 2003). Thus the biological activity of a particular compound(s) could be enhanced if it is separated from an antagonist. Alternately, because of the weak statistical power in this study, it was not possible to make a true differentiation in cancer inhibiting potential between the two navy bean fractions—the aqueous-ethanol soluble components (NBEM) and the aqueous-ethanol insoluble components (NBR).

In conclusion, the mouse model used in this study did not have the desired sensitivity to AOM to be recommended for widespread use to test dietary ingredients for colon cancer inhibiting potential. Nevertheless, the compounds contained in the aqueous-ethanol extract of navy beans (NBEM) reduced tumor incidence by 100% compared to the control diet.

CHAPTER V. DIETS CONTAINING NAVY BEAN (*PHASEOLUS VULGARIS* L.) OR NAVY BEAN FRACTIONS INHIBIT AZOXYMETHANE (AOM)-INDUCED COLON CANCER IN *OB/OB* MICE BY MODULATING THE TGF-β SIGNALING PATHWAY

A. ABSTRACT

Understanding the molecular, biochemical, cellular and genetic alterations occurring at different stages during colon cancer development may lead to chemoprevention strategies, targets for chemotherapy drugs and early biomarkers representing cancer risk. Diet is an important lifestyle factor that can be modified to reduce colorectal cancer risk.

Food components not digested and absorbed in the upper gastrointestinal tract come in direct contact with the colonic epithelial cells and may modify gene expression. Previous studies have found that feeding dry beans reduces colon tumor incidence in AOM injected rats by modulating gene expression involved in regulating colon crypt cytokinetics and in reducing inflammation. Consistent with those findings, disruptions in the regulation of crypt cytokinetics (i.e. cell proliferation, migration, differentiation, or apoptosis) can contribute to the development of colorectal cancer.

Transforming growth factor (TGF-β) is a cytokine that controls cell division, differentiation, migration, adhesion, extracellular matrix deposition and programmed cell death (apoptosis) in many cell types. Deregulation in the TGF-β signaling has been shown to play a role in human colorectal carcinogenesis and animal models of colorectal cancer. Since the biological actions and gene expression of TGF-β are consistent with

maintaining colon crypt homeostasis and since colon tumorigenesis is associated with perturbations in colon crypt homeostasis, it was hypothesized that reduced colon tumorigenesis observed when navy bean diets were fed would be associated with normalizing expression of genes involved in the TGF-β signaling pathway.

The results in this study suggest that feeding diets containing cooked navy bean and navy bean fractions — NBR and NBEM — inhibit AOM induced colon carcinogenesis in obese, mildly diabetic mice (*ob/ob* mice (B6.V-*Lep*^{ob} /J)) by modifying the expression of genes involved in the TGF-β signaling pathway. A downregulation in the gene expression of *c-fos* was observed in the colonic mucosa of bean fed mice and this was related to reductions in colon lesion incidence. *c-Fos* is required for normal cell cycle progression. Studies have found *c-fos* to be upregulated in some human cancers. Thus feeding navy beans and its fractions to AOM injected *ob/ob* obese mice inhibits colon carcinogenesis by maintaining colonic crypt cell homeostasis by modulating cytostatic processes involved in preventing uncontrolled cell proliferation.

B. INTRODUCTION

Carcinogenesis is a multi-step process wherein a series of genetic events are necessary to drive the transformation of normal cells to malignant cancers (Hanahan and Weinberg, 2000). Understanding the genetic, molecular, cellular and biochemical alterations occurring at the different steps in this process may lead to chemoprevention strategies through diet, the discovery of targets for chemotherapy drugs and early biomarkers representing cancer risk. Diet is an important lifestyle factor that can be modified to reduce CRC risk. Diet can alter metabolism and blood constituents (i.e., hormones, growth factors) that in turn alter carcinogenesis. In addition, food components not digested and absorbed in the upper gastrointestinal tract come in direct contact with the colonic epithelial cells. Because colon carcinogenesis is a multi-step process requiring a number of molecular alterations to fully manifest, it is logical that dietary-induced changes in metabolized and undigested food components might alter colon cancer development.

The large intestine is made up millions of crypts, which are around 50 cells deep (see Chapter 1, Figure 4) (Lipkin, 1999; Heavey et al, 2004, Humphries and Wright, 2008). Colonic cells are under constant renewal, with the entire human colonic epithelium being replaced every 3 to 8 days (Lipkin, 1999). Stem cells located at the bottom of the crypt replicate resulting in the original stem cell and a new daughter cell. The daughter cells continue to replicate and push upward toward the top of the crypt. About two-thirds up the crypt, the proliferative activity ceases and cells differentiate into mature epithelial cells. Once at the lumenal surface the mature cells undergo apoptosis and are exfoliated into the fecal stream. The crypt design provides a high probability that

damaged or mutated cells will not influence the integrity of the crypt (Heavey et al., 2004). Crypt cell kinetics or the molecular processes involved in maintaining the balance between new cell production and cell death are under tight regulation in highly renewing tissues like the colon (Lipkin, 1999). Disruptions in any one of these processes (cell proliferation, migration, differentiation, or apoptosis) can contribute to the development of colorectal cancer.

Rondini (2006) fed defatted soybean flour and black beans (75g per 100 g of diet) to rats injected with AOM. Feeding soy reduced tumor incidence by 67% and feeding black bean reduced tumor incidence by 56% compared to rats fed the casein diet. Based on cDNA microarray technology, the consumption of beans inhibits AOM induced colon tumorigenesis by modulating the expression of genes involved in regulating colon crypt cytokinetics as well as genes involved in reducing inflammation. The expression of several genes involved in the cell's progression through the cell cycle, were significantly downregulated in the colonic mucosa of bean fed rats (Rondini, 2006). This is consistent with the idea of food components coming in contact with the colonic epithelial cells, inducing changes in the expression of genes responsible for maintaining crypt cytokinetics, and consequently, altering colon cancer development.

Transforming growth factor (TGF-β) is a member of a large superfamily of cytokines that controls cell division, differentiation, migration, adhesion, extracellular matrix deposition and programmed cell death (apoptosis) in many cell types (Harradine and Akhurst, 2006; Massagué et al., 2006). Whether TGF-β inhibits or stimulates cell proliferation is dependent on cell type and the presence or absence of growth factors. However it is recognized as a potent inhibitor of proliferation in epithelial, endothelial

and hematopoietic cells (Hartsough and Mulder, 1997). TGF-β inhibits the proliferation of cells (Massagué et al., 2006; Seoane, 2006) primarily by preventing cell progression through the G1 phase of the cell cycle. In the normal colonic crypt, the expression of TGF-β increases from the bottom of the crypt to top, with the highest amounts expressed at the lumenal surface where the mature, differentiated cells are located (Shao et al., 1999). TGF-β expression is inversely related to proliferation. Since the biological actions and gene expression of TGF-β are consistent with maintaining colon crypt homeostasis and since colon tumorigenesis is associated with perturbations in colon crypt homeostasis, it was hypothesized that reduced colon tumorigenesis observed when navy bean diets were fed would be associated with normalizing expression of genes involved in the TGF-β signaling pathway.

C. MATERIALS AND METHODS

Experimental animals

Described in Chapter IV, section C. Material and Methods.

Preparation of Navy Beans and Fractions

Preparation of the navy bean flour and the fractions derived from it is described in Chapter III, C. Material and Methods.

Diet Preparation

Preparation of the four diets fed to the *ob/ob* mice; 1) control diet — modified AIN-93G diet; 2) NB — cooked navy bean diet, 3) NBR — navy bean residue diet; and 4) NBEM—navy bean extract mix diet are described in Chapter IV, C. Material and Methods. The composition of each diet is listed in Table 7 (Chapter IV).

Initiation of Colon Cancer

Described in Chapter IV, C. Material and Methods.

Necropsy and Histology

Described in Chapter IV, C. Material and Methods.

RNA Isolation

RNA was isolated and purified from frozen (-80°C) colonic mucosa scrapings of the mid-colon segment from *ob/ob* mice (n = 3 per dietary treatment). The scraped section had no macroscopic lesions or tumors.

The RNeasy Mini Kit (Qiagen, Valencia, CA) was used to isolate and purify total RNA according to the manufacturer's protocol. Briefly, a portion of colonic mucosa was added to a 2ml microcentrifuge tube containing 1ml of glass beads (1.0 mm in diameter, Biospec Products Inc., Bartleville, OK) and 1 ml of Buffer RLT. The tissue was homogenized by mixing on a Mini-bead beater-1 (Biospec Products Inc., Bartleville, OK) for 20 sec at 46 rpm. After centrifuging at full speed for 3 min, the supernatant (lysate) was transferred to a new 2ml microcentrifuge tube. One ml of 70% ethanol was added to the lysate (700µl) and mixed. Lysate + 70% ethanol mix (1700µl) was transferred to an RNeasy spin column, placed into a collection tube, centrifuged for 15 s at >8,000 x g and the flow through was discarded. The previous step was repeated. Then 350µl of Buffer RW1 was added to the RNeasy spin column, centrifuged for 15 s at >8,000 x g and the flow through was discarded. To prevent DNA contamination, 80µl of RNase free DNase I incubation mix was added to the RNeasy spin column and incubated on the benchtop (20-30°C) for 15 min. Buffer RWI (350µl) was added to RNeasy spin column, centrifuged for 15 s at >8,000 x g, and the flow through was discarded. The DNase I

digest step was performed 2 times. Buffer RPE (500µl) was added to the RNeasy spin column, centrifuged for 15 s at >8,000 x g, and the flow through was discarded. Buffer RPE (500µl) was again added to the RNeasy spin column, this time it was centrifuged for 2 min at >8,000 x g, and the flow through was discarded. The RNeasy spin column was placed into a new collection tube and centrifuged for 1 min at full speed. The RNeasy spin column was placed into a new 1.5ml collection tube, 25µl of RNase free water was added and centrifuged for 1 min at >8,000 x g, and the cleaned up RNA was eluted. The prior step was repeated and RNA eluates were combined.

Quantitative Real Time PCR

First strand cDNA synthesis was performed using the RT^2 PCR Array First Strand Kit (SABiosciences, Frederick, MD). Briefly, 2.5µg of RNA plus 1µl of P2 (Primer and External control mix) was added to a sterile PCR tube, mixed with a pipettor followed by brief centrifugation, this was termed the annealing mixture. The annealing mixture was placed into a thermocycler at 70°C for 3 min and then chilled on ice for 1 min. While the annealing mixture was incubating the RT Cocktail (contains the reverse transcriptase) was prepared according to the manufacturer's protocol. 10µl of the RT Cocktail was added to the chilled annealing mixture, mixed well with a pippettor and incubated at 37°C for 60 min in the thermacycler. The cDNA synthesis reaction (annealing mixture plus RT Cocktail) was heated to 95°C for 5 min. Then 91µl of ddH_2O was added to the PCR tube, the tube was mixed and placed on ice until Real Time-PCR was performed.

Prior to Real Time-PCR, the Experimental Cocktail was prepared following the manufacturer's protocol. Briefly, 1325µl RT^2 Real Time™ SYBR-Green / Fluroescein PCR master mix (contains the polymerase, SABiosciences, Frederick, MD), 1219µl of

ddH_2O and 106µl first strand cDNA synthesis reaction were added to a 10 ml sterile conical tube and gently mixed using a pipettor. The Experimental Cocktail was then carefully poured into a reservoir. Using an eight-channel pipettor, 25µl of the Experimental Cocktail was added to the 96 well RT^2 Profiler™ PCR Array: Mouse TGF-β / BMP Signaling Pathway (SABiosciences, Frederick, MD), 3 plates per dietary treatment; 1 animal per plate. The RT^2 Profiler™ PCR Array: Mouse TGF-β / BMP Signaling Pathway profiles the expression of 84 genes related to the TGF-β / BMP-mediated signal transduction and contains a 96 well primer set. Each plate contains a genomic DNA contamination control, Reverse Transcription control, Positive PCR control and Five housekeeping genes. Real-Time PCR was performed using the Bio-Rad iQ™5 Real-Time PCR Detection system (Bio-Rad, Hercules, CA). Real-time conditions were step as follows: Initial enzyme (Hotstart DNA polymerase) activation step of 10 min at 95°C followed by 40 cycles of denaturation/melting for 15 sec at 95°C followed by annealing / extension for 1 min at 60°C (Real time PCR step). A melting curve was generated by decreasing the set point temperature 95°C to 55°C over 1 min, followed by 81 cycles for 30 sec at 55°C.

Statistical Analysis and Biological Significance Criteria

Excel based Data Analysis Template was downloaded from the SABiosciences Website (http://www.sabiosciences.com/pcr/arrayanalysis.php). Differences in normalized gene expression — $2^{-\Delta Ct}$, (C_t is threshold cycle; $\Delta C_t = C_t(GOI) - C_t(HK)$; GOI = gene of interest, HK = housekeeping gene) — due to dietary treatment were determined using the Student's 2 tailed t-test. Changes in gene expression were

considered statistically significant when $P \leq 0.05$. A strong trend for a changes in gene expression was when $P > 0.05$ but ≤ 0.15.

In addition to meeting statistical criteria, changes in gene expression had to reflect lesion incidence and tumor incidence to be considered biologically significant. Thus, gene expression important in reducing hyperplasia and dysplasia must be significantly increased or decreased in mice fed NBEM and NBR and there must be a significant or a strong trend for an increase or decrease in mice fed NB. To be considered relevant for reducing tumor incidence, gene expression must be significantly increased or decreased in mice fed NBEM and there must be a significant or strong trend for a difference in gene expression for mice fed NBR

D. RESULTS

Total RNA was isolated and purified from the colonic mucosa with no grossly visible lesions from three mice per dietary treatment. The RT^2 Profiler™ PCR Array: Mouse TGF-β / BMP Signaling Pathway array includes the 84 genes listed in Table 13.

Out of the 84 genes only *fos* (FBJ osteosarcoma oncogene; D12Rfj1/*c-fos*) met the pre-established criteria for statistical and biological significance for reducing hyperplasia, dysplasia, and tumor development. The changes in gene expression were: NB -3.10; NBR -2.15; and NBEM -3.97 compared to mice fed the control diet. All of these changes were statistically significant ($P<0.05$).

Lefty 1 met the criteria for down regulation of tumor development. The gene expression for Lefty 1 was significantly down regulated in mice fed NB (-1.47, $P = 0.02$)

Table 13. Genes in the Mouse TGF-β-BMP Signaling Pathway PCR Array

Symbol	Gene Name	Gene Description
Acvr1	ALK2/ActR-I	Activin A receptor, type 1
Acvr2a	ActRIIa/Acvr2	Activin receptor IIA
Acvrl1	AI115505/AI427544	Activin A receptor, type II-like 1
Amh	MIS	Anti-Mullerian hormone
Amhr2	MISIIR/Misrii	Anti-Mullerian hormone type 2 receptor
Bambi	2610003H06Rik	BMP and activin membrane-bound inhibitor, homolog (Xenopus laevis)
Bglap2	OG2/mOC-B	Bone gamma-carboxyglutamate protein 2
Bmp1	TLD	Bone morphogenetic protein 1
Bmp2	AI467020/Bmp2a	Bone morphogenetic protein 2
Bmp3	9130206H07/9530029I04Rik	Bone morphogenetic protein 3
Bmp4	Bmp2b/Bmp2b-1	Bone morphogenetic protein 4
Bmp5	AU023399/se	Bone morphogenetic protein 5
Bmp6	D13Wsu115e/Vgr-1	Bone morphogenetic protein 6
Bmp7	OP1	Bone morphogenetic protein 7
Bmper	3110056H04Rik/CV-2	BMP-binding endothelial regulator
Bmpr1a	1110037I22Rik/ALK3	Bone morphogenetic protein receptor, type 1A
Bmpr1b	AI385617/ALK-6	Bone morphogenetic protein receptor, type 1B
Bmpr2	2610024H22Rik/AL117858	Bone morphogenic protein receptor, type II (serine/threonine kinase)
Cd79a	Ig-alpha/Iga	CD79A antigen (immunoglobulin-associated alpha)
Cdc25a	D9Ertd393e	Cell division cycle 25 homolog A (S. cerevisiae)
Cdkn1a	CAP20/CDKI	Cyclin-dependent kinase inhibitor 1A (p21)
Cdkn2b	AV083695/INK4b	Cyclin-dependent kinase inhibitor 2B (p15)
Chrd	Chd	Chordin
Col1a1	Col1a-1/Cola-1	Procollagen, type I, alpha 1
Col1a2	AA960264/AI325291	Procollagen, type I, alpha 2
Col3a1	AW550625/Col3a-1	Procollagen, type III, alpha 1
Dlx2	AW121999/Dlx-2	Distal-less homeobox 2
Eng	AI528660/CD105	Endoglin
Evi1	D630039M04Rik/Evi-1	Ecotropic viral integration site 1

Table 13. (cont'd)

Fkbp1b	AW494148	FK506 binding protein 1b
Fos	D12Rfj1/c-fos	FBJ osteosarcoma oncogene
Fst	FST	Follistatin
Gdf1	AI385651/Gdf-1	Growth differentiation factor 1
Gdf2	Bmp9	Growth differentiation factor 2
Gdf3	C78318/Gdf-3	Growth differentiation factor 3
Gdf5	CDMP-1/bp	Growth differentiation factor 5
Gdf6	BMP13/GDF16	Growth differentiation factor 6
Gdf7	BMP12	Growth differentiation factor 7
Gsc	GSC	Goosecoid
Id1	AI323524/D2Wsu140e	Inhibitor of DNA binding 1
Id2	AI255428/C78922	Inhibitor of DNA binding 2
Igf1	C730016P09Rik/Igf-1	Insulin-like growth factor 1
Igfbp3	AI649005/IGFBP-3	Insulin-like growth factor binding protein 3
Il6	Il-6	Interleukin 6
Inha	AW555078	Inhibin alpha
Inhba	INHBA	Inhibin beta-A
Inhbb	INHBB	Inhibin beta-B
Itgb5	AA475909/AI874634	Integrin beta 5
Itgb7	Ly69	Integrin beta 7
Jun	AP-1/Junc	Jun oncogene
Junb	JUNB	Jun-B oncogene
Lefty1	AI450052/Leftb	Left right determination factor 1
Ltbp1	9430031G15Rik/9830146M04	Latent transforming growth factor beta binding protein 1
Ltbp2	AW208642	Latent transforming growth factor beta binding protein 2
Ltbp4	2310046A13Rik	Latent transforming growth factor beta binding protein 4
Myc	AU016757/Myc2	Myelocytomatosis oncogene
Nbl1	D4H1S1733E/DAN	Neuroblastoma, suppression of tumorigenicity 1
Nodal	Tg.413d	Nodal
Nog	NOG	Noggin

Table 13. (cont'd)

Nr0b1	AHX/Ahc	Nuclear receptor subfamily 0, group B, member 1
Pdgfb	PDGF-B/Sis	Platelet derived growth factor, B polypeptide
Plat	AU020998/AW212668	Plasminogen activator, tissue
Plau	u-PA/uPA	Plasminogen activator, urokinase
Runx1	AI462102/AML1	Runt related transcription factor 1
Serpine1	PAI-1/PAI1	Serine (or cysteine) peptidase inhibitor, clade E, member 1
Smad1	AI528653/Madh1	MAD homolog 1 (Drosophila)
Smad2	Madh2/Madr2	MAD homolog 2 (Drosophila)
Smad3	AU022421/Madh3	MAD homolog 3 (Drosophila)
Smad4	D18Wsu70e/DPC4	MAD homolog 4 (Drosophila)
Smad5	1110051M15Rik/AI451355	MAD homolog 5 (Drosophila)
Smurf1	4930431E10Rik/mKIAA1625	SMAD specific E3 ubiquitin protein ligase 1
Sox4	AA682046/Sox-4	SRY-box containing gene 4
Stat1	2010005J02Rik/AA408197	Signal transducer and activator of transcription 1
Tdgf1	CR1/cripto	Teratocarcinoma-derived growth factor
Tgfb1	TGF-beta1/Tgfb	Transforming growth factor, beta 1
Tgfb1i1	ARA55/Hic5	Transforming growth factor beta 1 induced transcript 1
Tsc22d1	AA589566/AW105905	TSC22 domain family, member 1
Tgfb2	BB105277/Tgf-beta2	Transforming growth factor, beta 2
Tgfb3	Tgfb-3	Transforming growth factor, beta 3
Tgfbi	68kDa/AI181842	Transforming growth factor, beta induced
Tgfbr1	ALK5/AU017191	Transforming growth factor, beta receptor I
Tgfbr2	1110020H15Rik/AU042018	Transforming growth factor, beta receptor II
Tgfbr3	1110036H20Rik/AU015626	Transforming growth factor, beta receptor III
Tgfbrap1	3110018K12Rik/AU024090	Transforming growth factor, beta receptor associated protein

and NBEM (-1.42, P = 0.01). There was only a trend for down regulation of Lefty 1 (-0.24, P = 0.11) in mice fed NBR.

E. DISCUSSION

The biological actions and gene expression of TGF-β, a potent inhibitor of epithelial cell growth, are consistent with maintaining colon crypt homeostasis. Since colon tumorigenesis is associated with perturbations in colon crypt homeostasis the objective of this study was to determine if the inhibition of colon carcinogenesis observed in AOM-injected obese, mildly diabetic mice fed navy bean and navy bean fractions — NBR and NBEM —is associated with normalizing the expression of genes in the TGF-β signaling pathway. Feeding navy beans and its fractions downregulated the expression of c-*fos* mRNA (2 to 4 fold) compared to control fed *ob/ob* mice. These changes in gene expression were correlated with reductions in colon lesion incidence (see Chapter IV).

c-Fos, a direct target of TGF-β, is a proto-oncogene and member of the Fos family of transcription factors (in humans: c-Fos, FosB, Fra-1 and Fra-2). Normally it is not highly expressed (Milde-Langosch, 2005), but can be transiently and rapidly induced by a variety of stimuli including growth factors, cytokines, TPA, and UV irradiation (Angel and Karin, 1991). Fos proteins heterodimerize with members of the Jun family of transcription factors (in humans: c-Jun, JunB, JunD) to form the protein complex: activator protein-1 (AP-1). This complex can bind TPA-responsive elements (TRE) and other similar AP-1 like binding motifs, within the promoter region of target genes. AP-1 responsive genes are involved in the regulation of cell proliferation, differentiation and apoptosis; angiogenesis; hypoxia; invasion and metastasis (Busslinger and Bergers, 1994;

Milde-Langosch, 2005). The partnering between different Jun—Jun or Jun—Fos protein homodimers or heterodimers, respectively, in the AP-1 complex determines the AP-1target gene response and are cell type specific (Angel and Karin, 1991). Fos proteins are required for normal cell cycle progression and entry into S phase (Kovary and Bravo, 1991), yet an overexpression in *c-fos* has been associated with tumorigenesis and correlated with the expression of several cell cycle regulatory proteins in different cancer cells (Bamberger et al., 2001; Milde-Langosch et al., 2002; Milde-Langosch, 2005). In cervical carcinoma cells, the c-Fos protein was found to replace Fra-1 in AP-1 dimers, and this shift in expression was positively associated with malignancy (Soto et al., 2000). Additionally, the expression of *c-fos* is required for the progression of mouse skin papillomas to malignant tumors (Saez et al., 1995). These experimental studies suggest a role for *c-fos* in the transformation of normal cells to malignant cancers in some tissues.

Furthermore, in the colons of AOM injected rats, ACF were found to express higher levels of *c-fos* (mRNA and protein) than normal crypts (Stopera et al., 1992). The expression of *c-fos* mRNA followed the pattern of cell proliferation in colonic crypts, with expression being greatest in the lower compartment of the crypt where highly proliferating cells are located (Stopera et al., 1992). Additionally, the advanced lesions, ACF exhibiting dyplasia, expressed higher levels than early lesions exhibiting hyperplastic (Stopera et al., 1992). This evidence suggests that an overexpression of *c-fos* may contribute to the increased cell proliferation observed in early and advanced colonic lesions, which may help promote their progression to more invasive, malignant tumors. Thus *c-fos* overexpression may represent an early genetic change occurring in AOM induced colon carcinogenesis.

Feeding navy bean and navy bean fractions — NBR and NBEM was found to inhibit AOM induced colon carcinogenesis in *ob/ob* mice and this inhibition was accompanied by a downregulation in the expression *c-fos* in the colonic mucosa. In normal cells *c-fos* expression is required for cell cycle progression and cell proliferation, however its overexpression is associated with tumorigenesis. Thus the downregulation of *c-fos* observed in the colonic mucosa of navy bean and navy bean fraction fed AOM injected *ob/ob* mice play a role in maintaining normal crypt cell proliferation.

Additionally, feeding navy bean and it fractions downregulated the expression of Lefty 1 mRNA in the colonic mucosa of AOM injected *ob/ob* mice compared to the control fed group. This change in gene expression was only significant in animals fed the NBEM and navy bean diet, but not mice fed the NBR. Lefty negatively regulates different TGF-β family members i.e. TGF-βs, BMPs and nodal, and plays a critical role in implantation and embryogenesis (Ulloa and Tabibzadeh, 2001; Tabibzadeh and Hemmati-Brivanlou, 2006). It blocks TGF-β signaling by antagonizing pathway specific co-receptors (Cheng et al., 2004) and by inhibiting the phosphorylation and activation of receptor activated SMAD transcription factors (Ulloa and Tabibzadeh, 2001). Many epithelial derived tumors (≥85% of all human cancers) and several human colon carcinoma cell lines no longer respond to the antigrowth signals induced by TGF-β (Hoosein et al., 1989; Hsu et al., 1994; Elliott and Blobe, 2005) and an overexpression in Lefty has been observed in certain forms of human cancer, including colon cancer (Ulloa and Tabibzadeh, 2001). Thus an overexpression in Lefty 1 may assist initiated cells in evading growth inhibition, a recognized hallmark of cancer (Hanahan and Weinburg, 2000), and provide them with a selective growth advantage compared to surrounding

normal cells. Thus the downregulation in Lefty 1 may preserve epithelial cell sensitivity to TGF-β antigrowth effects and maintain colon crypt cytokinetics in navy bean and navy bean fraction fed AOM injected *ob/ob* mice.

In conclusion, feeding diets containing cooked navy bean and navy bean fractions — NBR and NBEM — inhibits AOM induced colon carcinogenesis in mildly diabetic, obese mice (*ob/ob*) by modifying the expression of genes involved in the TGF-β signaling pathway. In navy bean and navy bean fraction fed mice, a significant downregulation in the expression of *c-fos* mRNA was found in the colonic mucosa of AOM injected *ob/ob* mice when compared to the control diet fed mice and these changes correlated with the observed reductions in colon lesion incidence. This change in gene expression may prevent colonic epithelial cells from gaining the ability to proliferate without regulation, an early change found to occur in colon tumorigenesis (Lipkin, 1983) and recognized hallmark of cancer. Thus feeding navy beans and its fractions to AOM injected *ob/ob* obese mice inhibits colon carcinogenesis by maintaining colonic crypt cell homeostasis by modulating cytostatic processes involved in preventing uncontrolled cell proliferation.

CHAPTER VI. CONCLUSION

The consumption of dry beans has been associated with the inhibition of colon cancer and phenolic compounds in dry beans could be partly responsible for the observed protection. Dry beans must undergo some form of heat-treatment before they are safe for human consumption and heat treatments will induce physical and chemical compositional changes in beans, altering their nutrient and phenolic content. Thus it is important to determine the phenolic compounds and content in cooked common beans in order to further investigate their potential health benefits. In Chapter III, we identified several classes of phenolic compounds in the aqueous-alcohol extracts of four cooked dry common beans (*Phaseolus vulgaris* L.)—black, pinto, red, and navy beans— as well as in two fractions of cooked navy beans 1) navy bean extract (NBEM)—aqueous-ethanol soluble components (concentrated in phenolic compounds) and 2) navy bean residue (NBR)—aqueous-ethanol insoluble solid components (i.e. dietary fiber and protein), treated with and without acid and alkaline hydrolysis. Hydroxybenzoic acids and hydroxycinnamic acids were identified in all four cooked dry common beans, this included *p*-hydroxybenzoic acid, vanillic acid, and syringic acid. Protocatechuic acid was only identified in black, pinto, and red beans. The hydroxycinnamic acids identified in all beans were *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid. Ferulic acid was the predominant hydroxycinnamic acid in all four bean types. In general, acid hydrolysis liberated greater quantities of hydroxybenzoic acids while alkaline hydrolysis liberated greater quantities of hydroxycinnamic acids. Only black, pinto, and red beans contained the flavan-3ol (+)-catechin and the flavonols quercetin and kaempferol. The

partitioning of cooked navy beans into two fractions—NBEM and NBR—using aqueous-ethanol extraction (~60% ethanol) was more efficient in extracting hydroxycinnamic acid conjugates than hydroxybenzoic acid conjugates.

Identifying which components in dry common beans protect against the development of CRC and the molecular mechanism by which they exert these biological activities are of high priority. Partitioning beans into different fractions and determining the anti-cancer activity for the various fractions is one approach to narrowing the search for the anti-cancer components of beans. In our lab navy beans have been shown to inhibit chemically induced colon cancer in rats (Hangen and Bennink, 2002). Thus in Chapter IV, we fed diets containing cooked navy beans and its fractions—the aqueous-ethanol soluble components in cooked navy beans (NBEM) and the aqueous-ethanol insoluble components (NBR), or a control diet to AOM injected mildly diabetic, obese mouse (*ob/ob*; B6.V-*Lep*^{ob} /J) to determine whether one fraction inhibits colon cancer more than another. The *ob/ob* obese mouse model has not been previously used to evaluate the effect of dietary ingredients on chemically induced colon carcinogenesis. Obesity and type 2 diabetes mellitus are independent risk factors for CRC and dry beans have been associated with preventing both conditions. We concluded that the *ob/ob* mouse does not have the desired sensitivity to the colon carcinogenic properties of AOM to be recommended for widespread use to test dietary ingredients for colon tumor inhibiting potential. *Ob/ob* mice are susceptible to the hepatotoxic effects of AOM even when administered at subtoxic doses and the consequences of AOM hepatotoxicity manifest before colon tumors can fully develop. The low tumor (adenoma plus adenocarcinoma) incidence in the *ob/ob* mice fed the control diet seriously compromised

the ability to distinguish dietary induced differences. Nevertheless, the NBEM fraction significantly reduced tumor incidence by 100%. The NBR reduced tumor incidence by 69%, however it was not significant, further demonstrating the weak statistical power in this experiment.

Experimental studies have shown that feeding bean based diets to rats injected with AOM can reduce colon tumor incidence by modulating the expression of genes involved in regulating colon crypt cytokinetics and in reducing inflammation (Rondini, 2006). In Chapter V we investigated whether the reduction in colon tumorigenesis observed when navy bean and its fractions are fed to AOM-injected *ob/ob* mice was associated with normalizing the expression of genes involved in the TGF-β signaling pathway. The biological actions and gene expression of TGF-β are consistent with maintaining colon crypt homeostasis and disruptions in colon crypt homeostasis can contribute to the development of colorectal cancer. The results in Chapter V suggest that feeding diets containing cooked navy bean and navy bean fractions —NBR and NBEM— inhibit AOM induced colon carcinogenesis in mildly diabetic, obese mice by modifying the expression of genes involved in the TGF-β signaling pathway. A downregulation in the gene expression of *c-fos* was observed in the colonic mucosa of bean fed mice and this reflected the reductions in colon lesion incidence. *c-Fos* is required for normal cell cycle progression and studies have found it to be upregulated in some human cancers. Thus feeding navy beans to AOM injected *ob/ob* obese mice may inhibit colon tumorigenesis by inhibiting uncontrolled proliferation in colonic epithelial cells.

Collectively, Chapter III was one of the first studies to identify and quantify phenolic compounds in cooked common beans and to separate cooked navy beans into

two fractions 1) the aqueous-ethanol soluble components (NBEM; rich in phenolic compounds) and 2) the aqueous-ethanol insoluble solid components (NBR), to allow long-term colon cancer studies to be conducted in order to narrow the search for the anti-cancer components in beans. In Chapter IV it was determined that the mildly diabetic, obese mouse (*ob/ob* mice; B6.V-*Lep*^{ob} /J) did not have the desired sensitivity to AOM to be recommended for widespread use to test dietary ingredients for colon cancer inhibiting potential. Nevertheless, feeding cooked navy bean and navy bean fractions — NBR and NBEM — inhibited AOM induced colon carcinogenesis in mildly diabetic, obese mice (*ob/ob*) by maintaining colonic crypt cell homeostasis by modulating cytostatic processes involved in preventing uncontrolled cell proliferation (Chapter V).

CHAPTER VII. FUTURE STUDIES

I would recommend the following studies to further investigate and narrow the search for the anti-cancer components of cooked dry common beans and the possible mechanisms responsible for the inhibition of colon cancer.

Study 1:

1. Using more sensitive detection systems, i.e. MS/MS and NMR, in an attempt to identify unknown phenolic compounds in cooked dry common beans.
2. Identify and quantify anthocyanins and their aglycones in cooked dry common beans with blue and blue-violet colored seed coats, e.g. black beans.
3. Quantify the other non-nutrients found in cooked navy beans and in the navy bean fractions — aqueous-alcohol soluble components in cooked navy beans (NBEM) and in the aqueous-alcohol insoluble components in cooked navy beans (NBR)— with proposed anti-cancer properties such as: oligosaccharides, phytic acid, and saponins.

Study 2:

1. Repeat the study performed in Chapter IV using AOM injected rats, an accepted model of chemically induced colon carcinogenesis. Additionally, a fifth diet should be added to the study. This diet would consist of adding the NBEM fraction (concentrated aqueous-alcohol extract mix) back to the NBR fraction in an attempt to mimic the phenolic content in the cooked whole navy bean diet. This fifth diet would be another way to help distinguish which compounds in dry common beans possess anti-cancer properties.

Study 3:

1. Determine if c-fos protein expression levels corroborate the changes in *c-fos* mRNA expression observed in the colonic mucosa of AOM injected *ob/ob* mice fed the navy bean and navy bean fraction diets—NBR and NBEM.
2. Determine the location of c-fos protein within the colonic crypts and whether their expression correlates with cell proliferation patterns in the colon crypts.
3. Determine if these changes in mRNA expression also occur in AOM injected rats fed the cooked navy bean and navy bean fraction diets—NBR and NBEM— and the fifth diet discussed above (under Study 2).

APPENDICES

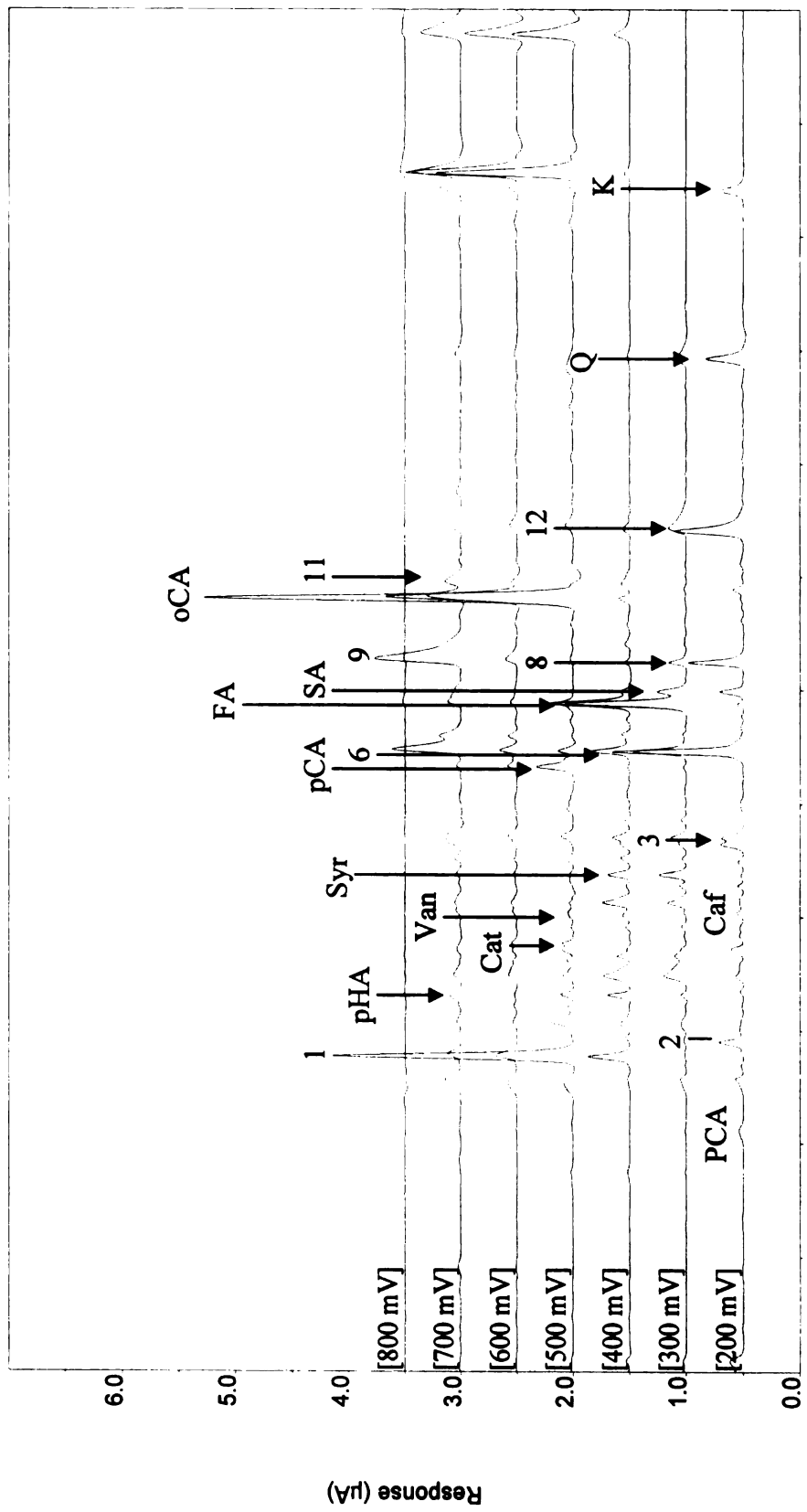


Figure 10a. Chromatogram of Non-hydrolyzed Black Bean
PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Cat, (+)-catechin; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Q, quercetin; K, kaempferol; numbers correspond to peaks of unknown compounds.

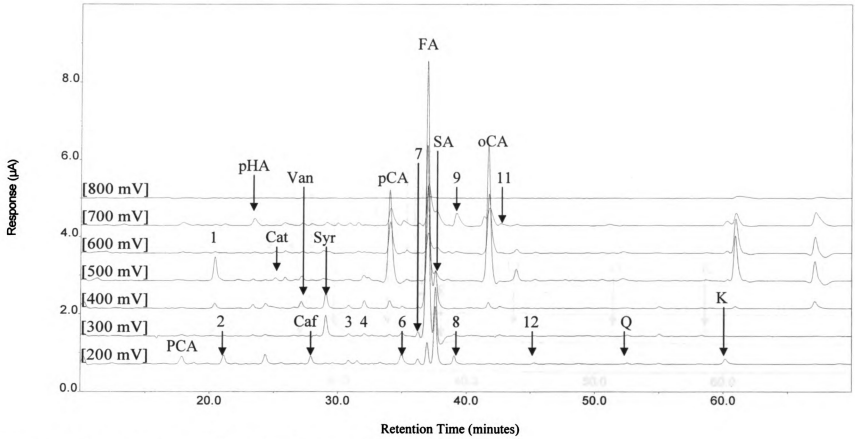


Figure 10b. Chromatogram of Alkaline Hydrolyzed Black Bean

PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Cat, (+)-catechin; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Q, quercetin; K, kaempferol; numbers correspond to peaks of unknown compounds.

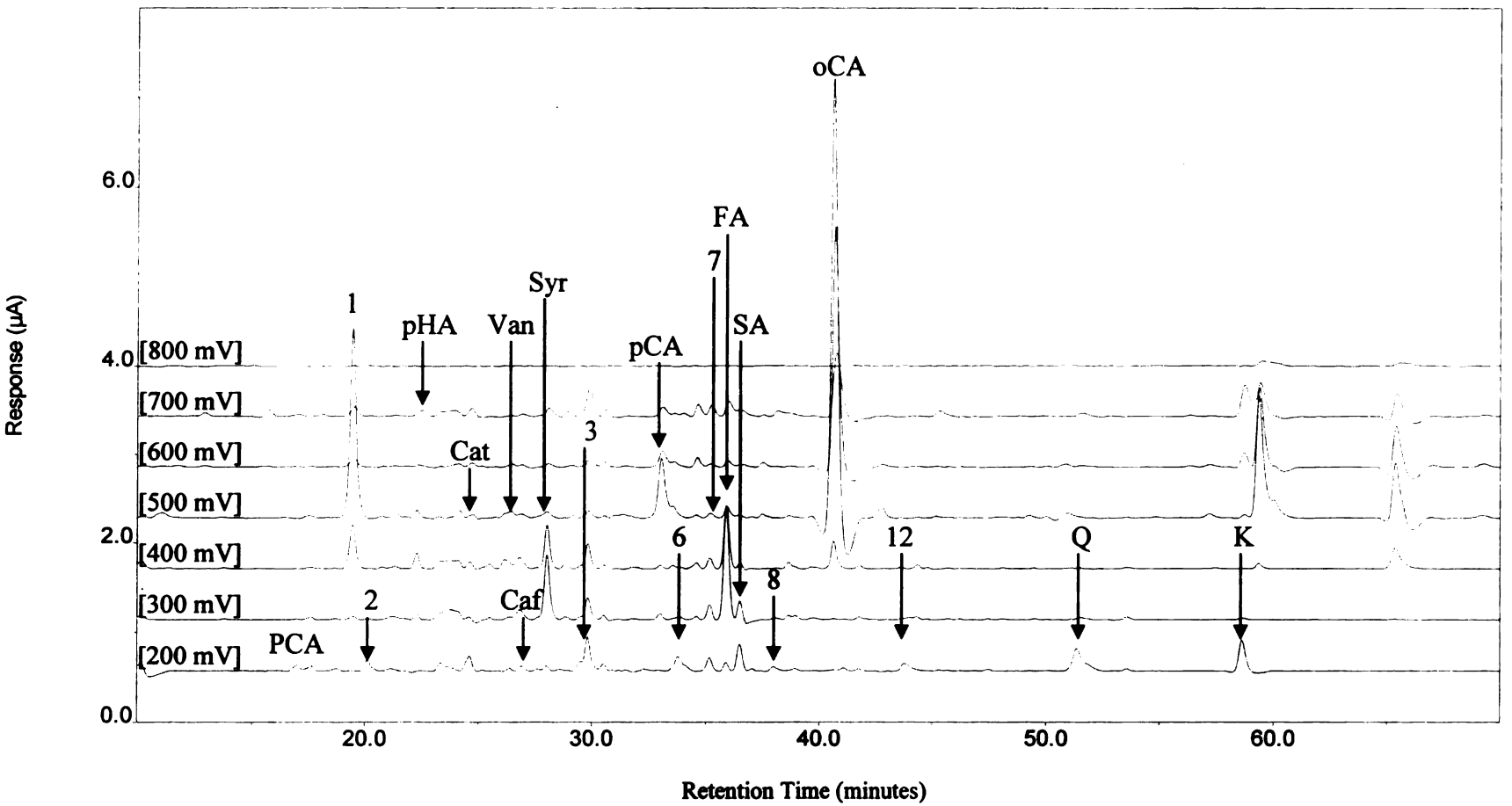


Figure 10c. Chromatogram of Acid Hydrolyzed Black Bean

PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Cat, (+)-catechin; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Q, quercetin; K, kaempferol; numbers correspond to peaks of unknown compounds.

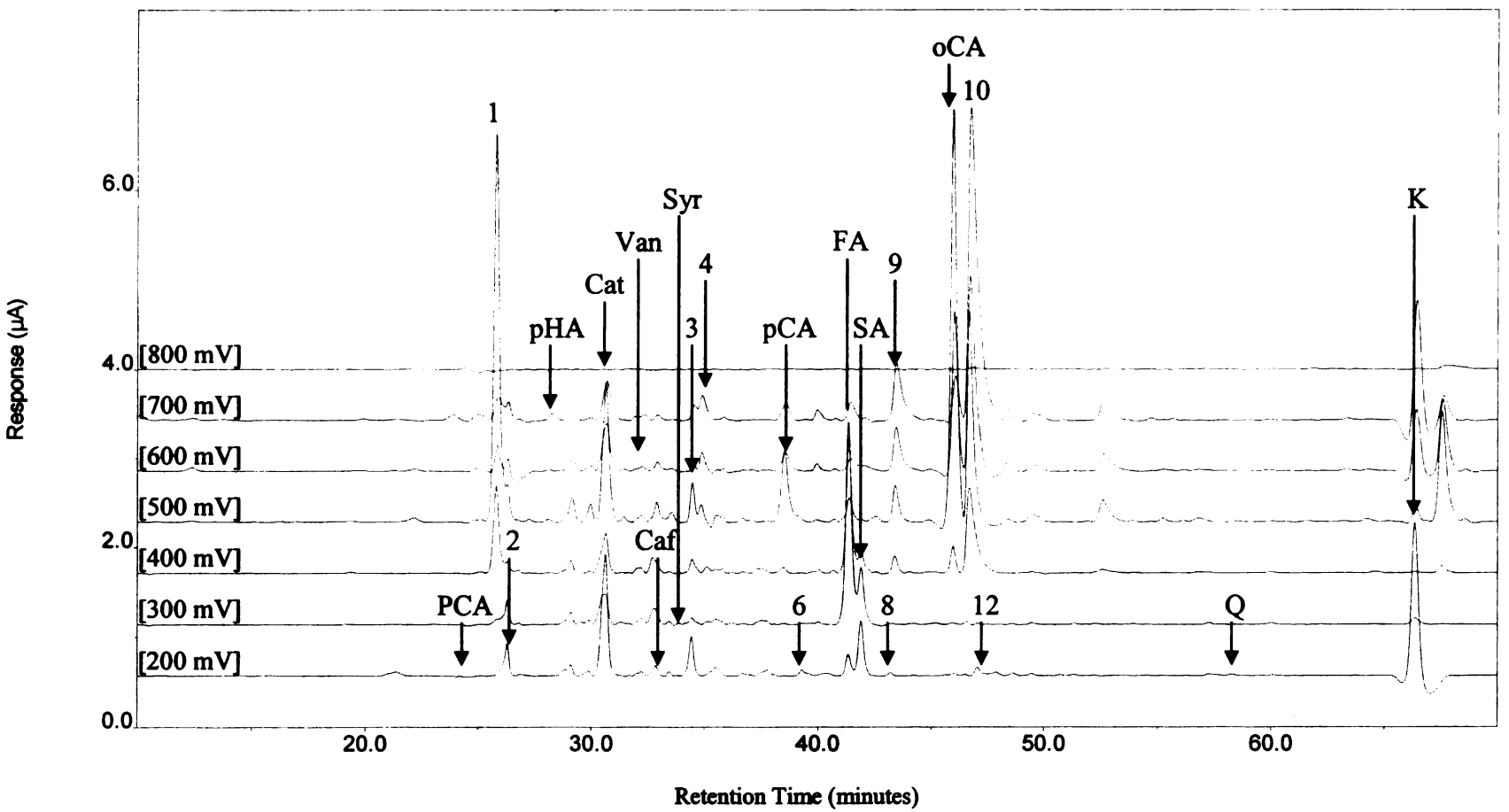


Figure 11a. Chromatogram of Non-Hydrolyzed Pinto Bean

PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Cat, (+)-catechin; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Q, quercetin; K, kaempferol; numbers correspond to peaks of unknown compounds.

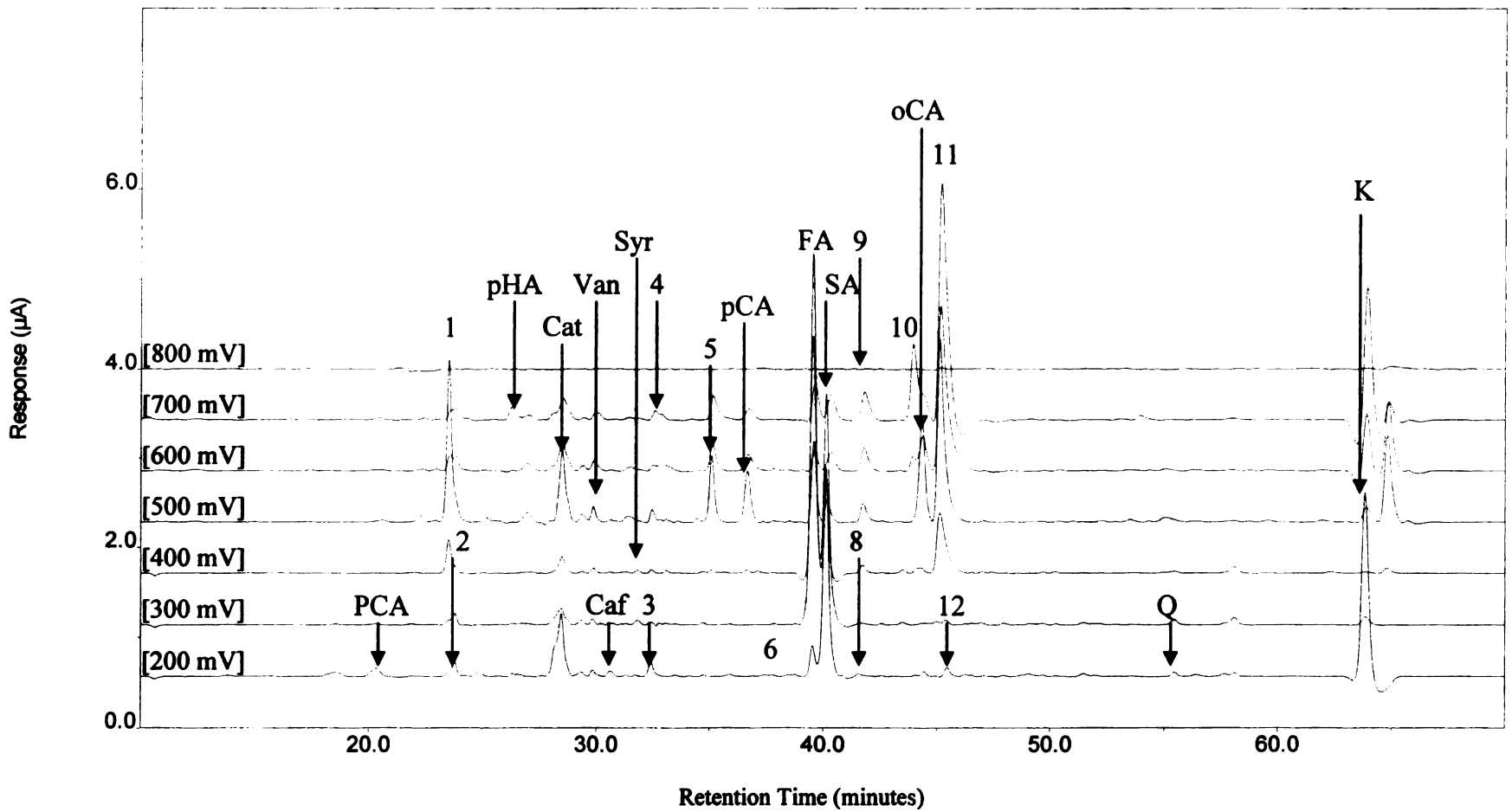


Figure 11b. Chromatogram of Alkaline Hydrolyzed Pinto Bean

PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Cat, (+)-catechin; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Q, quercetin; K, kaempferol; numbers correspond to peaks of unknown compounds.

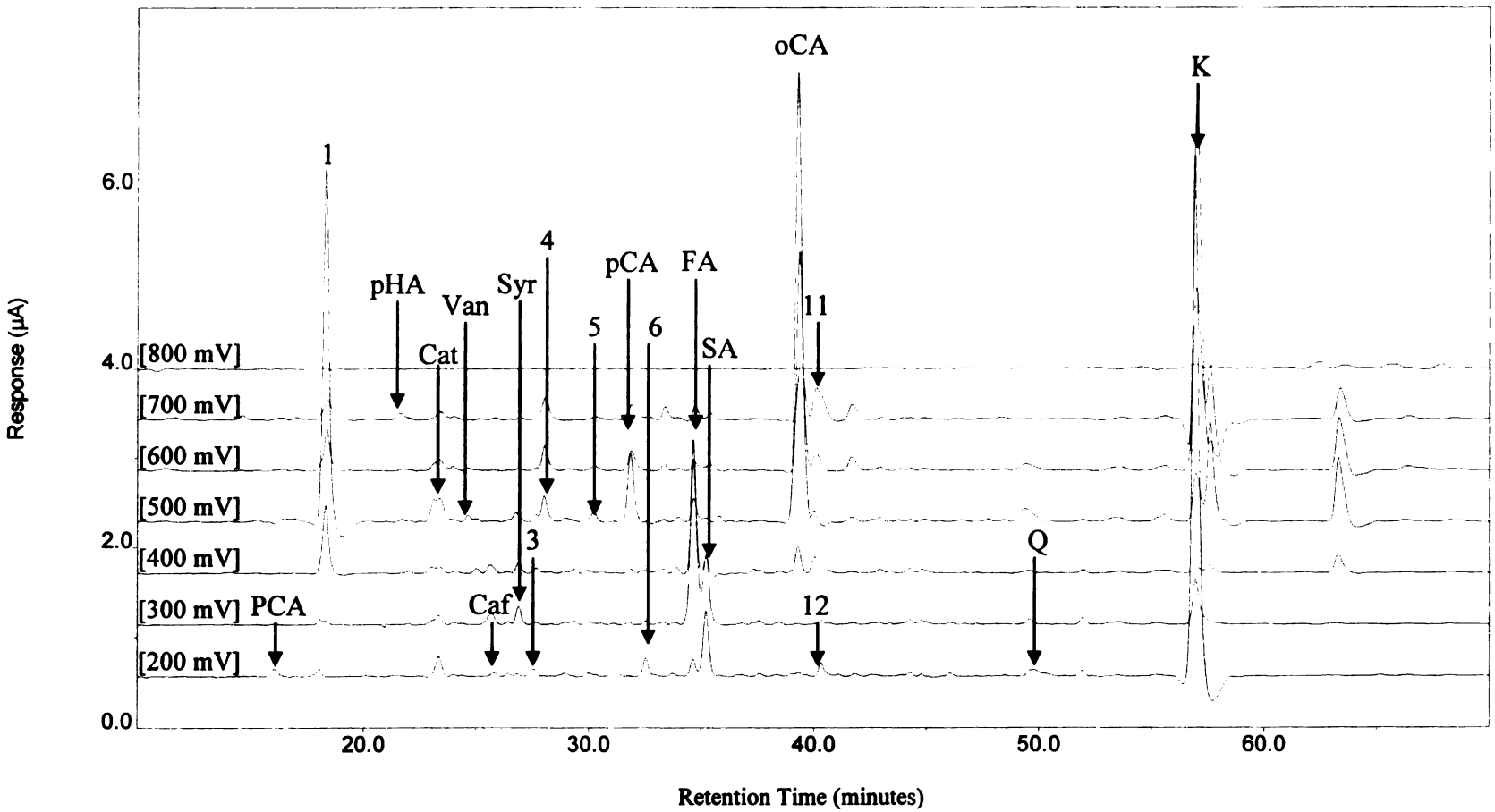


Figure 11c. Chromatogram of Acid Hydrolyzed Pinto Bean

PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Cat, (+)-catechin; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Q, quercetin; K, kaempferol; numbers correspond to peaks of unknown compounds.

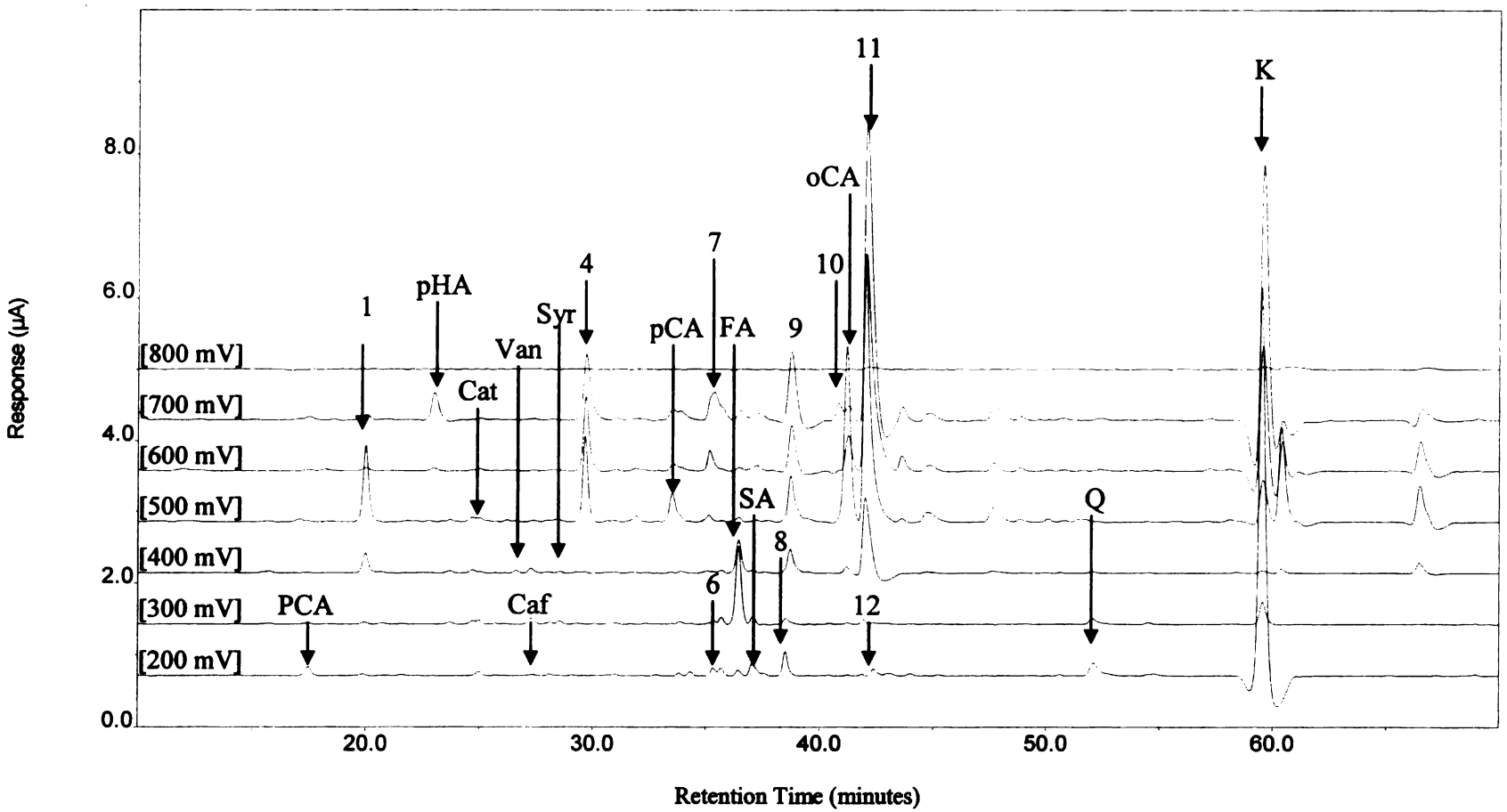


Figure 12a. Chromatogram of Non-Hydrolyzed Red Bean

PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Cat, (+)-catechin; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Q, quercetin; K, kaempferol; numbers correspond to peaks of unknown compounds.

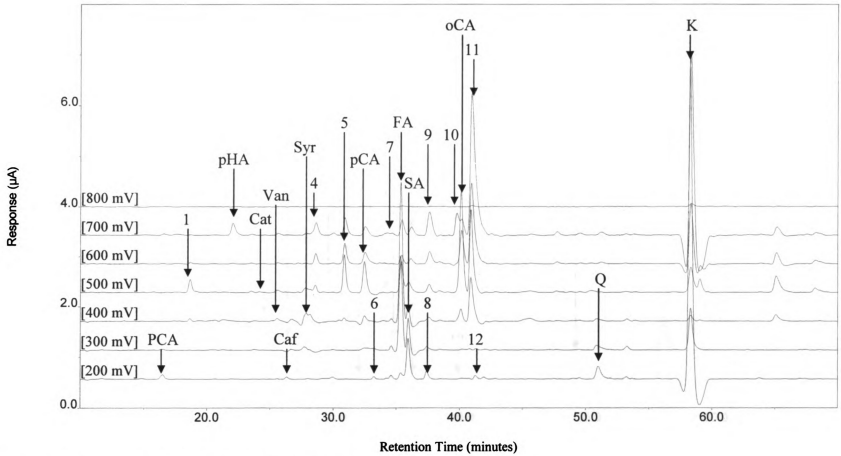


Figure 12b. Chromatogram of Alkaline Hydrolyzed Red Bean

PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Cat, (+)-catechin; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Q, quercetin; K, kaempferol; numbers correspond to peaks of unknown compounds.

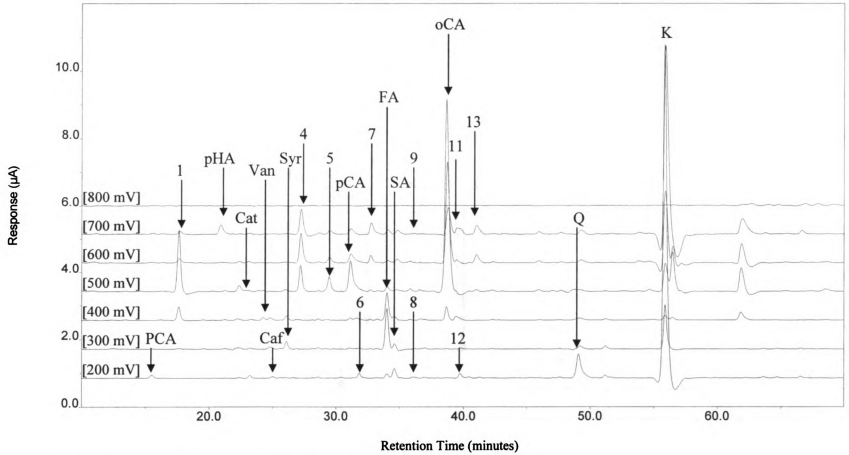


Figure 12c. Chromatogram of Acid Hydrolyzed Red Bean

PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Cat, (+)-catechin; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Q, quercetin; K, kaempferol; numbers correspond to peaks of unknown compounds.

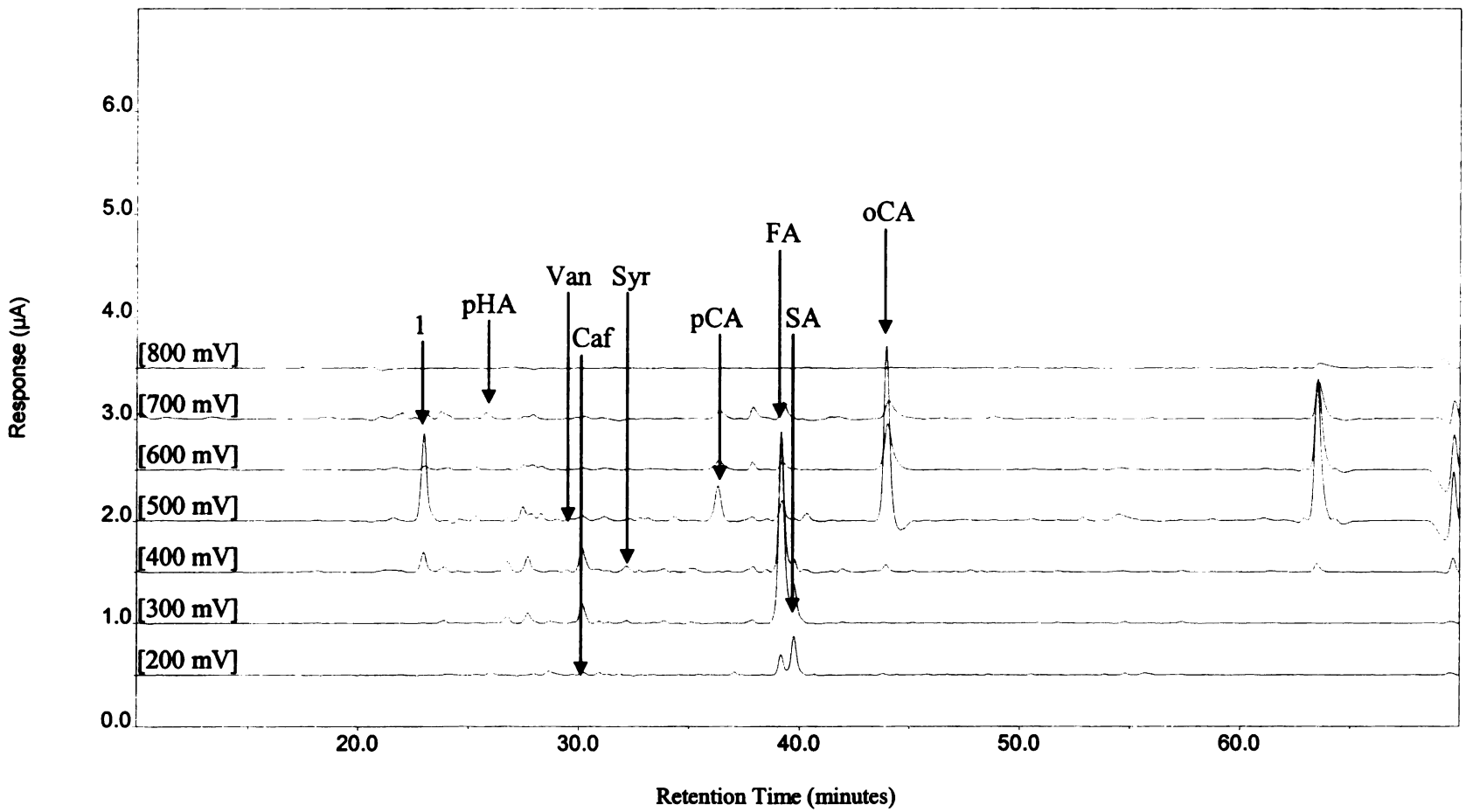


Figure 13a. Chromatogram of Non-Hydrolyzed Navy Bean
pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; numbers correspond to peaks of unknown compounds.

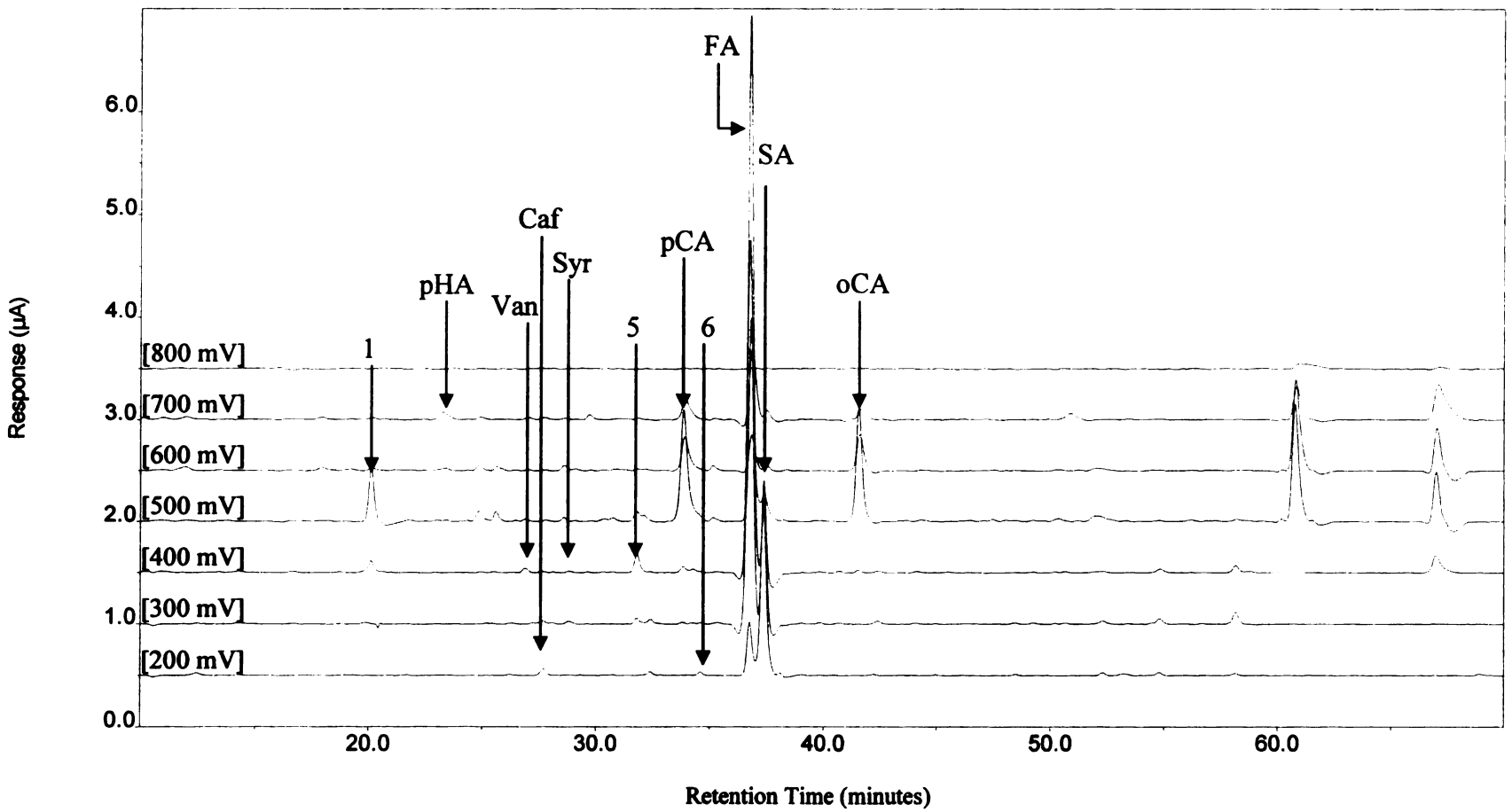


Figure 13b. Chromatogram of Alkaline Hydrolyzed Navy Bean
pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; numbers correspond to peaks of unknown compounds.

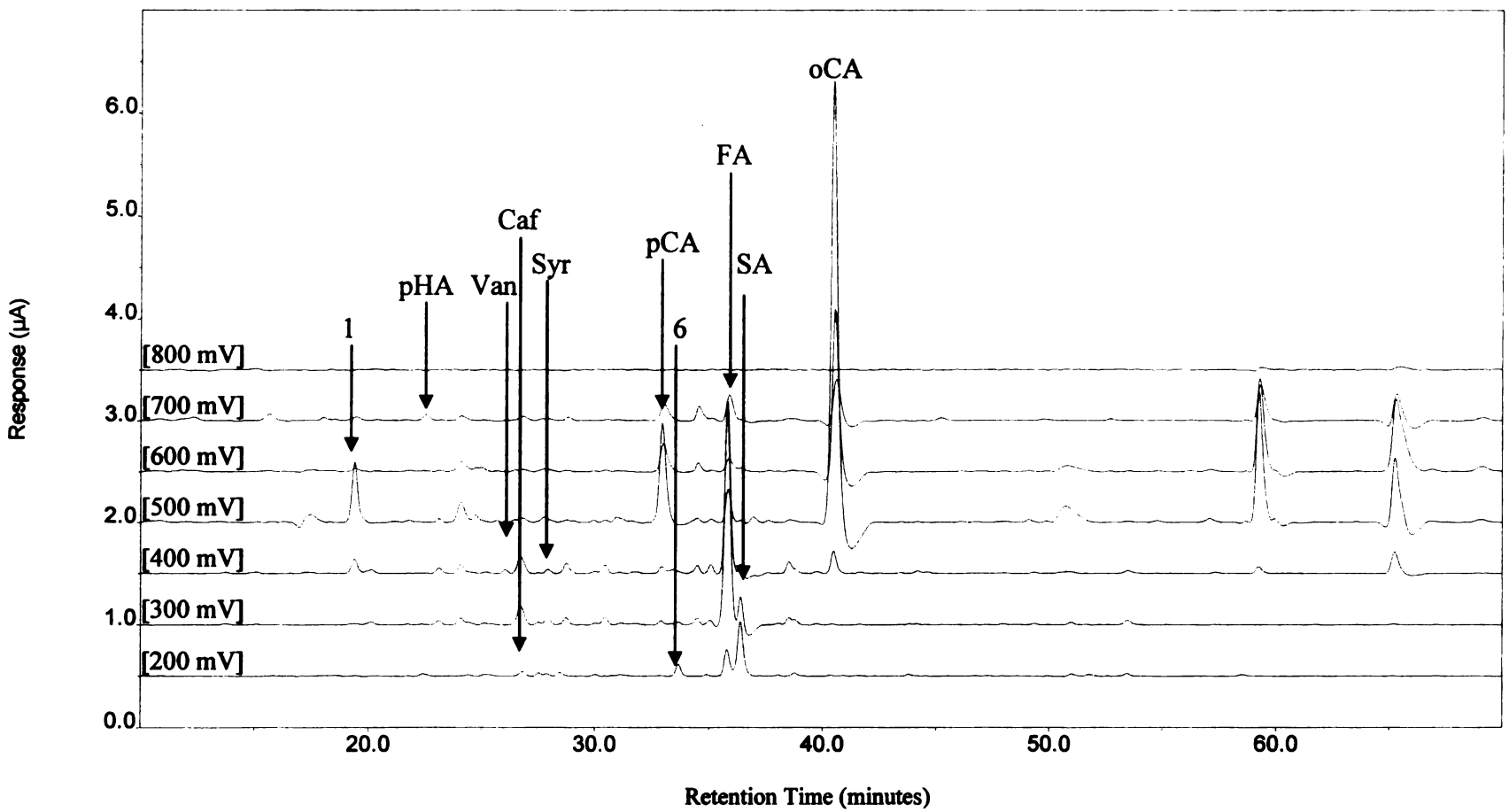


Figure 13c. Chromatogram of Acid Hydrolyzed Navy Bean
pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; numbers correspond to peaks of unknown compounds.

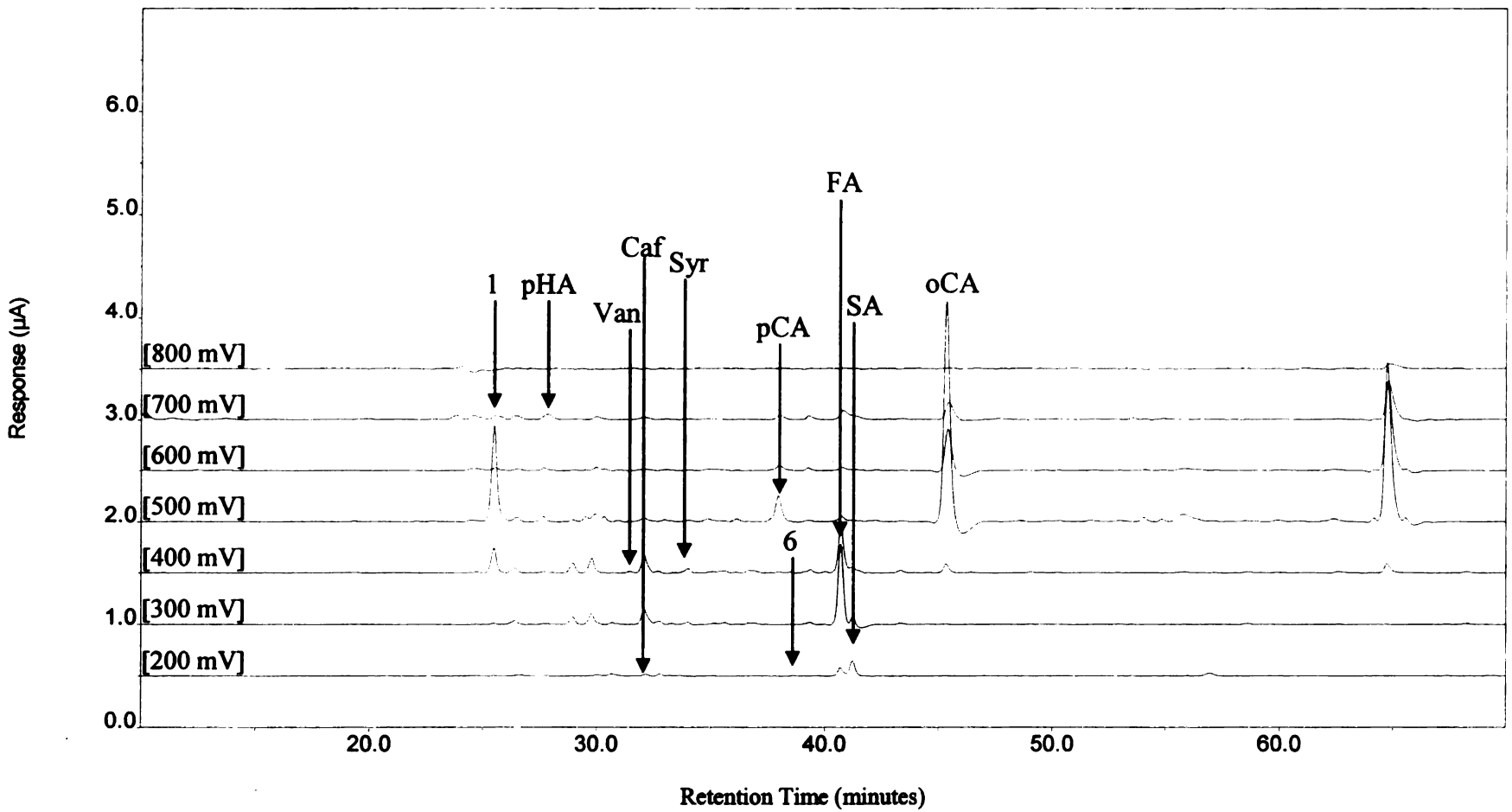


Figure 14a. Chromatogram of Non-Hydrolyzed Navy Bean Residue
pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; numbers correspond to peaks of unknown compounds.

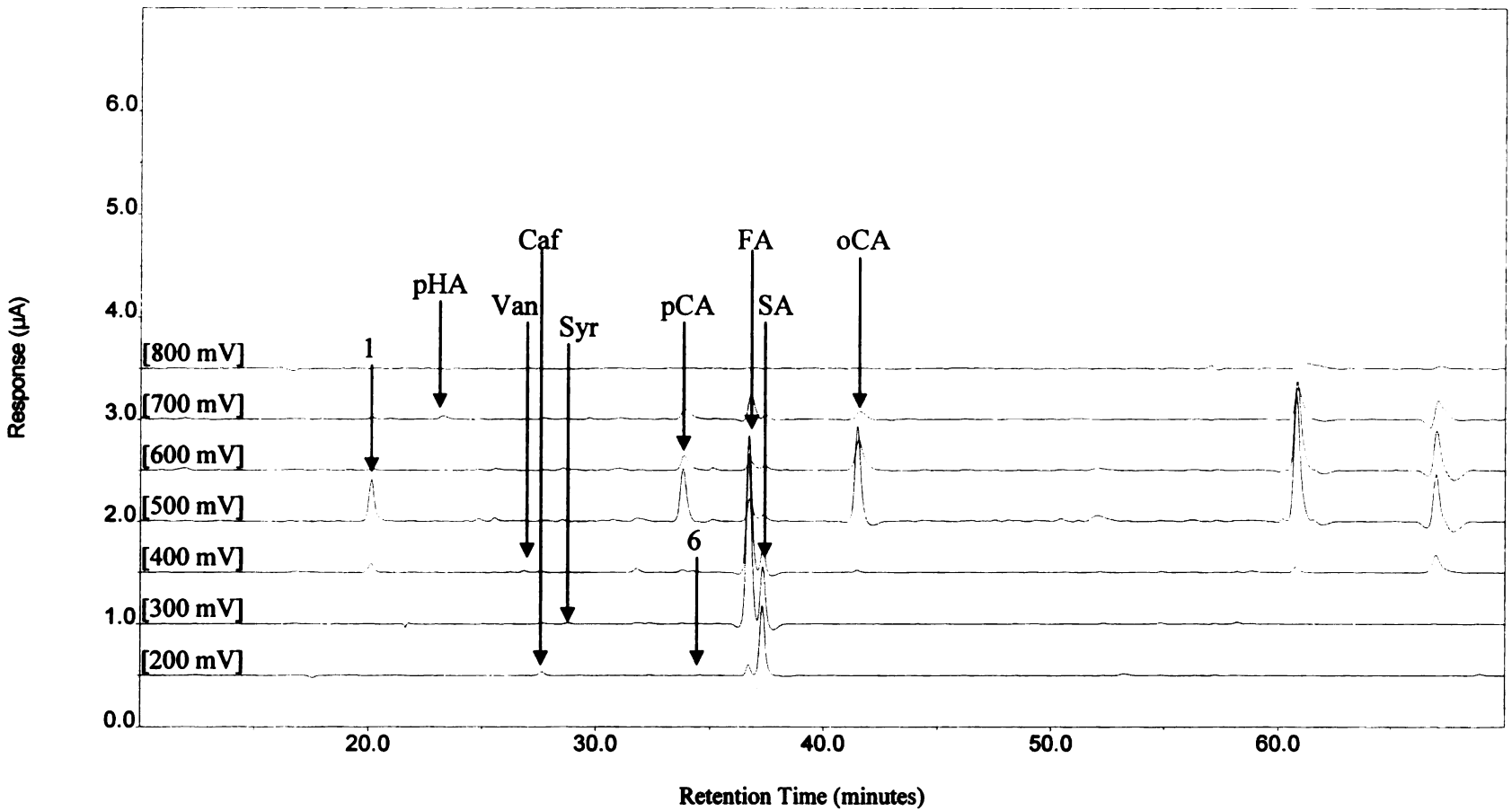


Figure 14b. Chromatogram of Alkaline Hydrolyzed Navy Bean Residue
pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; numbers correspond to peaks of unknown compounds.

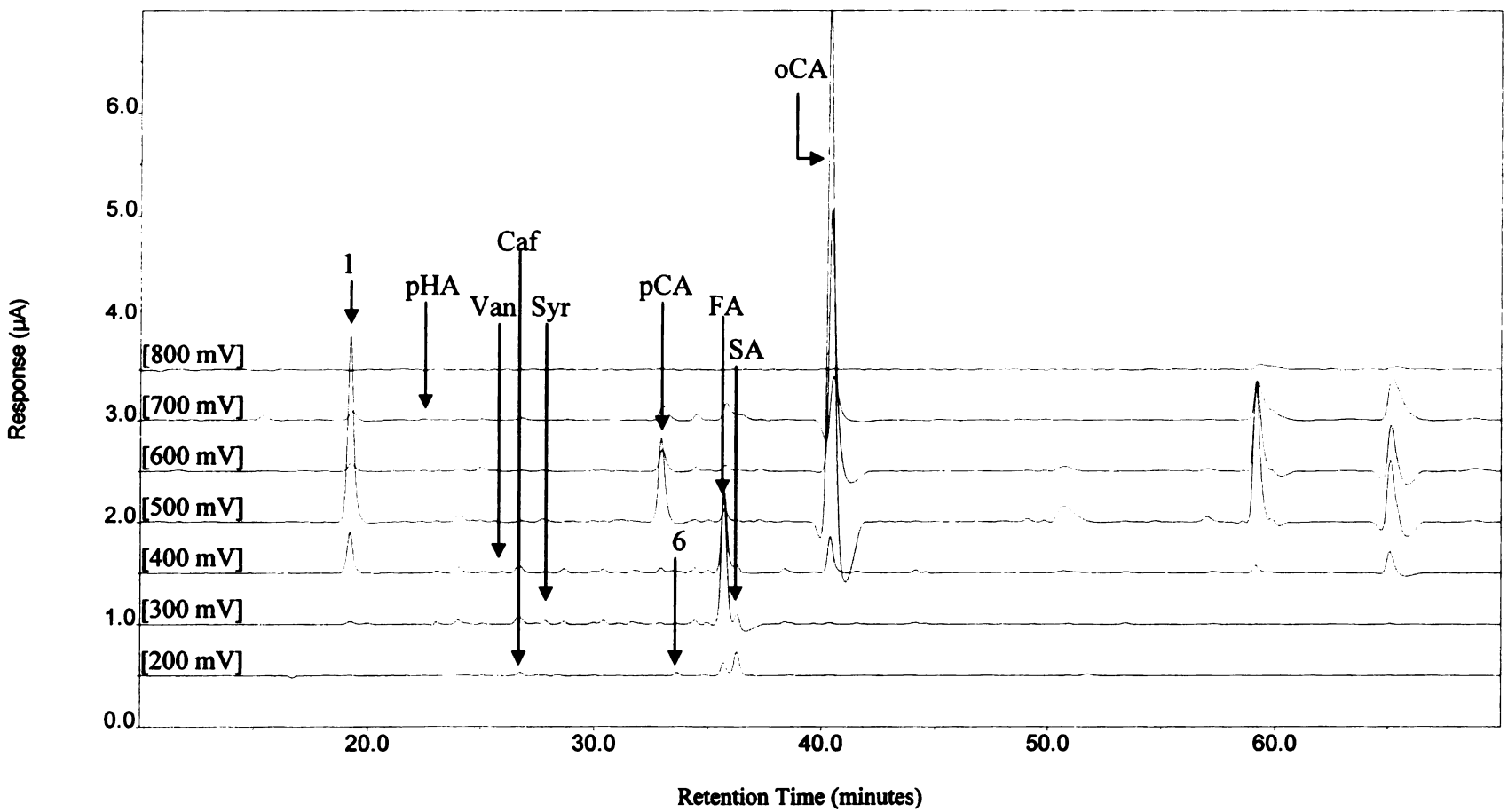


Figure 14c. Chromatogram of Acid Hydrolyzed Navy Bean Residue
pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; numbers correspond to peaks of unknown compounds.

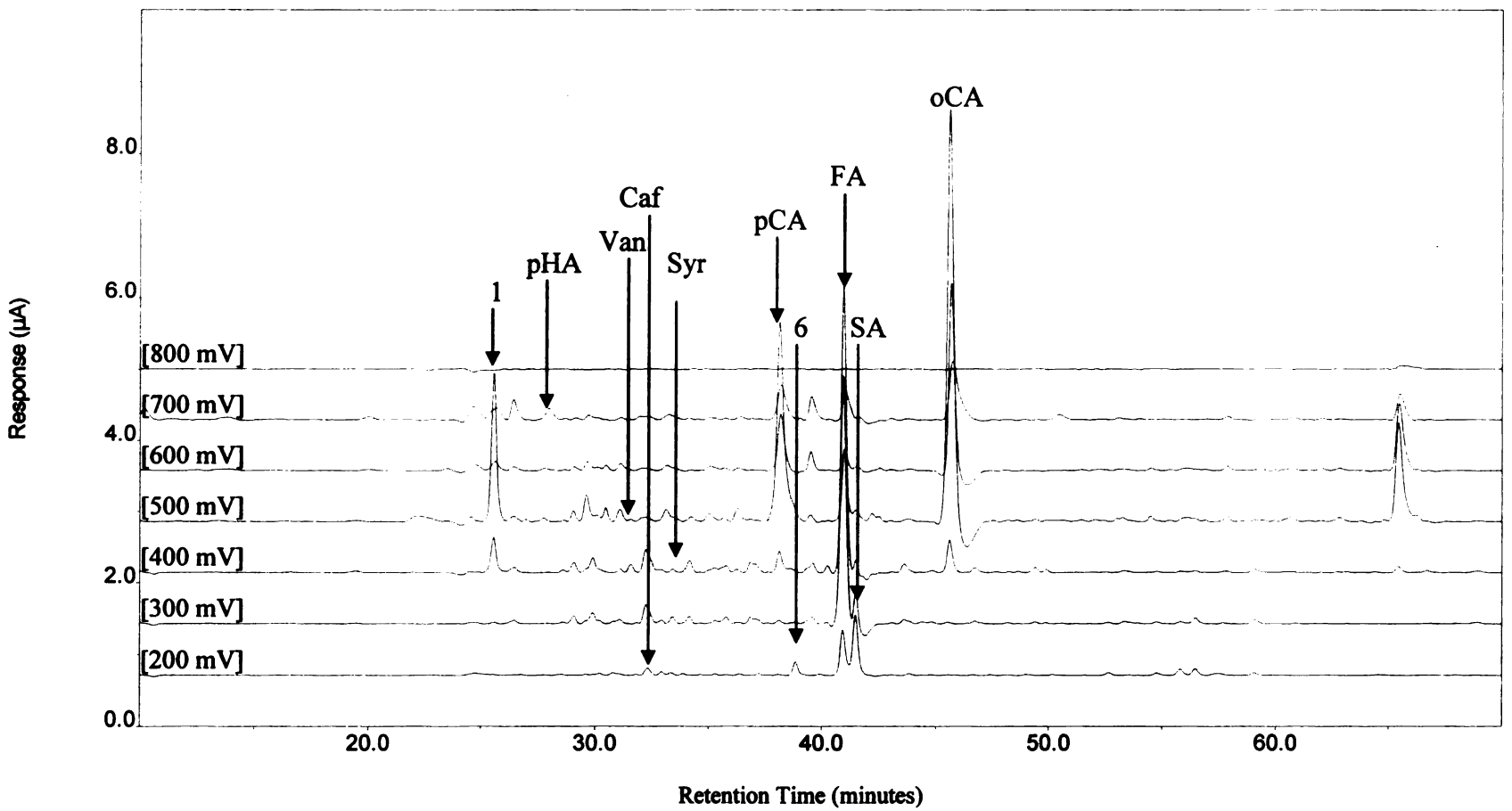


Figure 15a. Chromatogram of Non-Hydrolyzed Navy Bean Extract Mix
pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; numbers correspond to peaks of unknown compounds.

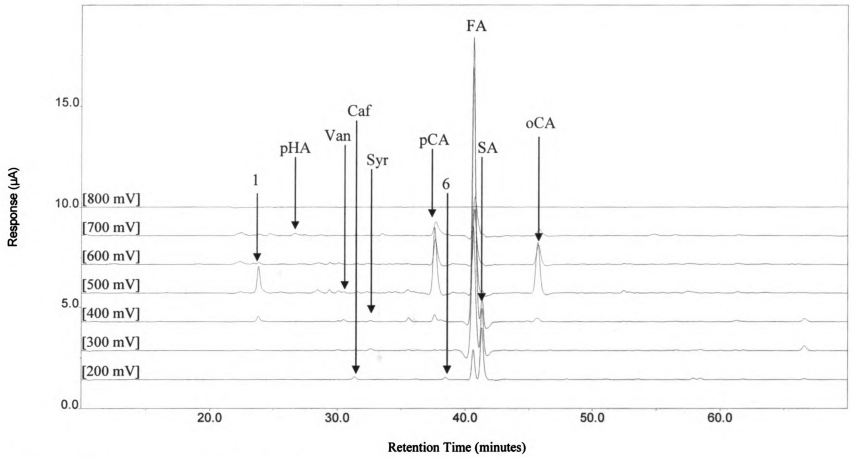


Figure 15b. Chromatogram of Alkaline Hydrolyzed Navy Bean Extract Mix
pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; numbers correspond to peaks of unknown compounds.

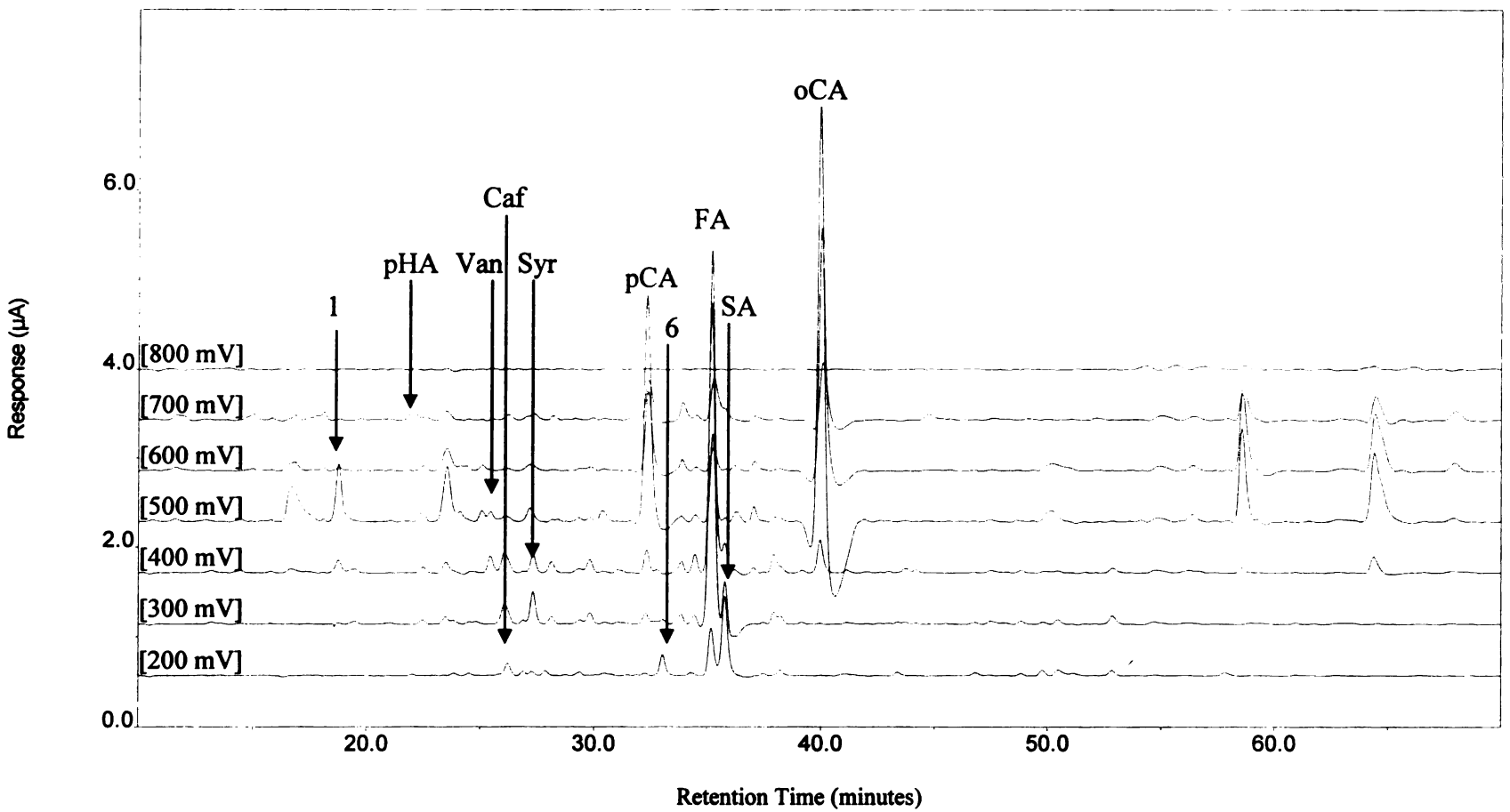


Figure 15c. Chromatogram of Acid Hydrolyzed Navy Bean Extract Mix
pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; numbers correspond to peaks of unknown compounds.

APPENDIX B

Table 3. Unconjugated Phenolic Compounds (mg /100 g of Bean Flour)*

	BB	PB	RB	NB
Hydroxybenzoic Acids				
PCA	1.63 ± 1.37	2.61 ± 3.13	5.48 ± 4.53	nd
pHBA	0.36 ± 0.27	0.90 ± 0.12	2.41 ± 0.68	0.37 ± 0.21
Van	0.69 ± 0.21	0.81 ± 0.21	0.55 ± 0.11	0.40 ± 0.11
Syr	0.93 ± 0.27	0.43 ± 0.27	0.53 ± 0.25	0.49 ± 0.23
Total	3.61	4.75	8.97	1.26
Hydroxycinnamic Acids				
Caf	0.17 ± 0.03	0.43 ± 0.15	0.50 ± 0.20	0.16 ± 0.03
pCA	0.28 ± 0.10	0.49 ± 0.21	0.44 ± 0.14	0.55 ± 0.22
FA	1.50 ± 0.47	3.06 ± 0.92	1.58 ± 0.55	2.93 ± 0.88
SA	0.40 ± 0.07	1.06 ± 0.18	0.61 ± 0.19	0.59 ± 0.17
Total	2.35	5.04	3.13	4.23
Flavan-3ols				
Cat	0.25 ± 0.14	2.65 ± 1.41	0.31 ± 0.13	nd
Total	0.25	2.65	0.31	nd
Flavonols				
Q	2.66 ± 0.82	0.86 ± 0.55	3.86 ± 1.50	nd
K	0.64 ± 0.16	6.71 ± 2.02	25.70 ± 9.99	nd
Total	3.30	7.57	29.56	nd
TIP[a]	9.50	20.00	41.96	5.49
TPC[b]	259.06 ± 9.11	274.69 ± 4.87	230.10 ± 6.16	149.45 ± 7.10

* All values are dry basis. Values are means of n= 5-8 determinations ± standard deviation adjusted for loss due to Sep Pak. BB, black bean; PB, pinto bean; RB, red bean; NB, navy bean; PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Cat, (+)-catechin; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Q, quercetin; K, kaempferol; nd, not detected. a. TIP; Total phenolic compounds identified by HPLC-ECD. b. TPC = Total Phenolic Content = mean mg of (+)-Catechin Equivalent per 100 g of bean flour ± standard deviation (n = 4-6) determined by Folin-Ciocalteu's Assay.

Table 4. Unconjugated Phenolic Compounds (mg /100 g Bean Fraction)*

	NBEM	NBR
Hydroxybenzoic Acids		
PCA	nd	nd
pHBA	0.66 ± 0.37	0.34 ± 0.05
Van	0.86 ± 0.34	0.27 ± 0.13
Syr	0.55 ± 0.21	0.53 ± 0.35
Total	2.01	1.14
Hydroxycinnamic Acids		
Caf	0.27 ± 0.09	0.09 ± 0.05
pCA	1.65 ± 1.18	0.29 ± 0.07
FA	5.90 ± 1.46	1.19 ± 0.30
SA	1.25 ± 0.37	0.27 ± 0.05
Total	9.07	1.84
TIP[a]	11.15	2.99
TPC[b]	328.40 ± 15.44	66.75 ± 5.96

*All values are dry basis. Values are means of n= 5-8 determinations ± standard deviation adjusted for loss due to Sep Pak and alkaline hydrolysis. NBEM, navy bean extract mix; NBR, navy bean residue; PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; nd, not detected. a. TIP; Total phenolic compounds identified by HPLC-ECD. b. TPC = Total Phenolic Content = mean mg of (+)-Catechin Equivalent per 100 g of bean flour ± standard deviation (n = 4-6) determined by Folin-Ciocalteu's Assay.

Table 5. Unconjugated Phenolic Compounds + Phenolic Compounds Liberated by Alkaline Hydrolysis (mg /100 g of Bean Flour)*

	BB	PB	RB	NB
Hydroxybenzoic Acids				
PCA	2.29 ± 1.78	3.03 ± 1.77	4.32 ± 2.73	nd
pHBA	0.46 ± 0.37	1.06 ± 0.12	2.44 ± 0.67	0.44 ± 0.27
Van	1.85 ± 0.57	1.12 ± 0.41	0.58 ± 0.42	1.05 ± 0.70
Syr	2.06 ± 1.63	0.74 ± 0.63	0.84 ± 0.87	1.22 ± 1.12
Total	6.66	5.95	8.18	2.71
Hydroxycinnamic Acids				
Caf	0.99 ± 0.70	0.92 ± 0.57	0.92 ± 0.79	0.58 ± 0.48
pCA	2.40 ± 1.80	0.80 ± 0.46	0.69 ± 0.43	3.00 ± 1.79
FA	13.68 ± 4.87	10.62 ± 5.19	5.12 ± 2.28	26.49 ± 7.88
SA	6.60 ± 1.70	7.75 ± 2.34	3.82 ± 0.96	5.93 ± 1.23
Total	23.67	20.09	10.55	36.00
Flavonols				
Q	5.24 ± 1.87	1.70 ± 0.28	6.36 ± 0.81	nd
K	1.99 ± 0.51	15.82 ± 2.93	55.65 ± 9.63	nd
Total	7.23	17.52	62.01	nd
TIP[a]	37.55	43.55	80.76	38.72

* All values are dry basis. Values are means of n= 5-8 determinations ± standard deviation adjusted for loss due to Sep Pak and alkaline hydrolysis. BB, black bean; PB, pinto bean; RB, red bean; NB, navy bean; PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Q, quercetin; K, kaempferol; nd, not detected. a. TIP; Total phenolic compounds identified by HPLC-ECD.

Table 6. Unconjugated Phenolic Compounds + Phenolic Compounds Liberated by Alkaline Hydrolysis (mg /100 g of Bean Fraction)*

	NBEM	NBR
Hydroxybenzoic Acids		
PCA	nd	nd
pHBA	0.67 ± 0.39	0.26 ± 0.22
Van	2.26 ± 1.66	0.27 ± 0.06
Syr	2.79 ± 2.22	1.36 ± 1.07
Total	5.72	1.89
Hydroxycinnamic Acids		
Caf	0.88 ± 0.79	0.32 ± 0.30
pCA	5.83 ± 5.37	0.83 ± 0.46
FA	45.45 ± 14.32	5.41 ± 1.95
SA	10.03 ± 2.30	2.73 ± 0.71
Total	62.19	9.29
TIP[a]	67.90	11.18

*All values are dry basis. Values are means of n= 5-8 determinations ± standard deviation adjusted for loss due to Sep Pak and alkaline hydrolysis. NBEM, navy bean extract mix; NBR, navy bean residue; PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; nd, not detected. a. TIP; Total phenolic compounds identified by HPLC-ECD.

Table 7. Unconjugated Phenolic Compounds + Phenolic Compounds Liberated by Acid Hydrolysis (mg /100 g of Bean Flour)*

	BB	PB	RB	NB
Hydroxybenzoic Acids				
PCA	4.00 ± 3.18	5.02 ± 2.49	7.00 ± 4.06	nd
pHBA	0.79 ± 0.56	1.22 ± 0.97	2.98 ± 2.40	0.34 ± 0.23
Van	1.63 ± 0.89	1.28 ± 0.55	0.88 ± 0.79	1.29 ± 1.10
Syr	2.50 ± 2.09	1.05 ± 0.91	0.81 ± 0.81	0.76 ± 0.59
Total	8.92	8.57	11.67	2.39
Hydroxycinnamic Acids				
Caf	0.41 ± 0.30	0.51 ± 0.45	0.67 ± 0.69	0.33 ± 0.23
pCA	0.74 ± 0.30	0.84 ± 0.33	0.85 ± 0.43	1.45 ± 0.30
FA	4.10 ± 1.60	5.70 ± 1.94	3.31 ± 1.18	8.75 ± 3.08
SA	1.09 ± 0.38	1.92 ± 0.46	1.22 ± 0.40	1.27 ± 0.30
Total	6.34	8.97	6.05	11.80
Flavonols				
Q	4.45 ± 1.82	4.34 ± 1.53	7.88 ± 3.33	nd
K	1.57 ± 0.62	19.15 ± 8.91	58.60 ± 26.07	nd
Total	6.02	23.49	66.48	nd
TIP[a]	21.28	41.02	84.20	14.18

* All values are dry basis. Values are means of n = 5-8 determinations ± standard deviation adjusted for loss due to Sep Pak and alkaline hydrolysis. BB, black bean; PB, pinto bean; RB, red bean; NB, navy bean; PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; Q, quercetin; K, kaempferol; nd, not detected. a. TIP; Total phenolic compounds identified by HPLC-ECD.

Table 8. Unconjugated Phenolic Compounds + Phenolic Compounds Liberated by Acid Hydrolysis (mg /100 g of Bean Fraction)*

	NBEM	NBR
Hydroxybenzoic Acids		
PCA	nd	nd
pHBA	0.43 ± 0.31	0.56 ± 0.50
Van	2.13 ± 1.93	1.62 ± 1.12
Syr	2.59 ± 2.02	0.30 ± 0.24
Total	5.15	2.48
Hydroxycinnamic Acids		
Caf	0.44 ± 0.43	0.06 ± 0.03
pCA	4.36 ± 2.33	0.89 ± 0.47
FA	12.32 ± 4.26	3.88 ± 1.38
SA	2.19 ± 0.58	0.59 ± 0.14
Total	19.31	5.42
TIP[a]	24.46	7.90

*All values are dry basis. Values are means of n= 5-8 determinations ± standard deviation adjusted for loss due to Sep Pak and alkaline hydrolysis. NBEM, navy bean extract mix; NBR, navy bean residue; PCA, protocatechuic acid; pHBA, *p*-hydroxybenzoic acid; Van, vanillic acid; Caf, caffeic acid; Syr, syringic acid; pCA, *p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; nd, not detected. a. TIP; Total phenolic compounds identified by HPLC-ECD.

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